UNIVERSITY OF
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Thesis submitted in accordance with the requirements of
The University of Liverpool for the
Degree of Doctor in Philosophy

Development and validation of liquid chromatography mass spectrometry methods to study the effects of nitisinone in Alkaptonuria

by:

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Dedicated to the memory of my parents:

Thomas George Hughes (09/02/1926 – 12/03/2003)

Elizabeth June Hughes (26/06/1928 – 17/02/1994)

“I would rather be exhausted from the hard times that breed success, than be well rested from achieving nothing”

(AT Hughes, 2013)

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Acknowledgements

Many say that doing a full time PhD is tough. Agreed. I had the fortune and pleasure of doing this PhD whilst working as a full-time employee of 30 years in the NHS running the non-automated laboratories of Biochemistry at the Royal Liverpool University Hospital. Squeezing this degree around managing the laboratories, running assays, developing new methods, presenting lectures, chairing meetings, supervising projects, dealing with “difficult”, “self-serving”, “unreliable”, and “entitled” staff, troubleshooting, writing documents, ploughing through constant red tape and individuals who were intent on making life demoralising and unpleasant, training, leading, managing, teaching, procuring, liaising, accrediting, appraising, interviewing, attempting to move the laboratories to a new-build hospital via more brick walls and appalling ineptitude, and doing all this plus overtime whilst being hit with several bouts of ill health, major surgery, moving house, and a global pandemic, we can maybe put into perspective any little quibbles a student may have regarding a failed assay, missing a lecture, meeting a deadline, feeling a little ‘stressed’, ‘anxious’, or not being able to contact their supervisor.

Tough times aside, none of this would be achievable without the support of my supervisors, Dr Anna Milan, Professor Lakshminarayan Ranganath and Professor Jim Gallagher. Many staff, co-workers, family, and friends over the years can be added to this list who have provided a positive support, especially from all the AKU international team as well as our very own National Alkaptonuria Centre here at the Royal Liverpool University Hospital.

Opportunities do not come by very often, to not only make a positive difference in the science community but having that difference make such a direct impact for the patients of this debilitating disorder. A difference that is here to stay and will continue to be expanded upon from all the international partners involved. We had to start somewhere and this whole project may never have got off the ground had it not been for the tenacity and dedication

of Robert Gregory, an AKU patient who was determined he should not remain another statistic and his voice should be heard, and Professor Ranganath, who listened and acted. Hopefully this will be the catalyst where more rare disorders may receive the same attention that AKU has. I would like to add my own personal note of thanks to Professor Ranganath for having the confidence in my technical ability to bring me on-board to the AKU group and for providing the funding and support for me to achieve this PhD.

An extra special note of thanks must indeed go to Anna Milan who has maintained a high level of patience and tolerance throughout this write-up by preventing me from quitting my job on countless occasions, putting up with my rants, maintaining my sanity to within acceptable parameters, providing considerable assistance throughout, particularly with the nightmarish task that is ‘formatting’, but mostly ensuring that the previously mentioned day to day issues did not result in any cosmic supernova on my part.

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Sadly, my parents are long gone, but I would like to dedicate this piece of work to their memory. I’m sure they would not have had a clue what I was banging on about….

Abstract

Alkaptonuria (AKU) is a rare inherited inborn error of tyrosine metabolism, resulting from a defect in the enzyme homogentisate 1,2-dioxygenase (HGD). Origins of this disorder can be traced back to ancient Egyptian times as a consequence of intrafamilial consanguinity. The major metabolic consequence from birth is the accumulation of the metabolite homogentisic acid (HGA) within the circulation, resulting in significant excretion in the urine. Clinical manifestations are typically observed from the third decade of life as a consequence of cumulative deposition of HGA within the cartilaginous and soft tissue which in turn undergoes a process known as ochronosis leading to destruction of load-bearing joints and multisystemic damage. Treatment, until recently has been only palliative, however, investigations using the drug nitisinone (NTBC) have been shown to significantly reduce the concentrations of HGA with promising effect.

The aims of this thesis were two-fold, firstly to develop and validate sensitive and specific liquid chromatography mass spectrometry (LC-MS/MS) methods that were able to measure all the major metabolites within the tyrosine (TYR) pathway linked to AKU plus the HGA-reducing drug NTBC. Then these methods were utilised in both international clinical trials and long-term monitoring of our own patients at the national AKU centre (NAC) in the Royal Liverpool University Hospital, to determine the effect of NTBC treatment in these patients.

The SONIA-1 (Suitabiity of NTBC in AKU 1) short-term clinical trial yielded a dose-dependent decrease in HGA concentrations with 8 mg NTBC proving the most effective. A 10 mg dose of NTBC was chosen for the 4-year SONIA-2 trial due its regularity of manufacture. Over this longer trial, the HGA remained suppressed and s-TYR was elevated. Development of a sensitive s-HGA assay enabled accurate measurement even at higher NTBC doses. The other consequence of treatment was a significant rise in metabolites 4-hydroxyphenylpyruvic acid (HPPA) and 4-hydroxyphenyllactic

acid (HPLA) in both urine and serum, which may pose less of a detrimental effect at higher concentrations than HGA which accumulates and initiates the ochronotic process.

Novel and robust LC-MS/MS methods have enabled quantitation of all key AKU metabolites in both serum and urine, as well as the drug NTBC enabling clinicians to provide diagnosis and on-going monitoring of AKU status and treatment. The data obtained from these LC-MS/MS methods has provided a valuable insight into the metabolic mechanisms taking place both pre- and post-NTBC treatment and will also enable monitoring of any TYR-lowering nutritional adjustments for patient wellbeing, long-term. From a time when the only care available was palliative and later surgical, methods presented within this thesis and subsequent application to AKU cohorts has supported the licensing of NTBC for AKU treatment. An international collaboration has given patients a new hope for treating, reducing and potentially if treated early, prevention of the painful and life-threatening consequences attached to AKU. The success of this work will hopefully be a catalyst for a more universal attention to rare disorders.

List of Abbreviations

ACN	Acetonitrile
AKU	Alkaptonuria
AKUSSI	AKU severity score index
ALCAP	Association for the Fight Against Alkaptonuria
BCE	Before common era
BQA	Benzoquinone acetic acid
C	Celsius
CV	Coefficient of variation
Da	Dalton
d-	deuterated
DC	Direct current
ERNDIM	(European Research Network for evaluation and improvement of screening, Diagnosis and treatment of Inherited disorders of Metabolism
EMA	European Medicines Agency
ESI	Electrospray ionisation
FA	Formic acid
FDA	Food and Drug Administration
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid
HETP	Height equivalent to a theoretical plate
HGA	Homogentisic acid
HGD	Homogentisate 1,2-dioxygenase
HILIC	Hydrophilic interaction liquid chromatography
HPLA	4-Hydroxyphenyllactic acid
HPPA	4-Hydroxyphenylpyruvic acid
HPLC	High Performance Liquid Chromatography
HPPD	4-Hydroxyphenylpyruvate dioxygenase
hr	Hour(s)
HT-1	Hereditary Tyrosinaemia type 1
HT-2	Hereditary Tyrosinaemia type 2
HT-3	Hereditary Tyrosinaemia type 3
IMP	Investigational medicinal product
IS	Internal standard

LC	Liquid chromatography
LC-MS/MS	Liquid chromatography mass spectrometry
LLMI	Low Level of Measuring Interval
LLOQ	Lower limit of quantification
LOD	Limit of detection
MeOH	Methanol
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
µ	Micro-
m/z	Mass to charge ratio
n=	Number
NAC	National alkaptonuria centre
NHS	National Health Service
nm	Nanometre
NTBC	Nitisinone
OA	Osteoarthritis
OMIM	Online Mendelian Inheritance in Man
PHE	Phenylalanine
psi	Pounds per square inch
Q1	Quadrupole 1
Q2	Quadrupole 2
Q3	Quadrupole 3
QC	Quality control
rf	Radio frequency
RT	Retention time
SD	Standard deviation
SIM	Selective/single ion monitoring
S/N	Signal to noise ratio
SOBI	Swedish Orphan Biovitrum International
SOFIA	Sub-ochronotic features in AKU
SONIA-1	Suitability of Nitisinone in alkaptonuria-1
SONIA-2	Suitability of Nitisinone in alkaptonuria-2
SOP	Standard operating procedures
SPE	Solid phase extraction

SRM	Selected reaction monitoring
TYR	Tyrosine
u-HGA_{24}	24hr excretion of urine HGA
u-$HPLA_{24}$	24hr excretion of urine HPLA
u-$HPPA_{24}$	24hr excretion of urine HPPA
UK	United Kingdom
UKAS	United Kingdom accreditation scheme
ULMI	Upper Level of Measuring Interval
u-PHE_{24}	24hr excretion of urine PHE
UPLC	Ultra-performance liquid chromatography
u-TYR_{24}	24hr excretion of urine TYR
UV	Ultra violet

Chapter 1

Introduction

1.1 Alkaptonuria (AKU) – History and Background

We begin in the new kingdom of Egypt, 1500 BCE; a middle-aged man is in great pain as he struggles with every step, not understanding why his urine is black and with every day that passes his condition progressively gets worse. His name was Harwa and he was the custodian of a granary and little did he know that several thousand years later his mummified body and his condition would be investigated. The discovery of X-rays in 1895 by Wilhelm Conrad Roentgen paved the way for the non-invasive investigation of many mummified bodies, and Stenn et al., (1977 and 1979) and Lee and Stenn (1978) reported the findings, stating Harwa was in his early thirties, cause of death was unknown, but he had obvious ochronotic depositions in the pelvic femoral head (figure 1.1). Pigmented and unpigmented shavings were taken and analysed revealing that the ochronotic pigment found in Harwa's joints was derived from homogentisic acid (HGA). They went on to further conclude that this condition was a frequent occurrence in ancient Egypt as a consequence of intra-familial marriage.

Prior to this paper, Wells and Maxwell published an article in 1962 where they discussed a paper the previous year from Simon and Zorab referring to an X-ray on an Egyptian mummy, a young woman from the Roman period (figure 1.2). Wells and Maxwell chose a 26th Dynasty (700 BCE) mummy that had been described in extensive detail by Dawson (1929) and was that of a middle-aged woman. They concluded that there was ochronosis present and so it seems that a debilitating bone condition was still present and more common than once thought in Egypt nearly a millennial after the death of Harwa.

O'Brien et al., (1963) provided a literature review of AKU in which he described Scribonius (1584), as the earliest known documentation of an individual passing a dark urine, "a schoolboy, who, although in good health, excreted urine as black as ink". Schenck (1609), reported a Carmelite monk who "although in perfect health, had passed black urine his entire life", and Lusitanus (1649) described a similar case of a fourteen-year old boy who passed black urine, and went on to live a long and healthy life.

Marcet, (1822) a physician at Guy's hospital, London, described a case of a child in good health but excreting urine that became dark like "a port wine" on standing, although he could not accurately determine the compounds present. All these previous papers suggested the same condition without expanding on any explanation. However, O'Brien et al., (1963) and Stenn et al., (1979) stated that it was Boedeker in 1859 who first named the condition AKU after noting the addition of alkaline solution to a patients urine caused the dark colouration, which he named 'alkapton'. His patient was 44 years old with severe lumbar pains and limited mobility, symptoms which are only too common in AKU.

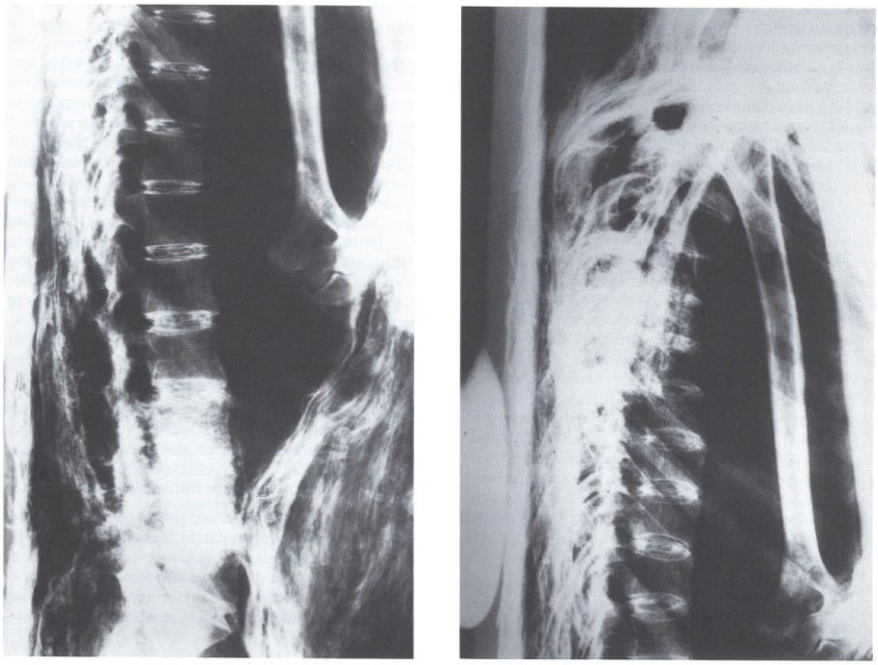


Figure 1.1: X-ray of Harwa showing wafer-like plaques in the intervertebral spaces. (Stenn et al., 1979).

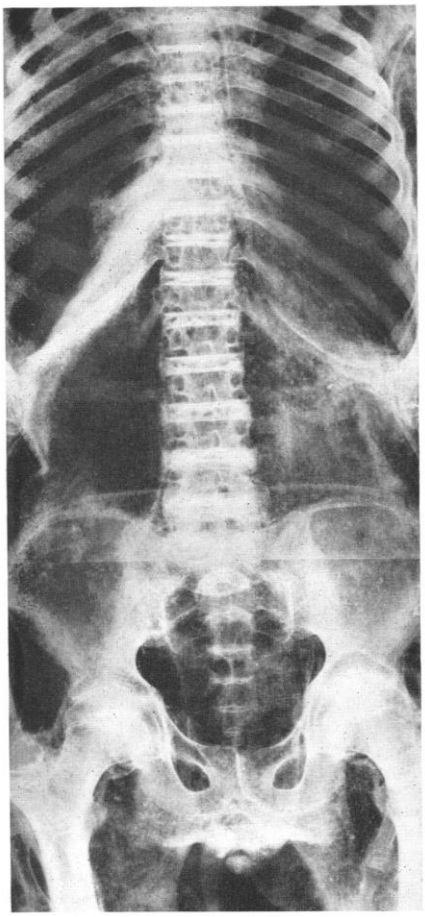


Figure 1.2: X-ray of Egyptian mummy showing dense calcification of intervertebral discs (Simon and Zorab, 1960).

Virchow in 1866 first used the term “ochronosis” (derived from the Greek terms yellow and disease) after noting cartilage discolouration in patients who had this urine abnormality as it showed a ‘brown ochre’ and in 1891 Wolkow and Baumann identified HGA as the responsible agent for this condition. Phocas et al., (1963) stated it was Albrecht in 1902 who tied together the condition of ochronosis and AKU as representing the same disorder, whilst that same year Archibald Garrod identified AKU as an inborn error of metabolism, the first disorder to be identified as such (figure 1.3 provides a timeline of the significant discoveries in AKU). In 1904 Osler was the first to publish a paper describing AKU in two living brothers describing discolouration of ears and sclera which he termed ‘Oslers sign’. In 1941 came another breakthrough when Beadle and Tatum postulated a defective gene results in the metabolic block of a single enzyme reaction. In the case

of AKU, this was identified by La Du in 1958 as homogentisate 1,2-dioxygenase (HGD, E.C.1.13.11.5), an enzyme involved in the metabolism of tyrosine (TYR) (figure 1.4).

To summarise, AKU, (OMIM 203500) is a rare inherited metabolic disorder of the tyrosine (TYR) metabolic pathway, characterised by elevated concentrations of circulating and urinary HGA (2,5-dihydroxyphenyllactic acid). This phenolic compound and metabolite of the TYR pathway is the primary antagonistic compound in AKU which, as a consequence of the inherent enzyme deficiency of HGD, accumulates from birth and over the subsequent decades with the biochemical process of ochronosis causes the destruction of connective tissue and cartilage. Its prevalence varies globally and was reported by Zatkova (2011) as 1 in 100,000 and Sacks in 1951 as rare as 1 in 10 million.

Symptoms of this disorder manifest themselves from birth with darkening of urine. It is not until about the third decade of life when ochronosis of the cartliagenous joints starts to take hold. Weight bearing joints such as the vertebrae, knee, ankle, hip and elbow become severely and painfully compromised from middle-age. Ochronotic pigmentation can also appear in the eyes, ear cartilage, skin, cardiac valves; and kidney and prostate stones have also been found (Ranganath and Cox, 2011; Ranganath et al., 2013; Sharma et al., 2021). Bone fractures and achilles tendon ruptures are not uncommon (Baca et al., 2019). Quality of life is therefore affected as joint destruction progresses, but there can be significant variability amongst patients and even siblings (Zatkova et al., 2020).

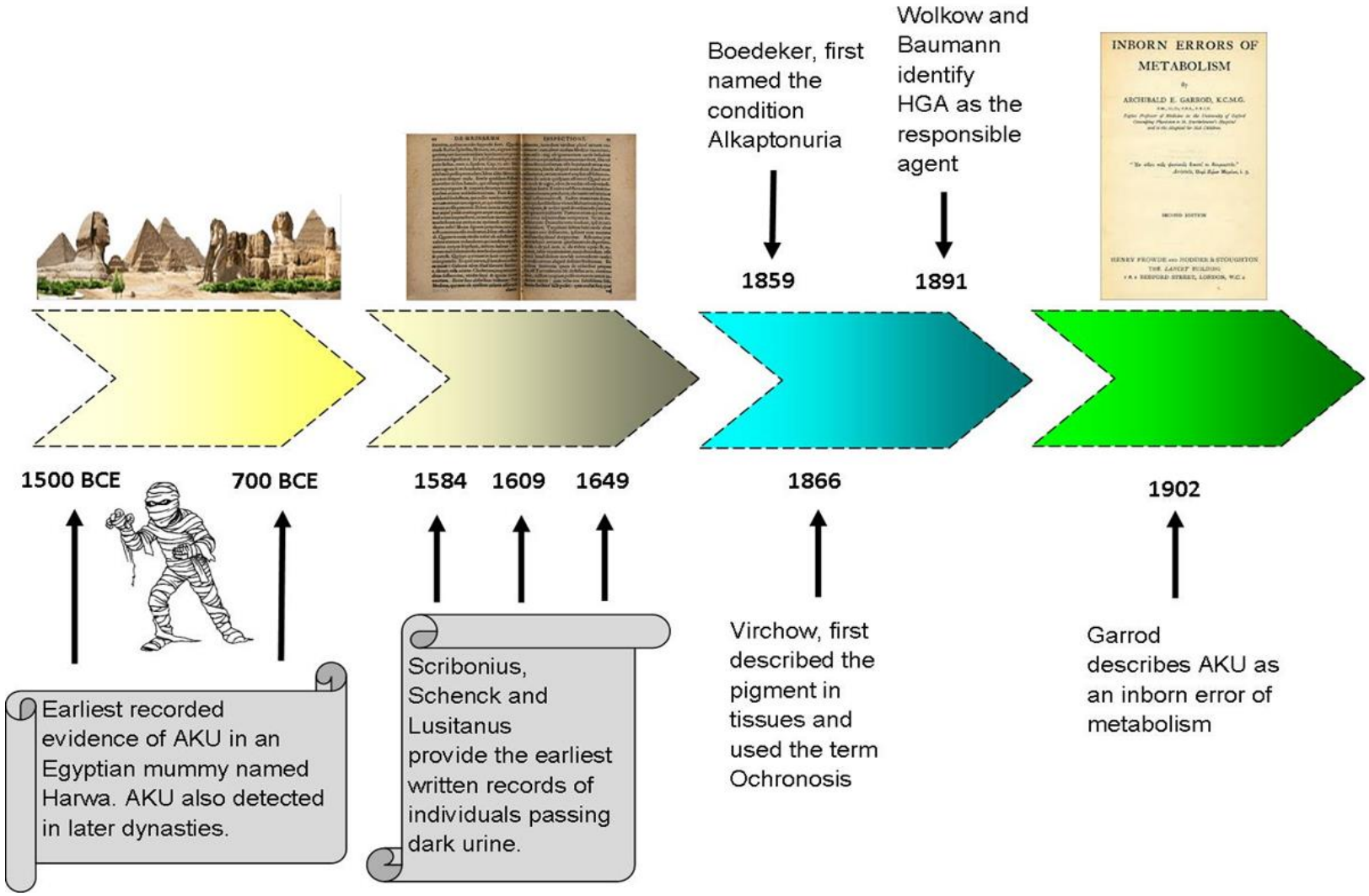
However, as in the case of consanguineous marriages (intrafamilial inbreeding) which was very much the case in ancient Egypt and certain pockets of the world where the genetic pools are weakened, then incidences of inborn errors of metabolism become less rare.

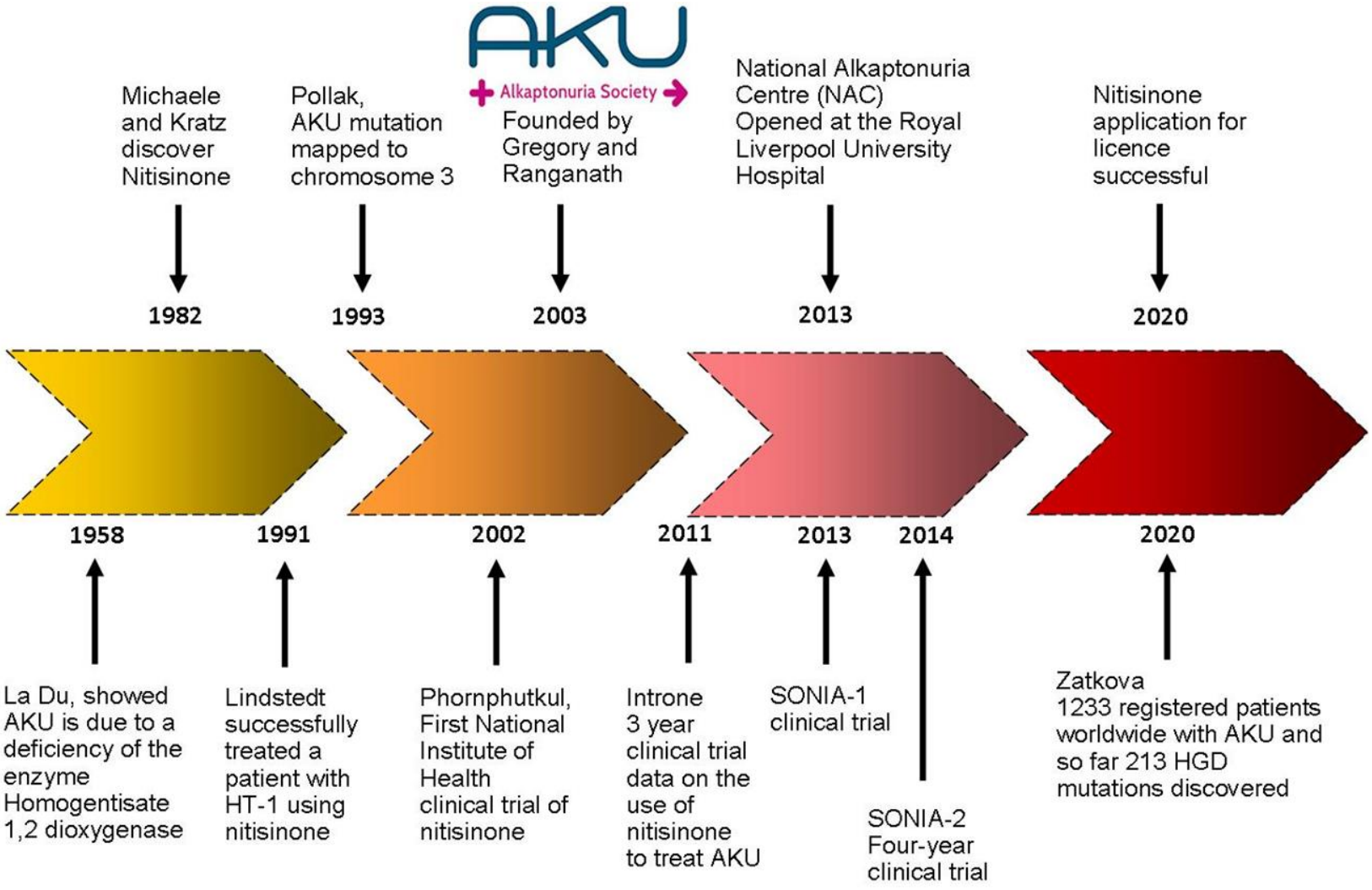
1.2 Genetic basis of AKU

AKU is a rare monogenic (only a single gene is affected) autosomal recessive disorder characterised by abnormally high circulating concentrations of HGA (figure 1.4).

It was Archibald Garrod (1857-1936), an English physician widely regarded as the father of inborn errors of metabolism, who first recognised AKU as a disease which followed Mendelian inheritance. His work with William Bateson noted that AKU followed the classic pattern of inheritance known as autosomal recessive and was most likely to occur in the children of first cousins. In his 1902 paper, Garrod referred to AKU as attributable to specific biochemical causes. Although DNA was first isolated by Friedrich Miescher in 1869, it wasn't until 1909 that Danish botanist Wilhelm Johannsen coined the word gene to describe the Mendelian units of heredity. He also made the distinction between the outward appearance of an individual (phenotype) and its genetic traits (genotype). Four years earlier, William Bateson, Garrod's working partner and a proponent of Mendel's ideas, had used the word 'genetics' in a letter as he felt the need for a new term to describe the study of heredity and inherited variations. But the term did not start spreading until Wilhelm Johannsen suggested that the Mendelian factors of inheritance be called genes (https://www.genome.gov).

Figure 1.3: Scientific progress of AKU




AKU
Alkaptonuria Society
1958
La Du, showed AKU is due to a deficiency of the enzyme Homogentisate 1,2 dioxygenase
1982
Michaele and Kratz discover Nitisinone
1991
Lindstedt successfully treated a patient with HT-1 using nitisinone
1993
Pollak, AKU mutation mapped to chromosome 3
2002
Phornphutkul, First National Institute of Health clinical trial of nitisinone
2003
Founded by Gregory and Ranganath
2011
Introne 3 year clinical trial data on the use of nitisinone to treat AKU
2013
SONIA-1 clinical trial
2013
National Alkaptonuria Centre (NAC) Opened at the Royal Liverpool University Hospital
2014
SONIA-2 Four-year clinical trial
2020
Zatkova 1233 registered patients worldwide with AKU and so far 213 HGD mutations discovered
2020
Nitisinone application for licence successful

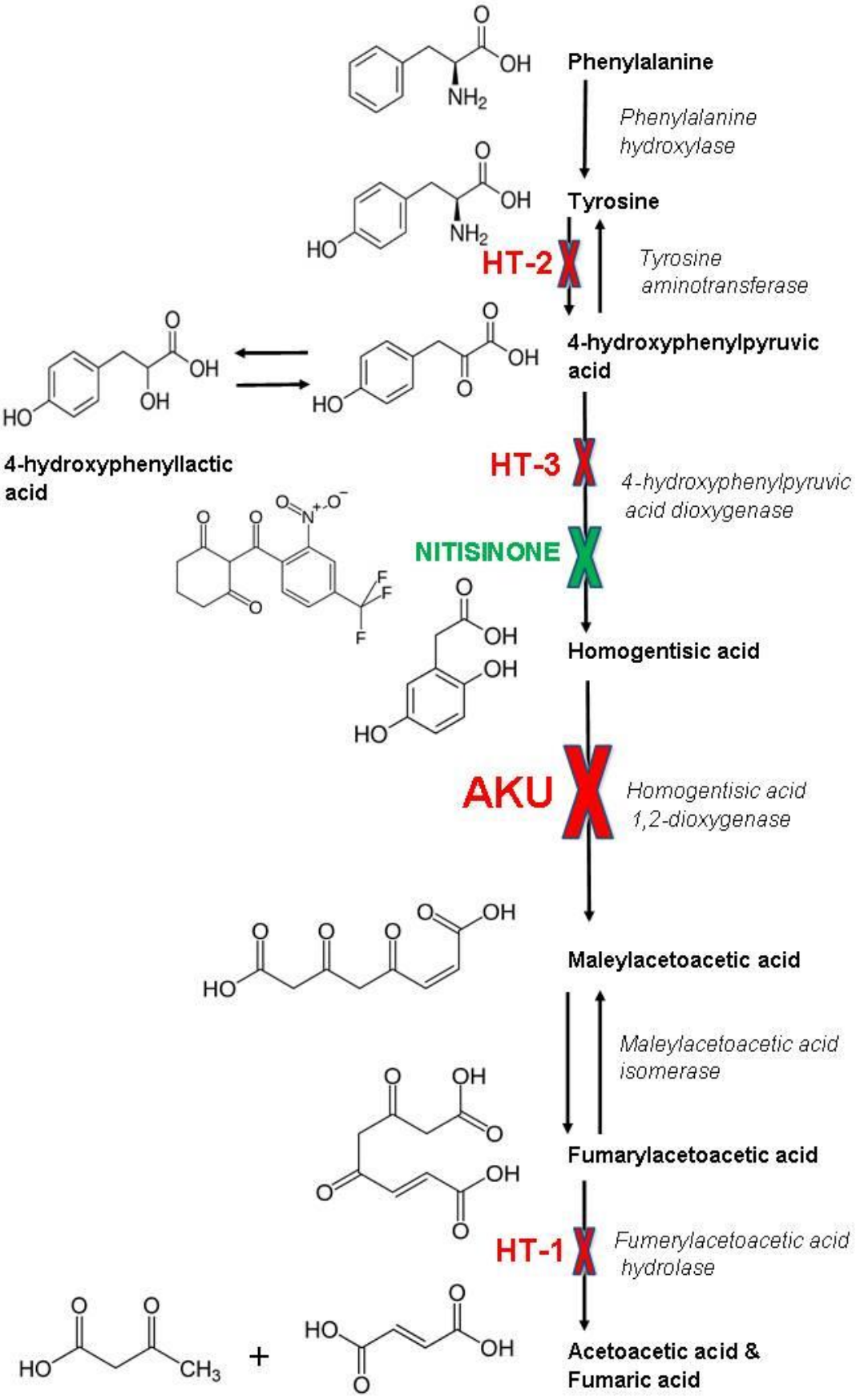


Figure 1.4: The TYR pathway: Major metabolites and prominent inborn disorders

The specific genetic cause of AKU is bi-allelic mutations in the homogentisate 1, 2-dioxygenase (HGD) gene (meaning the mutation has to occur in both the paternal and maternal gene for it to be represented in the offspring). The HGD gene encodes the enzyme homogentisate 1,2-dioxygenase, its absence resulting in a block along the TYR pathway (La Du 1958), causing an accumulation of HGA and the consequences associated with this multisystemic disorder (figure 1.4).

AKU prevalence is generally accepted to be in the region of 1 in 250,000 to 1 in 1,000,000 in the population, however, in certain regions of the world namely Slovakia (Srsen et al., 2002), India (Sakthivel et al., 2014), Jordan (Al-sbou and Mwafi 2012) and Dominican Republic (Goicoechea et al., 2002), its prevalence increases to as much as 1 in 19,000 (Mistry et al., 2013, Nemethova et al., 2016).

The HGD gene is located on chromosome 3 (region: 3q21-q23), and comprises 14 exons, encodes a protein of 445 amino acids and spans 54,363 base pairs (Fernandez-Canon et al., 1996). The functional HGD enzyme is a dynamic hexamer composed of two disc-like trimers (Titus et al., 2000) and is responsible for metabolising ingested phenylalanine (PHE) and TYR, surplus to dietary requirements and during protein metabolism. Hepatocytes and renal proximal tubular cells are the major sites of HGD activity (Zatkova et al., 2020, Hughes et al., 2020). To date there has been a reported 213 human HGD mutations, (http://hgddatabase.cvtisr.sk/), the most frequent are missense variants (67%), followed by splicing (12.2%), frameshift (12.2%) and stop codons (6%).

Zatkova et al., (2011) discussed the prevalence of HGD mutations which seem to be distributed throughout the entire gene, with some having been introduced into Europe with founder populations, and others having a more widespread distribution. Several mutations are also region specific, for example India and Jordan pointing towards a consanguinity within the population resulting in weaker gene pools, or just a result of community isolation. The founder effect is also a consequence of the high incidence of

AKU in the Dominican Republic (a reduced genetic diversity which results when a population is descended from a small number of colonizing ancestors).

As of writing Zatkova et al., (2020) has stated there are 1233 patients identified worldwide with AKU. With the recent advances in genetic profiling for the many HGD mutations, Zatkova et al., (2020) stated that there was no direct effect of mutation type on the variability of the phenotype and therefore other factors such as the dietary intake of TYR and efficiency of renal function determined the circulating concentration of HGA. Phornphutkul et al. (2002) also noted that there is no correlation between particular HGD mutations and HGA excretion or disease severity.

The discovery of global AKU hotspots has heightened further interest in this disorder and it is likely there are many other undiagnosed regions that will eventually come to the attention of the AKU society and DevelopAKUre (https://akusociety.org/) who are several consortia discussed about later in this introduction.

1.3 Disease progression and Ochronosis

Elevated concentrations of HGA become the direct causative agent in a process referred to as ochronosis. Zannoni et al., (1969) described this process as the oxidation of HGA to an intermediate compound called benzoquinone acetic acid (BQA). A further polymerisation occurs resulting in the yellow-brown-black pigment which accumulate in tissues.

Cartilage and connective tissues are particularly susceptible to ochronotic pigmentation which can lead to a form of osteoarthritis (OA) particularly in joints resulting in the cartilage to become brittle and break down. This process occurs from birth as a consequence of a genetic defect and does not usually manifest itself until the third decade of life, by which time significant damage has been done (Ranganath and Cox, 2011). Early signs, of AKU as mentioned previously is the darkening of urine on standing over a period of days (figure 1.5).

Bluefarb (1958) presented a case history of a 42 year-old male displaying ochronotic features and disc degeneration. Many case histories have followed; (O'Brien et al., 1963, Phornphutkul et al., 2002, Helliwell et al., 2008; Ranganath et al., 2013; Akbaba et al., 2020; Kisa et al., 2021) all of which have discussed the debilitating features of AKU which are multisystemic, ranging from cardiovascular, osteoarthropathy, osteopenia, renal and prostate stones to muscular, tendon and ligament ruptures, particularly the achilles tendon (Jebaraj et al., 2006, Alajoulin et al., 2015). Ochronotic pigmentation can also be observed in the sclera of the eye and the pinna of the ear as blue-black patches (figure 1.6). Petit et al., (2011) reported upto a 40% prevalence in aortic valve disorders by the 5th decade of life as a consequence of ochronosis leading to calcification and the risks involved in such necessary surgery replacing the valves.

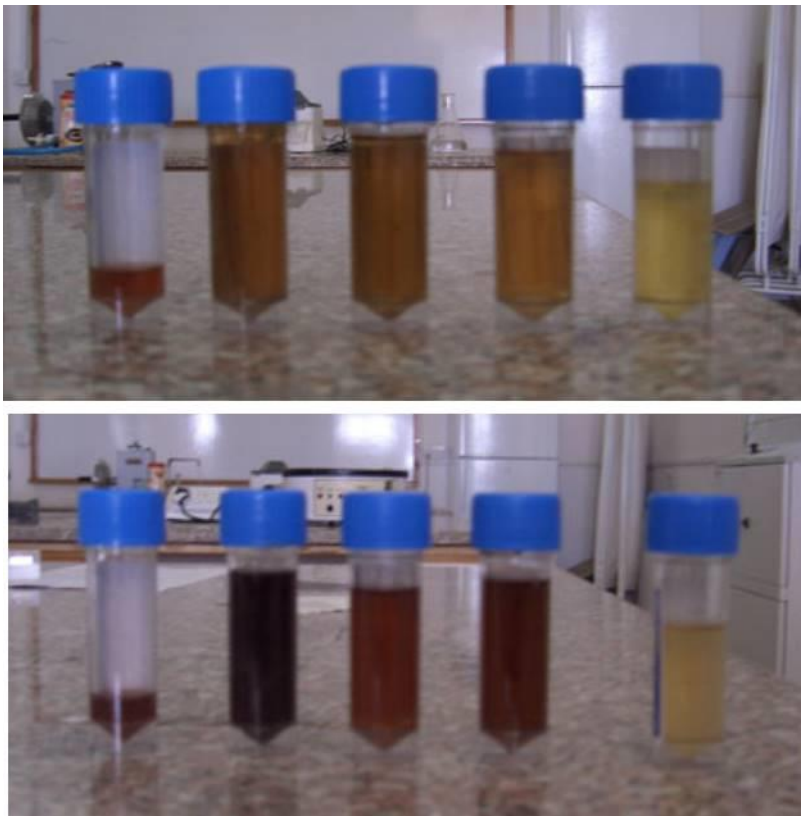
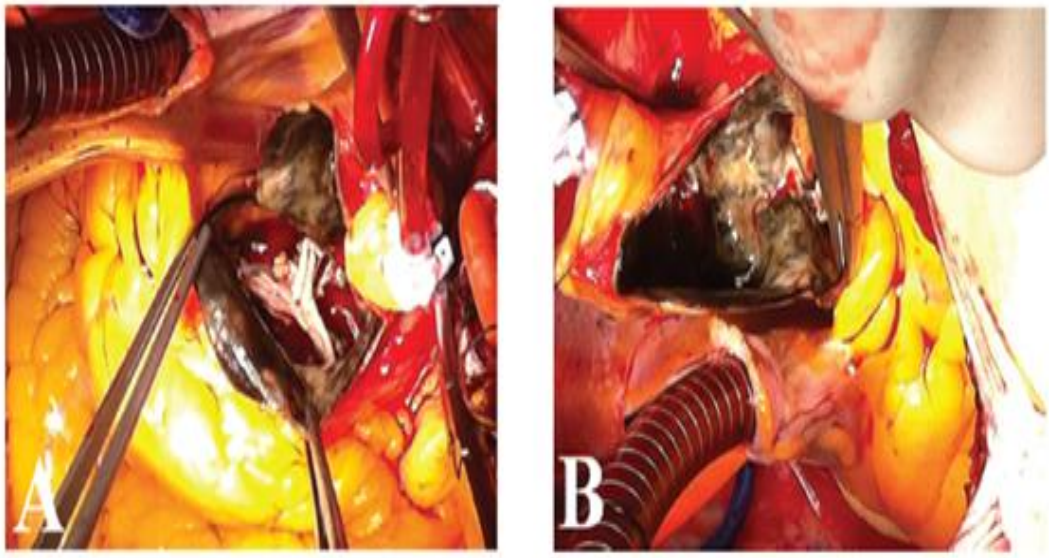


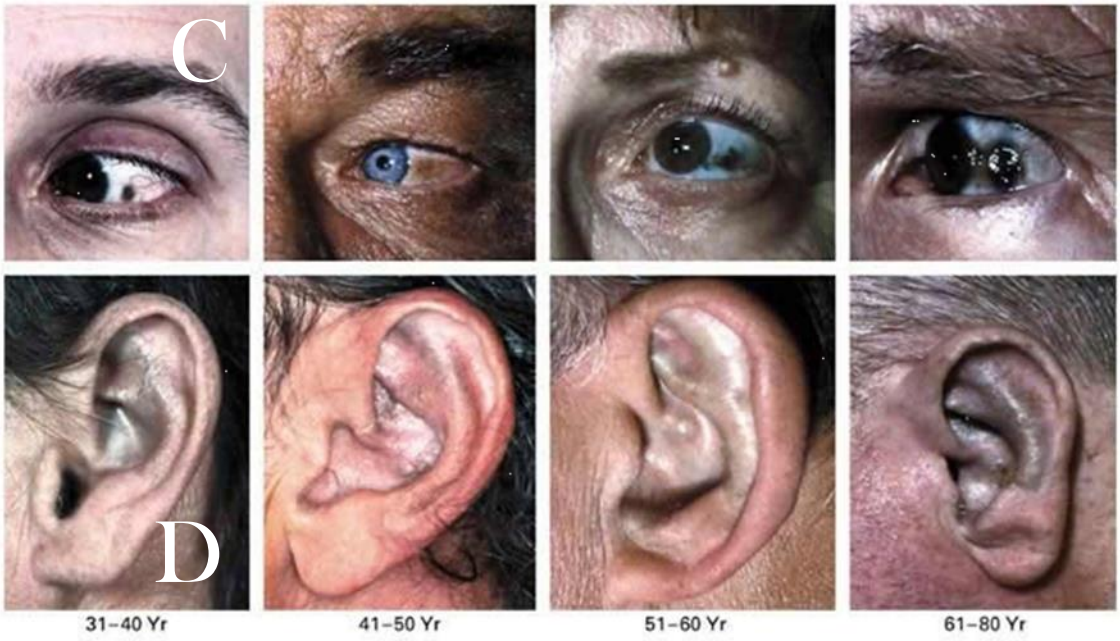
Figure 1.5: Fresh urine samples from four AKU patients and one healthy individual.

The top picture depicts the urine at the time of sampling and the bottom picture, 48hr standing at room temperature. The urine on the right of the picture is that of the healthy individual**.**

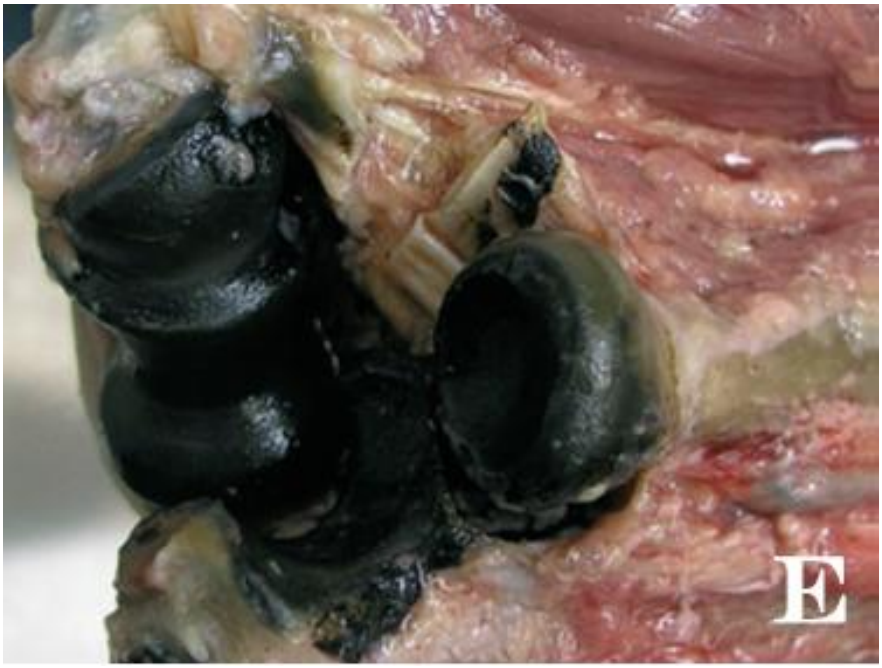
Figure 1.6: Examples of the debilitating physical effects of AKU on various patients



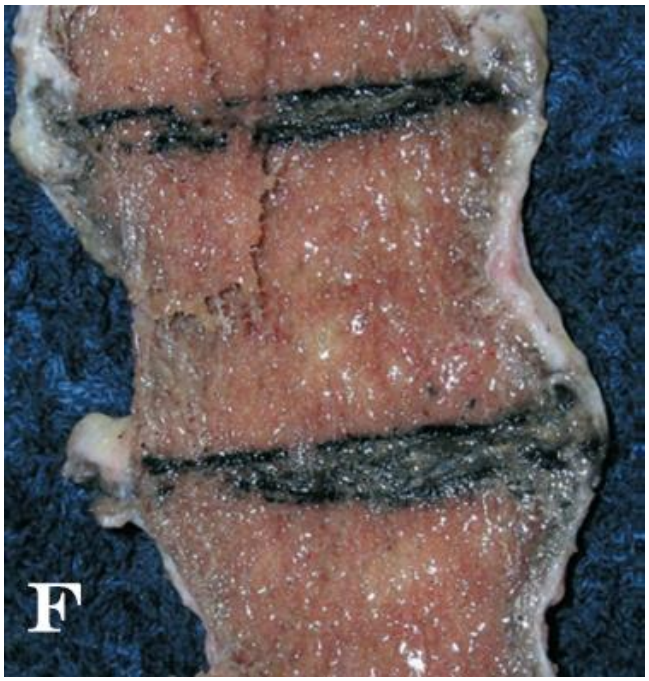
Black aortic wall and calcification [**A** and **B**], (Capuano et al., 2014)



Ochronotic pigmentation of sclerae and ear cartilage at various ages. [**C** and **D**], (Phornphutkul et al., 2002).



Generalized black pigmentation of the articular cartilages of elbow joint, [**E**], (Helliwell et al., 2008).



Black pigmentation of intervertebral discs with bony bridges at the edges of the discs, [**F**], (Helliwell et al., 2008).

Capuano et al., (2014) reported a case of an AKU patient with Heyde's syndrome (a condition of aortic stenosis, linked in with gastrointestinal bleeding and impaired haemostasis) who presented with an aortic valve that on surgery was severely calcified with ochronotic patches (figure 1.6).

Ochronotic osteoarthropathy is progressive, degenerative and presents itself initially in the 4th decade of life as lower back pain with load bearing joints such as knees, hips and spine all significantly affected by this disorder. The consequence of this is significant pain for the patient but also limited mobility and as stated by Mueller et al., (1965), no satisfactory specific treatment. However as surgical techniques improved then severely affected patients with total joint failure required arthroplasty around age 50 years in more than 50% of patients (Ranganath et al., 2013).

AKU disease progression and severity increases into the 5th decade of life onwards, likely as a consequence of age-related impairment of renal function having the knock-on effect of exposing organs and bone to continuously increasing concentrations of HGA, (Phornphutkul et al., 2002, Ranganath et al., 2020b). Renal failure markedly accelerates ochronosis and morbidity in AKU patients (Introne et al., 2002, Davison et al., 2016).

An autopsy described by Helliwell et al., (2008) confirmed the multisystemic nature of AKU but noted pigmentation present in cartilaginous and non-cartilaginous tissue if it had undergone stress or been damaged. The most damage still remained in the high impact regions of greatest stress such as the joints and vertebrae discs. However, other regions suffered pigmentation based on the type of system activity involved. For instance, tendons and ligaments suffer from tensile stresses; arterial, aortic and bronchial regions suffer from expansive and contraction pressure stress; ear cartilage and other peripheral extremities would more likely suffer from sleep or everyday activity stresses whereas the eye sclera pigmentation as a consequence of muscular contractions and pull on the eyeball during ocular movements. Furthermore, UV radiation damage of the conjunctiva and sclera also leaves these areas vulnerable to pigmental damage. The overall theory here, being

that HGA will deposit itself, and hence effect ochronosis in areas that have been damaged or most vulnerable to damage, but not indiscriminately everywhere.

This long-term ochronotic damage leads to more severe and progressive conditions such as osteopenia, spondylosis, fractures, spinal cord compression linked to nerve and muscle damage, ligament and tendon rupture, stenosis of the aortic valve requiring replacement, hearing loss from inner ear damage and glaucoma or astigmatism severely affecting eyesight (Phornphutkul et al., 2002, Helliwell et al., 2008, Cox and Ranganath, 2011; Mistry et al., 2013). Therefore, the progression of untreated AKU results in a varying array of unpleasant consequences for the unfortunate patient as summarised in table 1.1.

The various manifestations of AKU have been grouped and scored based on a Severity Score Index called an AKUSSI (Cox et al., 2011; Ranganath et al., 2011; Ranganath et al., 2013, Ranganath et al., 2015). The patients recruited underwent questionnaire analysis, clinical examinations, extensive imaging and medical photography. Scoring took into account the physical, medical, and mental manifestations of AKU and attempted for the first time to try and accurately trace the progression and predict the outcome (figure 1.7). Ranganath et al., (2015) categorised AKU into phases (table 1.1).

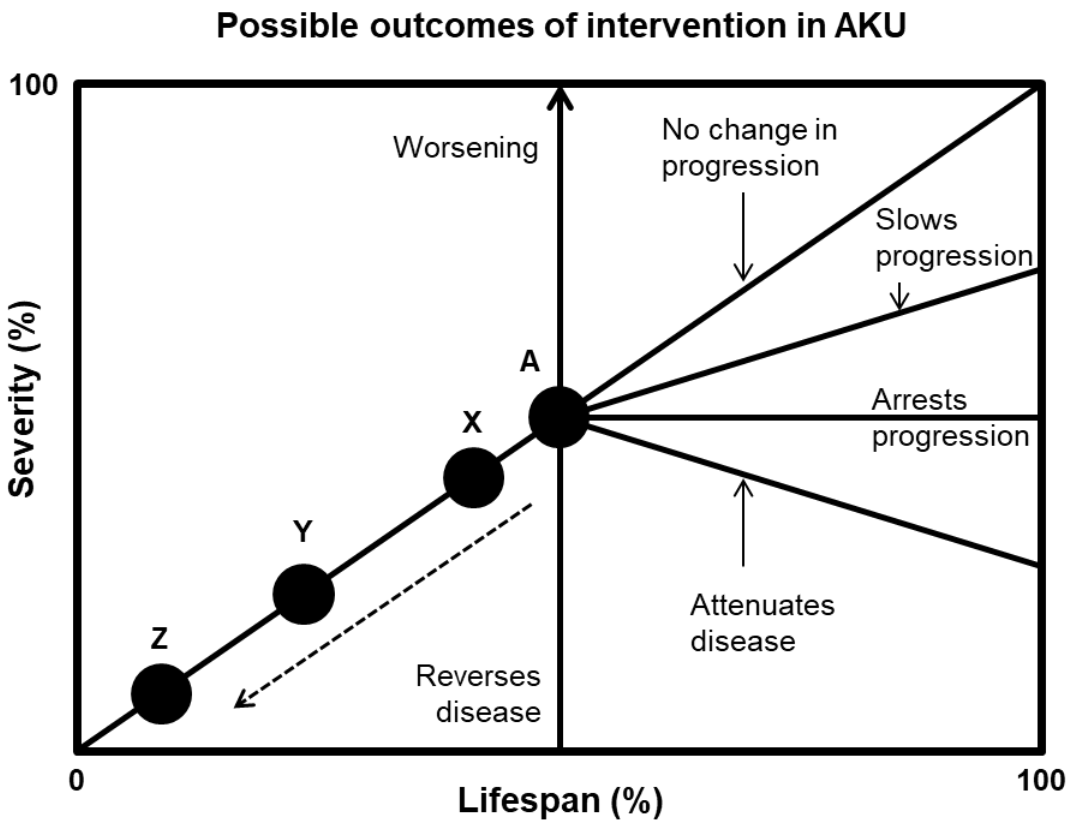


Figure 1.7: Possible outcomes of intervention in AKU. (Ranganath et al., 2015).

Ochronosis is assumed to progress continuously over a life time (solid diagonal line) with potential points of intervention at A, X, Y or Z. If intervention is started at A, then worsening, no change, slowing, or arresting of the disease is shown from point A assuming irreversibility of disease. If AKU proves to be reversible, (considered unlikely), the lines from point A also show potential decrease in disease or full reversal. If AKU is irreversible, intervention at earlier points X, Y or Z should lead to less residual disease.

Decades of life	Symptoms / features
1st	No obvious symptoms apart from excess HGA excretion.
2nd	
3rd	Ear pigmentation. Joint issues emerging. Onset appears a decade later in females.
4th	Ochronotic progression and joint issues increasing in severity with replacement often a consideration. Pain management required.
5th	
6th onwards	Other issues around major organs starting to manifest themselves. Joint replacement and life-saving cardiac surgery necessary.

Table 1.1: Summary of AKU progression.

As AKU is an autosomal recessive condition, this should suggest an equal distribution of affected males and females as they are both born with the condition, and excrete elevated concentrations of HGA from birth. However, an observation is that ochronosis appears earlier in males (around 30 years old) whereas in females it appears a decade later, initially showing slower progression but showing a steep rise after the age of 60 (Ranganath et al., 2011). There is a possibility that this could be hormonally related, implying that non-genetic factors may also influence the manifestations and development of the disease; only a larger clinical trial will aid in understanding the impact of environmental and external influences (Ranganath et al., 2015).

Therefore, it seems ochronosis is irreversible, but slowing or halting its progress certainly is an option and a consideration with treatment progression. Table 1.2 sums up the common long-term damaging effects of AKU on tissues and organs.

Tissue	Damaging factor	Long-term effect
Spinal intervertebral discs	Weight-bearing stress and movement	Ochronotic damage and significant pain, cord compression and loss of mobility
Joints	Weight-bearing stress and movement	Ochronotic damage and significant pain, loss of mobility
Tendons and ligaments	Tensile stresses	Ochronotic damage and rupture, e.g. achilles tendon
Aortic valve and root	Systemic blood pressure, expansile stress	Calcification, aortic stenosis, cardiac arrest
Bronchial cartilage	Expansion and contraction	Severe breathing difficulties
Ear cartilage	Pressure during sleep	Ear pain; ochronotic damage to inner ear and hearing damage
Sclera and conjunctiva	Expansion and contraction, UV light damage	Ochronotic damage, astigmatism, glaucoma and cataract formation

Table 1.2: Summary of damaging factors in AKU

1.4 Animal models

AKU is not limited to the human condition, it has been reported in many animals ranging from apes to dogs, horses, cattle and rabbits. Darkened urine was noted but not so, ochronosis or joint issues (Mistry, 2013).

The first murine (rodent) model was generated by Montagutelli et al., (1994) where they mapped the location of the HGD mutation to chromosome 16. Early reports stated there was no evidence of ochronosis or arthropathy, however Taylor et al., (2012) produced evidence of ochronosis in tissues and joints of mice with the AKU genotype, similar to that seen in humans. Although a similar progression of elevated but constant HGA levels and ochronotic development was observed, joint issues synonymous with humans were not, probably due to faster cellular turnover that clears HGA-adducted proteins before polymerization combined with shorter rodent life-span. Also, AKU mice treated with nitisinone (NTBC) showed an 88%

reduction in plasma HGA concentration confirming its effectiveness in slowing and halting the process of ochronosis (Preston et al., 2014, Keenan et al 2015) with reduction in pigmentation in chondrocytes. This murine work has provided valuable information in terms of pathology and treatment response and paved the way for the introduction of human clinical trials and the off-label use of the drug NTBC.

1.5 Existing treatments for AKU

When this PhD began, there was no licenced approved drug for the treatment of AKU and therefore palliative care (relieving pain without dealing with the cause of the condition) was the only option. With the contribution of the data presented in this thesis and the work of the DevelopAKUre consortium, by completion, the drug NTBC has been granted a licence.

Phornphutkul et al*.,* (2002) and Arnoux et al., (2015), noted that vitamin C (ascorbic acid) anti-oxidant therapy used to prevent the conversion of HGA to BQA and subsequently the ochronotic pigment, had limited success. A low protein diet which would reduce the loading capacity on the TYR pathway, strangely, again had no noticeable effect on the progression of ochronosis. However, a low protein diet from very early age could delay the ochronotic effects and reduce their severity but maintaining a strict diet throughout life poses its own challenges, most notably through the nutritional avenue. As a consequence of elevated HGA levels, stone formation is also common in AKU. Therefore, patients are encouraged to maintain a good state of hydration and thus minimise the formation of renal stones which can lead to renal impairment, damage and painful removal, especially noticeable in later life as age related renal impairment becomes an issue (Ranganath et al., 2020b).

Physiotherapy can be beneficial, but for long-term outside the NHS, it is expensive so patients attending the National AKU Centre (NAC) are provided with pain control options including transcutaneous electrical nerve stimulation, acupuncture and neuromuscular blocking drugs which can also provide long-term relief in AKU.

The final stage is inevitable surgery such as joint replacement, particularly knee, hip, and shoulder (Introne and Gahl 2003), spinal decompression surgery may also be needed when spinal compression complicates, and can return the patients mobility (Akeda et al., 2008).

Ochronotic pigment deposition within the connective tissues acts as a trigger for dystrophic calcification of the heart valves and within the coronary artery itself. Post mortem studies of individuals with AKU revealed pigmented calcified valvular cusps and obstruction of coronary arteries with pigmented deposits. Pettit et al., (2011) stated aortic valve disease was seen from the fourth decade and was more common with advancing age with aortic valve thickening and calcification seen in 50% of individuals older than 55 years. Phornphutkul et al., (2002) described cardiovascular involvement from a mean age of 54 years. Early identification of aortic valve disease and regular clinical assessment would delay surgical intervention and the prescription of angiotensin converting enzyme inhibitors may serve to modify disease progression and Pettit et al., (2011) further suggested that patients with AKU above the age of 40 years should undergo routine echocardiographic surveillance.

1.6 The hereditary tyrosinaemias

Normal plasma TYR concentrations in health are 30 to 120 µmol/L with values >200 µmol/L considered elevated. However, clinical manifestations of hypertyrosinaemia typically do not become apparent until plasma levels exceed 500 µmol/L.

Hereditary tyrosinaemia type 1 (HT-1) is another rare autosomal recessive inherited disorder of the TYR metabolic pathway (figure 1.4). The defective enzyme in this case is fumarylacetoacetate hydrolase which causes an accumulation of toxic metabolites downstream of HGA in the pathway. Elevated levels of fumaryl and maleyl-acetoacetic acid results in hepatorenal damage and an increase in succinylacetoacetate and its derivatives which leads to porphyria-like neurological issues. The symptoms are present from

birth and include diarrhoea, jaundice, cabbage-like odour, nosebleeds and a failure to thrive, further leading to hepatorenal failure, bone weakening, nervous system trauma and an increased risk of liver carcinoma, with death occurring in 90% of all cases before the age of 12 when only treated with dietary restrictions (Russo et al., 2001).

Treatment needs to be started as soon as the condition is diagnosed which includes a diet restricted in TYR and phenylalanine (PHE) along with the licenced drug nitisinone (2-[2-nitro-4-trifluoromethylbenzoyl]-cyclohexane-1,3-dione) (NTBC). This drug inhibits the enzyme HPPD and effectively halts the debilitating consequences of this disorder and is now the mainline treatment for this disorder (Holme and Lindstedt 2000). McKiernan (2006) stated that a dose of 1 mg/kg body weight of needs to be administered, taking the average weight of a newborn as 3.5 kg.

The deficient enzyme in hereditary tyrosinaemia type 2 (HT-2) is tyrosine aminotransferase, which is responsible for converting TYR to 4-hydroxyphenylpyruvic acid (HPPA). Symptoms present from birth and include photophobia, painful skin lesions on the palms and soles, with about 50% of individuals suffering intellectual disability. Treatment, similar to HT-1 needs to be started as soon as the condition is diagnosed which includes a diet restricted in TYR and PHE.

Hereditary tyrosinaemia type 3 (HT-3) is a consequence of the inherited deficiency of the enzyme 4-hydroxyphenylpyruvate dioxygenase (HPPD). Controlled from diagnosis with a low TYR diet, the symptoms are less severe than the other two tyrosinaemias but can still result in intellectual disability and seizures.

1.7 Nitisinone

Returning to the subject of AKU, and due to its promising effect in the inhibition of HPPD enzyme in the treatment of HT-1, NTBC was considered as a potential treatment for AKU.

NTBC is a triketone herbicide derived from leptospermone produced by the bottlebrush plant (figure 1.4). It was first discovered by Zeneca Agrochemicals in 1982 (Michaeley and Kratz 1986). Treating plants with triketone herbicides causes bleaching symptomatology due to reduced chlorophyll and carotenoid synthesis and prevents the growth of competing plants around. NTBC, despite belonging to this wide family, revealed a novel mechanism of action by inhibiting the enzyme HPPD which catalyzes the conversion of HPPA to HGA as part of the TYR degradation pathway (Aktuglu-Zeybek and Zubarioglu, 2017).

Lock et al., (2014) stated that during early toxicology testing in rats and dogs, prior to its use in the treatment of HT-1, NTBC was not acutely toxic following single large oral doses, (>1 mg/kg/day) however low dietary doses of <1 mg/kg/day resulted in eye lesions (keratopathy), which recovered on withdrawal of the drug. Lock (2017) covered extensively the discovery, mechanisms and uses for NTBC as well as discussing the work and contributions from Lindstedt and Holme within this field.

In effect, NTBC is blocking the enzyme that is deficient from birth in patients with HT-3 (HPPD), which left unchecked resulted in high concentrations of TYR accumulating and saturating in the eyes, leading to crystallisation and cellular damage. On the plus side, it was preventing the accumulation of toxic metabolites fumarylacetoacetate, maleylacetoacetate and succinylacetone seen in HT-1 by effective intervention blockage within the TYR metabolic pathway.

With this information at hand, the Zeneca group approached Sven Lindstedt at Gothenburg University, Sweden, who had isolated and purified HPPD from human liver and was looking for a suitable drug for the inhibition of HPPD and therefore a treatment for HT-1. After much deliberation in 1991 a seriously ill two-month old child was treated for HT-1 with NTBC with almost immediate dramatic effects, decreasing the toxic metabolites to undetectable concetrations within 12hr. Four further patients were treated with NTBC with similar success (Lindstedt et al., 1992).

In May 1995, NTBC (Ordafin) was designated an Orphan drug by the US Office for Orphan Product Development. An Orphan drug is defined as one that is intended for the treatment, prevention or diagnosis of a rare disease or condition which affects less than 200,000 people and is thus not marketed under standard regimens. NTBC received approval from the US FDA in 2002 and the EU Drug Agency in 2005, for the treatment of HT-1 and is marketed as Orfadin® by Swedish Orphan Biovitrum (SOBI), Stockholm, Sweden who had the worldwide rights to this drug.

Anikster et al., (1998) suggested NTBC as a potential treatment for AKU in 1998 and in the earliest study by Phornphutkul et al., in 2002, out of a total of 58 patients suffering with varying degrees of AKU severity two women in their 50's were given NTBC doses; one receiving 0.70 mg daily dose of NTBC for 7 days increased to 2.8 mg for a further three days and the other patient stayed on a daily dose of 0.7 mg for the full 9 days. This dose is significantly lower than previously mentioned, administered in HT-1 patients.

Urine HGA, and plasma TYR concentrations were measured in all patients pre- and post-treatment and showed significant decreases and increases respectively. Both patients remained asymptomatic throughout and following cessation of treatment, TYR levels returned to normal after two-three weeks. Therefore, this study concluded the effectiveness of NTBC on suppression of HGA levels in AKU but also concluded no correlation between the particular genetic mutation and level of HGA excretion. Furthermore, high dose ascorbic acid treatment proved ineffective on the urinary HGA concentrations of treated and untreated AKU patients, a theme that was also noted in much earlier studies by Sealock et al., (1940) and Wolff et al., (1989).

Further studies by Suwannarat et al., *(*2005) and Introne et al., (2011) using daily doses of 2 mg NTBC showed over a 95% decrease in urinary HGA. A protein restricted diet resulted in further decrease of HGA concentrations, however care was required not to decrease protein consumption to such a level (<20 g/day) that symptoms are exacerbated and muscle mass is compromised.

1.8 Measurement of the primary metabolites in AKU

Many papers have noted elevated levels of HGA (La Du, 1958; Phornphutkul et al., 2002; Suwannarat et al., 2005; Introne et al., 2011; Ranganath et al., 2016; Milan et al., 2017; Sloboda et al., 2019). With efficient renal clearance, a mean urine HGA excretion of 20,557 µmol/24hr was detected with a range from 18,446–22,669 µmol/24hr. In circulation, a mean value in serum of 30 µmol/L was noted with a range 11.9–75.2 µmol/L compared with <3.1 µmol/L in healthy subjects (Davison et al., 2015; Milan et al., 2017).

A recent review (Davison et al., 2020) covered a selection of significant methods for the detection and quantitation of HGA but most methods however, either measured serum or urine but not both (table 1.3). Early methods were dependent on the technology available at the time, tended to use noxious or highly toxic chemicals interspaced with laborious analytical processes (Zoutendam et al., 1976). Although sensitive down to the 1-10 µmol/L range, long run-times also rendered these methods inadequate for routine analytical use (Lustberg et al., 1971).

Akesson et al., (1987) and Bory et al., (1990) improved matters using high performance liquid chromatography (HPLC) and UV detection, but it wasn't until Hughes et al., (2014, 2015) where mass spectrometry became the important tool not only for rapid efficient measurement of HGA but also the other prominent compounds in AKU and the TYR degradation pathway, (table 1.3 and chapters 2, 3 and 4).

In the healthy population, HGA and TYR levels were investigated to achieve a normal reference range (Davison et al., 2015). A cross section of 11 males and 11 females, age range 25-63 were measured for serum and urine TYR and HGA using the methods previously described (Hughes et al., 2014 and 2015). HGA both in serum and urine were below the LLOQ of approximately 3 µmol/L. A later more sensitive method developed by Hughes et al., (in preparation for 2022, chapter 5) was able to measure HGA down to a concentration of 0.1 µmol/L in serum and showed concentrations in non-AKU individuals at ≤0.1 µmol/L. This provided a more up-to-date and accurate

representation of the concentrations of HGA that are present in non-AKU individuals and enabled a target for potential treatment options.

However, other studies (Lustberg et al., 1971, Suwannarat et al., 2005, Introne et al., 2011) reported detectably significant levels of urine HGA in normal individuals ranging from 59-779 µmol/24hr, inconsistent with Davison et al., 2015. This is most likely to be method related as liquid chromatography mass spectrometry (LC-MS/MS) is the gold-standard method and is validated to stringent guidelines whereas indirect colorimetric or electrophoresis methods are prone to interferences and perhaps less robust validation. Serum TYR concentrations ranged from 30–87 µmol/L, which were not too dissimilar to other studies (Suwannarat et al., 2005, Introne et al., 2011).

With significant interest gathering in not only the investigation of HGA, but also measurement of TYR and the drug NTBC, there was a need to be able to measure these compounds and monitor not only disease progression but to see if anything could be done to curtail the debilitating effects of AKU.

1.9 The AKU Society, DevelopAKUre and the National AKU Centre

The AKU society was set up in 2003 as a response to many dialogues between Robert Gregory (a long-term AKU patient) and Professor Lakshminarayan Ranganath. It was initially devised as a support base where UK patients could receive information and discuss their condition. As more global research was being conducted to better understand this disorder, the National AKU Centre (NAC) was established at the Royal Liverpool University Hospital in 2012.

Since 2012, AKU patients have been treated routinely using NTBC off-label at the NAC in the Royal Liverpool University Hospital. Funded by NHS England, this specialised service assisted through the AKU society, patients with confirmed AKU are administered 2 mg NTBC on alternate days for the first three months, which is then increased to 2 mg daily thereafter. Assessments are repeated on an annual basis to monitor response to

therapy (Milan et al., 2017, chapters 7 and 8). The NAC is supported by a multidisciplinary team of healthcare professionals to ensure the efficacy and safety of NTBC is monitored and the patients have regular contact, communication and assessment.

Research was expanded and DevelopAKUre (a European consortium) was established to investigate the use of NTBC for the treatment of AKU on a larger scale in order to obtain a licence. Duration of DevelopAKUre was 75 months, from 1st November 2012 to 28th February 2019. The first clinical trial undertaken by the DevelopAKUre consortium, funded by the European Commission as part of the Framework Programme 7 (FP-7) was entitled SONIA-1 (Suitability Of Nitisinone In Alkaptonuria-1). This was a dose ranging study where patients were administered 0, 1, 2, 4 or 8 mg NTBC over a 4 week period, with regular serum and urine samples taken. The conclusion was 8 mg NTBC proved to be the most effective at reducing HGA concentrations, with comparable safety (Ranganath et al., 2016, Chapter 9).

From the success of SONIA-1, a longer-term trial was formulated where the patients were administered 10 mg NTBC over a 4-year period (SONIA-2), leading to a submission to the European Medicines Agency in February 2020 to approve the use of NTBC in AKU (Ranganath et al., 2020a).

The main partners in DevelopAKUre were the Royal Liverpool University Hospital, the University of Liverpool, the AKU Society (UK) and ALCAP (France), who deal with patient groups for communications/dissemination, and help with patient recruitment; Biomarker analysis was undertaken at Liverpool, Nordic Biosciences (Denmark) and Siena (Italy). PSR (Netherlands) dealt with clinical trial coordination and Cudos (Netherlands) for medical monitoring, with the mid-sized pharma company Sobi (Swedish Orphan Biovitrum International) supplying the drug NTBC and regulatory advice.

Three universities, (Liverpool, Siena and the Slovak Institute of Molecular Physiology and Genetics) provided the analysis and interpretation of data, along with three clinical trial centres (Liverpool, Paris and Piestany). This project could only have been achieved through a Europe-wide collaboration, allowing recruitment of enough patients for an adequately powered trial and providing access to the elite among AKU researchers (https://akusociety.org/).

1.10 The plan comes together

Pre-treatment, AKU patients only have elevated HGA levels, and their TYR is normal like in non-AKU controls. When NTBC was administered, to the patients at the NAC (2 mg/day), as expected they showed significantly decreased urinary (>94%) and serum (>83%) HGA levels after 2 years (Milan et al., 2017, chapter 7) and as a consequence the cessation of further ochronotic events (Ranganath et al., 2018). However, the consequential significant rise in TYR levels did manifest itself as asymptomatic TYR keratopathy with the recommendation that AKU patients taking low dose NTBC should maintain a low protein diet of <1 g/kg body weight (Khedr et al., 2018).

However, due to the enzyme that NTBC is blocking, the patient is effectively being treated for AKU by inducing HT-3 with the resultant hypertyrosinaemia effects. These effects have been reported in many papers, including those previously mentioned, but regardless of the dose of NTBC given whether it be for HT-1 or AKU, the TYR increase is comparable as noted in table 1.4.

This effect was highlighted during the SONIA-1 trial (Ranganath et al., 2016) whilst noting the dose-dependent significant decrease in HGA concentration over varying doses of NTBC, TYR serum elevation was not dose-dependent and up to a 10-fold increase was observed, which was also noted in the earlier publication by Introne et al., (2011).

The SONIA-2 trial, where the AKU patients were administered 10 mg doses of NTBC over a 4-year period, demonstrated similar findings with decreased

urinary (>99.7%) and serum (>98.8%) HGA levels after 12 months and remaining over 95% at the end of the trial (Ranganath et al., 2020a).

The SOFIA (Sub-ochronotic features in AKU) study in young adults provided information on the age at which it might be most beneficial to begin treatment. In this study, 32 patients with AKU were recruited, two males and two females in each set of age groups ranging from 16-50 to investigate the degree of pigmentation with increasing age. The study concluded that biopsy ear pigmentation was discovered in a 20-year old and eye ochronosis shown in a 22-year old therefore suggesting early onset introduction of NTBC would be beneficial (Cox et al., 2019).

Considering the effects of hypertyrosinaemia on the central nervous system, more studies need to be undertaken to fully understand the knock-on effects as there is no doubting the symptoms observed in untreated infants suffering from HT-1, HT-2 and HT-3 but conversely, studies by Davison et al., (2019a) did not find any changes to monoamine neurotransmitters in brain tissue from NTBC-treated mice.

The future may lie in the realm of gene or enzyme replacement therapy to correct for the inborn error directly, but this is some way off yet and would most likely require a pill that would have to initially evade the destructive environments of the upper gastrointestinal tract before releasing its undenatured enzyme contents for absorption in the lower gastrointestinal tract.

Enzyme replacement would be an ideal therapy for AKU, consisting of immediate replacement of HGD in the TYR degradation pathway. However, there are potentially fatal complications associated with this therapy. It is imperative that the HGD enzyme is delivered to the exact location of TYR metabolism within the hepatocytes of the liver. If not, spontaneous formation of succinylacetone (formed from the production of maleylacetoacetic acid and fumarylacetoacetic acid in TYR metabolism) would occur, which is toxic and highly mutagenic (Mistry et al., 2013).

However, the progress that has been made in a relatively short time, with international collaboration has given hope to many patients worldwide not only in AKU but in any inborn error of metabolic disorder. One major leap forward is the ability to detect not only the metabolites of interest and the treating drug but also to be able to do this in a cheap, efficient and rapid way. The following sections deal with the processes and equipment involved in such feats and how they were adapted to perform the required tasks.

Table 1.3: Summary of targeted analytical methodologies reported for the measurement of HGA, TYR and NTBC in patients with AKU (Davison, et al., 2020).

Sample preparation	Analytical technique	Metabolites measured	Matrix	Analytical measuring range	Inter-assay CV (%)	Reference
Acidification with 5N sulphuric acid	LC-MS/MS using a 10 cm x 3.0 mm, C18 3 µm column. Gradient: 0.1%. FA in MeOH. RT 7 min	TYR, HGA, PHE IS: d_4-TYR, $^{13}C_6$-HGA, d_5-PHE	Urine*	TYR: 10-2000 µmol/L HGA: 2-4000 µmol/L PHE: 10-500 µmol/L	Not stated	Taylor et al., 2018
Sample dilution	Capillary electrophoresis with UV-Visible detection at 190 nm	HGA	Urine	0.02-0.16 mmol/L LOD 3.33 µmol/L	Not stated	Oztekin et al., 2018
30 µL urine on filter paper eluted with phosphate buffer	HPLC using a 7.5 cm x 4.6 mm C18 3 µm column. Gradient: phosphate buffer to MeOH. RT 18 min. Detection at 260-280 nm	HGA	Urine	20-800 µmol/L LOQ 5 µmol/L	Not stated	Jacomelli et al., 2017
Deproteinisation with perchloric acid	LC-MS/MS using a 10 cm x 3.0 mm C18 3 µm column. Gradient: 0.1 %. FA in MeOH. RT 7 min	TYR, HGA, NTBC IS: d_2-TYR, $^{13}C_6$-HGA, $^{13}C_6$-NTBC	Serum	TYR: 10-500 µmol/L HGA: 3-2000 µmol/L NTBC: 0.2-10 µmol/L	<5 <8 <10	Hughes et al., 2015 (Chapter 3)

Sample preparation	Analytical technique	Metabolites measured	Matrix	Analytical measuring range	Inter-assay CV (%)	Reference
Acidification with 5N sulphuric acid	LC-MS/MS using a 10 cm x 3.0 mm C18 3 µm column. Gradient 0.1 %. FA in MeOH. RT 7 min	TYR, HGA, IS: d_2-TYR, $^{13}C_6$-HGA	Urine	TYR: 20-4000 µmol/L HGA: 20-16,000 µmol/L	<10 <5	Hughes et al., 2014 (Chapter 2)
Deproteinisation with ACN and IS	LC-MS/MS using a 5 cm x 2.1 mm C18 1.7 µm column. Gradient: ammonium acetate in MeOH. RT 4 min	NTBC IS: 2-Nitro-4-(trifluoromethly) benzoic acid	Plasma	0.75-150 µmol/L LOD 0.15 µmol/L	<20	Davit-Spraul et al., 2012
Plasma - precipitation with ACN. Blood spot - elution with MeOH.	LC-MS/MS using a 15 cm x 4.6 mm C8 5 µm column. RT 12 min	NTBC IS: Mesotrione	Plasma Blood spots	Plasma: 0-100 µmol/L Blood spots: 0-50 µmol/L LOD 0.18 µmol/L	2.8-7.3 3.3-11.7	Prieto et al., 2011
Deproteinisation with ACN	Capillary electrophoresis with diode array detection at 278 nm	NTBC	Serum	25-200 µmol/L LOQ 10.6 µmol/L LOD 3.17 µmol/L	<2.43	Cansever et al., 2010
Deproteinisation with ACN	LC-MS/MS using a 15 cm x 2.0 mm C18 3 µm column. Gradient: 0.1 %. FA/0.01 % TFA in ACN. RT 7 min	NTBC IS: Mesotrion	Serum	2.5-40 µmol/L LOQ 0.35 µmol/L LOD 0.1 µmol/L	2.8-13.6	Herebian et al., 2009

Sample preparation	**Analytical technique**	**Metabolites measured**	**Matrix**	**Analytical measuring range**	**Inter-assay CV (%)**	**Reference**
Acidification with 1M HCl	Infra-red spectrometry	HGA	Urine	0-60 mmol/L	Not stated	Markus et al.,2001
Dilution with phosphate buffer pH2	HPLC using a 15 cm x 3 mm basic 3 µm column. Gradient: isocratic 50% ACN. Diode array detection at 278 nm	NTBC	Plasma	0.3-68 µmol/L	3.0-13.5	Bielenstein et al., 1999
Plasma - deproteinisation with perchloric acid. Urine - acidification with HCl	HPLC using a 10 cm x 4.6 mm, C18 3 µm column. Gradient: orthophosphoric acid in MeOH. RT 8 min. UV detection at 290 nm	HGA IS: 4-Amino-2 chlorobenzoic acid	Plasma Urine	Linear up to 0.15 mmol/L Linear up to 0.15 mmol/L LOD 3 pmol/L	<2.9 <3.5	Bory et al., 1990
Samples dried and derivatisation with TBDMS and ACN	Gas chromatography	HGA IS: 1,2-$^{13}C_2$ HGA	Plasma	5-10 ng/mL	Not stated	Deutsch et al., 1996
Unacidified urine diluted with 10mmol IS	1D NMR spectroscopy (89.99 MHz). RT 15 min	HGA IS: d_4-TSP	Urine	5-125 mmol/L	Not stated	Yamaguchi et al., 1986

Sample preparation	Analytical technique	Metabolites measured	Matrix	Analytical measuring range	Inter-assay CV (%)	Reference
Serum - deproteinisation with 50% TCA. Urine – acidification with acetic acid	HPLC using a 20 cm x 4 mm C18 5 µm column. Gradient: MeOH to 10 mmol acetic acid. RT 15 min. UV detection at 292 nm. Plasma and urine diluted in 10 mmol acetic acid with IS	HGA IS: 3,4-Dihydroxyphenyl-acetic acid	Plasma Urine	5.9-59 µmol/L 5.9-59 µmol/L LOD 2 ng/mL	<2.8	Akesson et al., 1987
Liquid – liquid extraction with ethyl acetate	TLC with HPLC electrochemical detection.	HGA	Serum Urine	10 ng/mL-100 µg/mL 20 µg/mL-4.8 mg/mL LOD 1 ng/mL	Not stated	Zoutendam et al., 1976
Serum - deproteinisation with 50%TCA. Urine – acidification with concentrated HCl.	High voltage electrophoresis. RT 70 min.	HGA	Serum Urine	Linear up to 15 µg/mL Linear up to 15 µg/mL	Not stated	Lustberg et al., 1971

***** Urine sample collected onto Mitra microsampling device, using method of Hughes et al., 2014.

TYR: tyrosine; HGA: homogentisic acid; PHE: phenylalanine; NTBC: nitisinone; IS: internal standard; MeOH: methanol; FA: formic acid; LOQ: limit of quantification; LOD: limit of detection; TSP: trimethylsilylpropanoic acid; RT: run-time; TBDMS: t-butyldimethylsilyl; TFA: trifluoroacetic acid; TCA: trichloroacetic acid; TLC: thin layered chromatography; ACN: acetonitrile; HPLC: high performance liquid chromatography; LC-MS/MS: liquid chromatography tandem mass spectrometry

Table 1.4: Key studies in the TYR metabolic pathway and the effects of NTBC in AKU (Davison, et al., 2020)

Trial design	n	Pre-NTBC concentrations	Dose	Duration	Overall results	Additional findings	Reference
Open, uncontrolled proof of concept. No dietary modifications	58*	[HGA]s 39.3±15.5 [HGA]u 2.4-73 [TYR]s 79±18	0.35-1.4 mg twice daily	9 and 10 days	73-95 % reduction in [HGA]u	Increased urinary HPPA 10-15 fold increase in [TYR]s	Phornphutkul et al., 2002
Open-label, uncontrolled, proof of concept. Reduced protein intake (last week only)	9	[HGA]s 10.2±6.2 [HGA]u 23.9±10.6 [TYR]s 68±18	0.35-1.05 mg twice daily	15 weeks (n=5)	94 % reduction in [HGA]u Serum HGA below LLOQ	[HGA]u reduced to ~97 % when protein restricted (<40 g/day) 10 fold increase in [TYR]s	Suwannarat et al., 2005
Randomised, parallel group, single blind	20	[HGA]s 18.7-62.4 (mean 34.1) [HGA]u 33.8±11.8 [TYR]s 60±13	No treatment, 2 mg daily	36 months	>95 % reduction in [HGA]u 95 % reduction in [HGA]s		Introne et al., 2011
Randomised, open label, parallel-group design SONIA-1 Clinical Trial	40	[HGA]u 14.4-69.5	No treatment, 1, 2, 4, 8 mg once daily	4 weeks	98.8 % reduction in [HGA]u (on 8 mg dose)	10-12 fold increase in [TYR]s from baseline	Ranganath et al., 2016

*Only 2/58 received NTBC. Note: All serum and urine HGA and TYR concentrations are expressed in µmol/L and mmol/24 h, respectively, and as mean ± SD where data were available otherwise a range is quoted.

Trial design	n	Pre-NTBC concentrations	Dose	Duration	Overall results	Additional findings	Reference
Off licence use of NTBC	28	[HGA]s 30.0±12.9 [HGA]u 20.6±5.5 [TYR]s 42±13.3	2 mg daily after 3 months (2 mg given every other day up to 3 months)	24 months	88.8 and 94.1 % reduction in [HGA]u at 3 months and 2 years. 83.2 % reduction in [HGA]s at 2 years	Serum NTBC 1.58±0.52 at 2 years	Milan et al., 2017
Off licence use of NTBC	3	Maximum [HGA]u 1211-27 624**	0.2 mg daily. Over duration of study this varied as 1 patient was pregnant and 1 patient was a child	36-60 months	Minimum [HGA]u 3-882, >90 % reduction	[TYR]s 305-464	Sloboda et al., 2019
SONIA-2 Clinical Trial, off licence randomised and no treatment-controlled parallel group study	138	[HGA]u 35.0±13.1 [HGA]s 30.4±11.0	10mg daily (n=69) or no treatment (n=69)	4 years	99.7% reduction in [HGA]u at 12 months 98.8% reduction in [HGA]s at 12 months	[TYR]s >500 in all on nitisinone Range 563-1530 at 12 months	Ranganath et al., 2020a

LOQ: lower limit of quantification; [HGA]s: serum HGA concentration; [HGA]u: urinary HGA concentration; [TYR]s: serum TYR concentration; [TYR]u: urinary TYR concentration.

1.11 Liquid chromatography tandem mass spectrometry (LC-MS/MS)

The technique of mass spectrometry (MS) has been around for over a century but has made most significant advancements over the last 50 years. For historical interest, table 1.5 shows the timeline of advancements in this field of work from its original inception by the renowned physicist, chemist, Nobel prize winner and discoverer of the electron, Joseph John Thomson.

In the field of LC-MS/MS, a lot of jargon and terminology is used which can be overwhelming to the novice. Murray et al., (2013) provides an alphabetical resume of many terms offering a reference baseplate for further exploration. The mass spectrometer is an instrument designed to separate and identify molecules based on their mass to charge ratios (*m/z*) and relative abundances. The choice of instrument for the purpose of this thesis was a triple quadrupole MS.

The process involves the conversion of the sample into gaseous ions, followed by a fragmentation step and detection. Since the motion and separation of ions is based on electrical and/or magnetic fields, it is the *m/z* ratio, and not only the mass, which is of importance. The analyser is operated under high vacuum, so that the ions can travel to the detector with a sufficient yield and not be hindered by obstructive molecules, where their signal is amplified.

Table 1.5: Historical developments in Mass Spectrometry

Investigator(s) and year	Developments
Thomson 1897–1911	First mass spectrometer
Dempster 1918	Electron ionization and magnetic focusing
Aston 1919	Atomic weights (isotopes) using MS
Stephens 1946	Time-of-flight mass analysis
Hipple, Sommer, and Thomas 1949	Ion cyclotron resonance
Johnson and Nier 1953	Double-focusing instruments
Paul and Steinwedel 1953	Quadrupole analyzers
Beynon 1956	High-resolution MS
Biemann, Cone, Webster, and Arsenault 1966	Peptide sequencing
Munson and Field 1966	Chemical ionization
Dole 1968	Electrospray Ionization (ESI)
Beckey 1969	Field desorption MS of organic molecules
Horning 1974	Atmospheric Pressure Chemical Ionisation (APCI)
MacFarlane and Torgerson 1974	Plasma desorption MS
Comisarow and Marshall 1974	Fourier-Transform Ion Cyclotron Resonance (FTIR) MS
Yost and Enke 1978	Triple quadrupole MS
Barber 1981	Fast atom bombardment
Vestel and Blakely 1983	Thermospray introduced
Tanaka, Karas, and Hillenkamp 1983	Matrix-assisted laser desorption/ionization
Fenn 1984	ESI on biomolecules
Paul 1989	Ion trap technique
Mann and Wilm 1991	MicroESI
Makarov 1999	Orbitrap
Siuzdak, Bothner, Fuerstenau,and Benner 1996–2001	Intact viral analysis
Takats 2004	Desorption ElectroSpray Ionisation
Balog 2013	iKnife

The LC-MS/MS consists of 3 major components:

1. **Ion Source:** where an ionised gas stream of the sample mix is produced
2. **Analyser:** separates ions based on their *m/z* ratio (quadrupole 1); fragments selected ions (quadrupole 2), and transports them to a third quadrupole for further selectivity.
3. **Detector:** measurement of the abundance of ions present in the sample, enhancement of signal and conversion to an electrical signal.

Other components of the LC-MS/MS system include:

- A vacuum pump
- A HPLC system
- Gas supply (nitrogen carrier gas and argon or high purity nitrogen collision gas)
- Computer software

The LC-MS/MS used for the purpose of this study was an Agilent 6490 of which a general overview is shown in figure 1.8.

The first step in the MS process is the formation of the ionic gas phase by the process of electron ionisation to form positive or negative charges. Neutral species can be analysed by a MS as long as they can be ionised either with the assistance of adducts or buffering of the mobile phases to create an ion rich environment.

Electrospray ionisation (ESI) involves three steps at the point of introduction of the sample mixture:

1. Creation and dispersal of a fine aerosol spray of charged droplets
2. Solvent evaporation
3. Ejection of ionic species into the MS

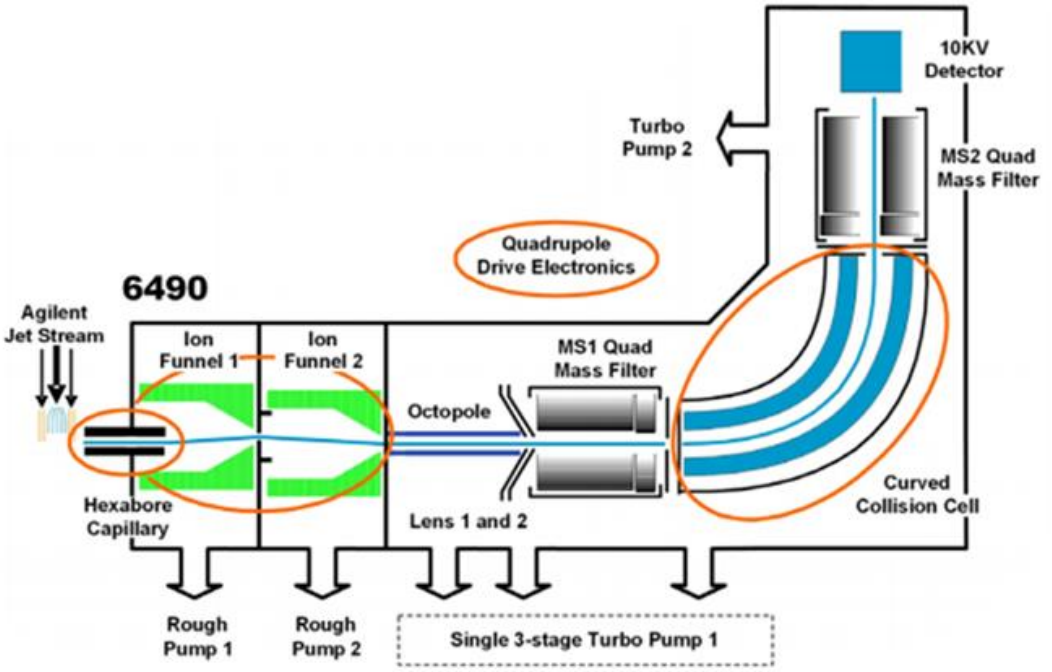


Figure 1.8: Overview of the Agilent 6490 Mass Spectrometer.
(Agilent 6400 Series Triple Quadrupole LC/MS system concepts guide, 2014).

A mist of highly charged droplets with the same polarity as the capillary voltage is generated. The majority of MS measurable compounds prefer the positive mode of ionisation, however acids prefer negative mode. The added advantage of using the Agilent 6490 was its ability to rapidly switch between modes enabling the simultaneous measurement of both ionic species within the same analytical run. The addition of the nebulising gas nitrogen maintains a constant sample flow rate, the source temperature is sufficiently high to prevent condensation of the droplets and an extra stream of nitrogen drying gas evaporates them to a small enough size whereby the ions at the droplet surface are ejected into the gaseous phase, reach the MS cone, a tapered region with an orifice used to guide the ions which are then accelerated into the MS for processing (figure 1.9).

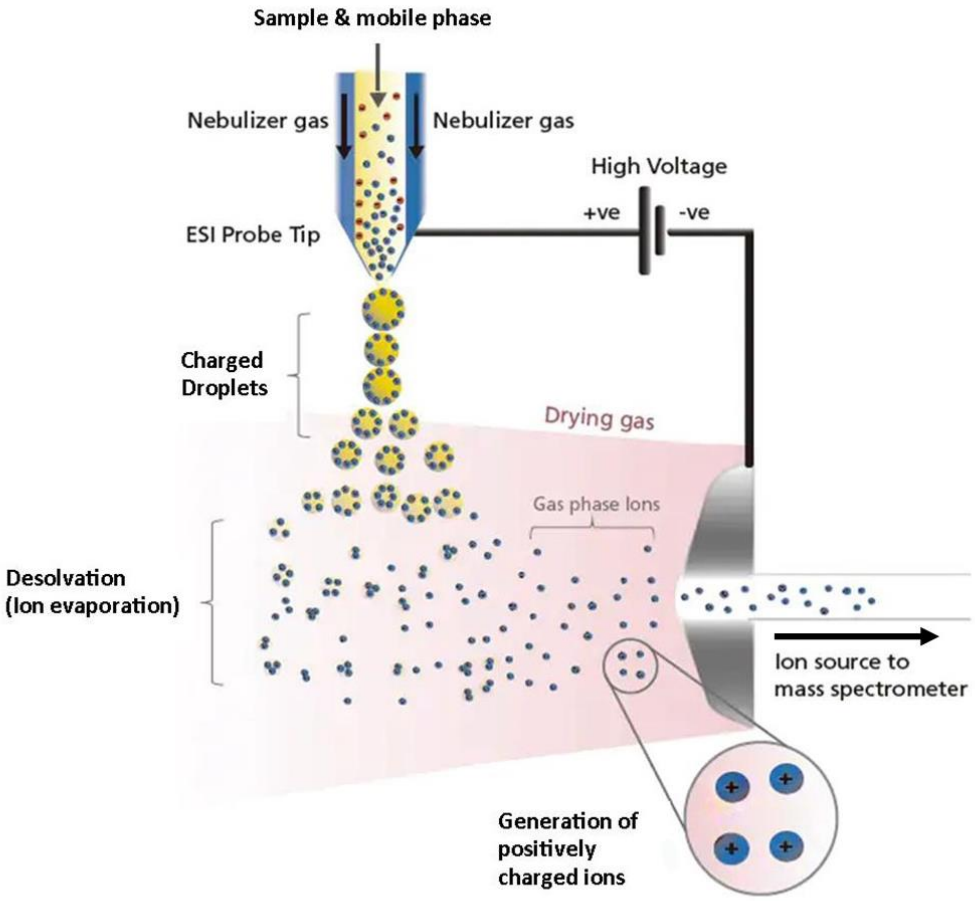


Figure 1.9: Generation of ions in the source chamber of a MS in positive mode.

(Shimadzu Primer - Fundamental guide to liquid chromatography mass spectrometry, 2019)

Ions then progress towards the first quadrupole which comprise of two pairs of metallic rods, one set positive, one set negative across which dc (direct current) and rf (radio frequency) voltages are applied. The positive pair of rods act as a high mass filter, the other pair as a low mass filter and resolution depends on the dc value in relationship to the rf value. For a given amplitude of the dc and rf voltages, only the ions of a set *m/z* ratio will resonate, having a stable trajectory to pass through the quadrupole for further processing. Other ions will be de-stabilized and hit the rods and dissipate. The performance (ability to separate two adjacent masses across the applicable range) depends on the quad geometry, the electronics,

voltage settings and the quality of the MS in use. Increasing the resolution means that fewer ions will reach the detector, and consequently impacts the sensitivity.

The first quadrupole is used to select a precursor (parent) ion, which is now fragmented in the collision cell (second quadrupole or Q2) by accelerating the ions in the presence of a collision gas (argon, helium or high purity nitrogen). The energy of the collision with the gas can be varied to allow different degrees of fragmentation and resulting fragments are analysed and selected for transport to the third quadrupole (Q3). The ion fragments reaching Q3 are now treated by the same selection process that occurred in Q1 before reaching the detector for analysis.

There are several operational modes available for a tandem mass spectrometer, (table 1.6) however, regimental steps must be adhered to when analysing an unknown compound or mixture of compounds for the first time as the analysist requires accurate mass of the parent ion, collision energies, and accurate mass of the product (daughter) ions. For an extra degree of specificity, the selection of two product ions (if available) is recommended. The most common and popular scan type is multiple reaction monitoring (MRM) mode, also sometimes referred to as selected reaction monitoring (SRM). The benefit of this is that many compounds and their corresponding internal standard (IS) can be measured in grouped segments both in positive and negative modes. In selective/single ion monitoring (SIM) mode, the parameters (amplitude of the dc and rf voltages) are set to observe only a specific mass, or a selection of specific masses. This mode provides the highest sensitivity for users interested in specific ions or fragments, since more time can be spent on each mass. That time can be adjusted and is called the dwell time.

Mode	Q1	Q3	Applications
Precursor scan (Parent ion)	Open scan	Fixed scan	Detection of structurally related analytes that produce common fragment ions
Product ion scan (Daughter ion)	Fixed scan	Open scan	Identification of unknown compounds, confirmation by spectral matching with standards
Selective/Single ion monitoring (SIM)	Fixed scan	Fixed scan	Specific but narrow mass range used
Multiple reaction monitoring (MRM)	Fixed scan	Fixed scan	Most common. Used to detect multiple compounds in a sample.

Table 1.6: Common operational modes within a tandem mass spectrometer

Therefore, the process involved when introducing an unknown sample mixture into a LC-MS/MS for the first time to obtain information about the compounds of interest, these steps are followed:

1. Set MS to precursor scan mode (MS2 scan) and introduce a volume (1-5 µL) at no greater concentration than 5-10 µmol (introducing millimolar concentrations into a MS will poison it and result in laborious cleaning procedures and angry analysts). This mode will allow all ions between the atomic mass unit range we set, e.g. 50-350 through to be scanned and registered. For optimum performance, positive and negative ions are scanned individually.
2. Once we detect our compounds of interest, for example TYR (182.2), HGA (167.2) and NTBC (330.2) we proceed to product ion detection step. Note: if the compound is detectable in the positive mode we add a proton (+H) and if it is an acid in the negative mode we remove a proton (-H) from the molecular weight.
3. Set MS to product ion scan mode where the samples are introduced again and subjected to a range of collision energies required to produce the most abundant product ions for the set precursor mass.

4. With two product ions for each compound selected the MS is then set to MRM mode where the sample is reinjected again to now determine the exact collision energies for these product ions.
5. All the information gathered is then introduced into the operational software and the MS for the method optimisation and validation can begin

With our compounds of choice optimised; What about the MS itself? This now needs to be optimised to its peak performance settings and this is a process that can take up to 24hr depending on the number of methods being set up at the time. But as a general rule of thumb, this process of optimising the MS settings when done for the first time, in most cases sets a peak performance for most assays to run satisfactorily from then on.

What settings do we need to optimise:

1. **Capillary voltage** – the potential required to maintain ion integrity
2. **Nebuliser gas flow** – the flow of nitrogen drying gas through the MS. Too low and you risk an uneven spray, too high and a risk of premature fragmentation and loss of ion integrity.
3. **Nebuliser gas temperature –** required at optimal level, too low and there is the risk of aerosol size and integrity loss, too high and ions can be lost to evaporation and fragmentation.
4. **Ion funnel** – an ion chamber, initially set at high radio frequency (rf) voltage and pressure to guide the unfocused ions to a sharp beam leading to the second chamber at lower pressure and voltage where the channelled ions are directed to the collision cell.

To summarise; a tandem MS is an instrument that acts as a separation device reducing the need for a perfect chromatographic separation. Its other use is accurate quantitation, having the ability to select a compound initially by its molecular mass and then even more precisely by its unique fragmentation. Further advantages of enhanced selectivity is a decrease in the signal to noise ratio enabling quantitative analysis on complex matrices like whole blood, serum, plasma and urine with a very short chromatographic

separation, and even with no separation at all. Study of mass spectral fragments can also provide structural information especially when categorising groups of compounds with very similar structures.

However, one should keep in mind that, when doing quantitation, the first important step is the ionisation, which takes place in the source. No ionisation means no results. The presence of interfering compounds in the source might also cause unexpected effects, like "ion suppression". Such effects impact the quantitation, whatever the MS analyser. Direct injection with no form of separation of compounds poses more problems than not, for starters, even the most sophisticated and sensitive MS would be overwhelmed with a direct injection of multiple compounds plus isotopic IS's to render the assay unworkable. To address this 'elephant in the room', MS works most effectively when used in conjunction with HPLC.

1.12 High performance liquid chromatography (HPLC).

Chromatographic techniques have been around arguably for centuries in one form or another and as the timeline in table 1.7 shows, the advancements have really taken-off since the 1970's particularly with the advancement in column technology providing increasing selectivity and when combined with MS systems provide the analyst with multiple options.

The general principle is the same for chromatographic separation in that a sample mix is separated into its constituent parts based on the affinities the molecules present have for the mobile or stationary phase; in effect "like is attracted to like". The outline of a standard HPLC system is shown in figure 1.10.

Therefore, our first considerations are:

What compounds do we need to separate?

What column would be suitable?

What mobile phase(s) would be most suitable?

Using an MS/MS system alone without a liquid chromatography (LC) sampler is counterproductive particularly when attempting to measure complex, high matrix or multiple-compound mixtures, as the MS can be overwhelmed with an influx of compounds and thus dramatically lose in all areas, ranging from specificity, selectivity, to resolution and sensitivity. This is where LC and effective sample preparation steps are necessary.

Table 1.7: Timeline history of chromatography

Investigator(s) and year	Developments
Runge 1850's	Capillary work with coloured chemicals on paper
Eichhom and Boecker 1870's	Ion exchange studies
Tswett 1903	Chromatographic separation of plant pigments through calcium carbonate
Lederer and Kuhn 1930	Separation of carotin and zeaxanthin
Brockmann 1934	Aluminium oxides standardised
James and Martin 1941	Gas-liquid chromatography: triggers development of analytical chromatographic principles
Horvath 1966	Developed HPLC
Since 1970's	Liquid chromatography takes over and reverse phase supersedes normal phase
Majors 1971	Microparticulate silica (5-10 µm)
Kirkland 1972	Spherical silica (7 µm) and bonded phases
1976	Rheodyne valve
1977 Hewlett Packard	Diode array detector UV-Vis
1981	First process scale HPLC system (Kiloprep)
Turnell and Cooper 1988	ASTED HPLC system
1992	3 µm particle size
2000's	Ever advancing column technologies
2003	1.8 µm particle size
2004 Waters	Acquity UPLC

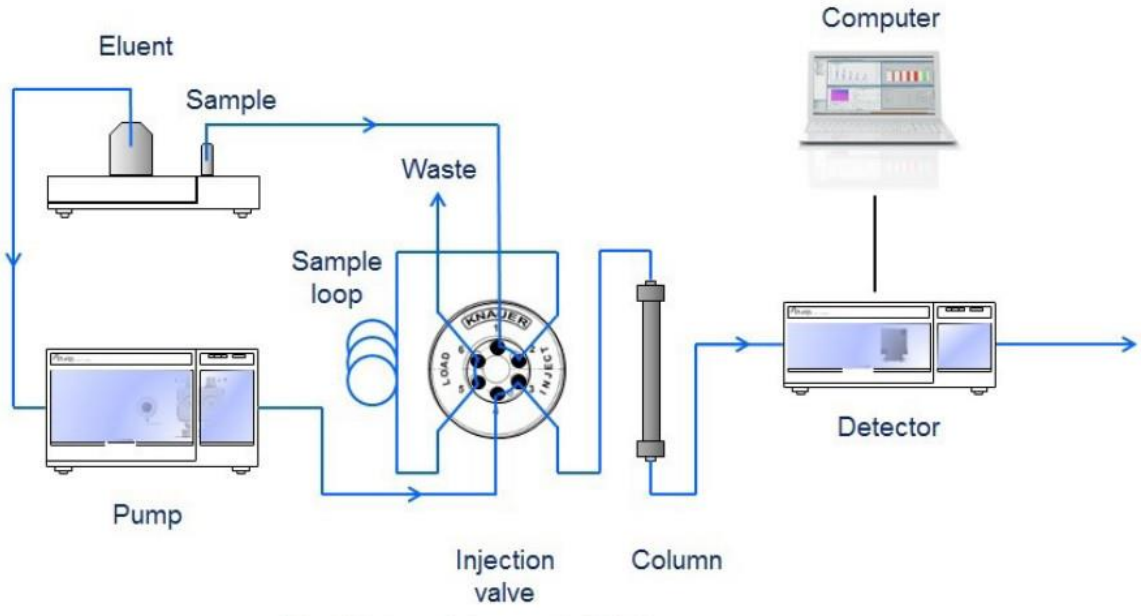


Figure 1.10: Schematic layout of a HPLC system

HPLC is utilised in two phases; mobile and stationary.

1. Mobile phase refers to the solvent(s), which are continuously pumped through the column during operation.
2. Stationary phase, or column material which acts as a carrier for the sample solution introduced via the injector port.

1.12.1 Mobile Phase

The mobile phase is dependent on the nature of the compounds to be separated in the mixture. It can be a single entity and recycled; a term phrased as **isocratic.** However, this method is dependent on the compounds all migrating at different rates in the chosen solvent and can often suffer from poor resolution and longer run-times. More commonly used is a method where two solvents of differing polarity are mixed at varying concentrations throughout the run; a term phrased as **gradient** chromatography. The most common solvents used are deionised water that is usually buffered and kept at a pH <4, with second solvents of methanol or acetonitrile. These solvents must be HPLC grade or preferably LC-MS grade and degassed before use to remove dissolved gases and particulates that can plug or contaminate the column.

As with most forms of HPLC methods, buffers and salts ranging from borates, formates or acetates may be added to the mobile phase to keep ionizable analytes in a single form and if used in conjunction with mass spectrometry, then formic acid is a necessity to create the ionizable matrix necessary for detection.

A standard HPLC pump schematic is shown in figure 1.11 depicting the steps required prior to the mobile phase reaching the column.

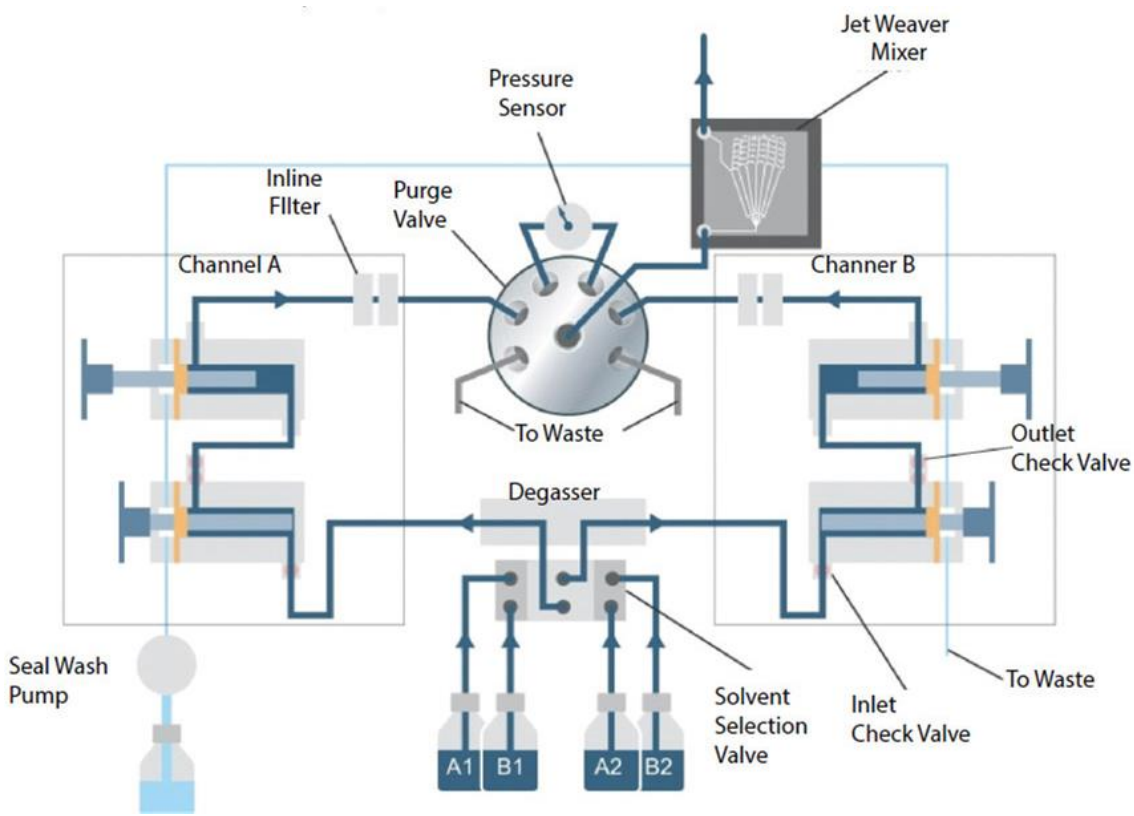


Figure 1.11: Schematic layout of an Agilent HPLC binary pump

1.12.2 Stationary Phase

This is the column and its packing material, which can vastly vary with the type of adsorbent material used. The stationary phase is vital for efficient separation of components in a mixture and can be chosen based on particle size and activity of the solid. The most popular adsorbent is silica as it is inert and anhydrous. There are thousands of different columns on the market and choosing one can be a minefield in its own right, each one having particular

characteristics especially in relation to particle size, bonded phase groups and properties which is why a basic knowledge of chemistry, chromatography and separation techniques (which come from experience) are an advantage. In fact, it would be a thesis in its own right to merely discuss reasoning behind stationary phase choices in HPLC.

The most popular type of HPLC in use is **reverse phase** which operates on the basis of hydrophilicity and lipophilicity. The stationary phase consists of silica-based packings with n-alkyl chains covalently bound. For example, C-8 signifies an octyl chain and C-18 an octadecyl ligand in the matrix. The more hydrophobic the matrix on each ligand, the greater is the tendency of the column to retain hydrophobic moieties. Thus, hydrophilic compounds elute more quickly as they are carried along the column with a polar mobile phase, than do hydrophobic compounds which are retained longer.

In gradient elution, different compounds are eluted by increasing the strength of the organic solvent (B). The sample is injected while a weaker mobile phase (A) is being applied to the system. The strength of the mobile phase is increased in increments by raising the organic solvent fraction, which subsequently results in elution of retained components. This is usually done in a stepwise or linear fashion. As the sample solution is transported through a column with the mobile phase(s), compounds present migrate according to the non-covalent interactions of the compounds with the column. Chemical interactions of the mobile phase and sample, with the column, determine the degree of migration and separation of components contained in the sample. For example, those samples which have stronger interactions with the mobile phase than with the stationary phase will elute from the column faster, and thus have a shorter retention time, while those with a greater affinity to the stationary phase are retained longer as shown in figure 1.12.

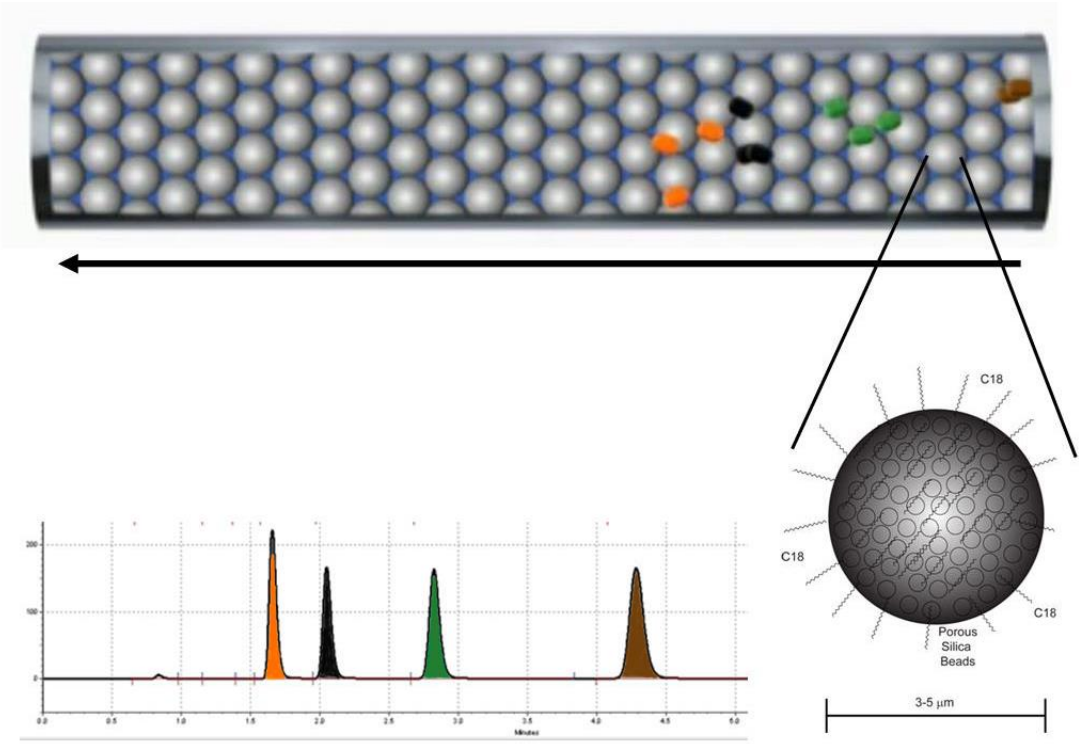


Figure 1.12: Principle of reverse phase HPLC depicting a C18 silica particle

If isocratic HPLC is insufficient to achieve optimum separation efficiency and resolution, many other factors need to be taken into consideration:

1. Gradient solvents: understand the chemistry of the compounds to be separated. Hydrophobic compounds will be retained on a reverse phase column for a significant time unless a strong solvent is used.
2. Gradient curve: ensure the flow of weak (mobile phase A) or strong (mobile phase B) solvents have a bearing on the chemistry of the compounds to be resolved.
3. Column choice: chemistry plays an essential role; C18 may not be suitable, is HILIC (hydrophilic interaction liquid chromatography) more suitable? Are specific columns with attached groups more suited to the job?
4. Column dimensions: increase in length provides greater resolution but may increase retention times and also back pressure and run-time.

5. Particle size: generally 3 µm and 5 µm are popular for sufficient separation. Less than 2 µm provide even better resolution but significantly higher back pressures, requiring UPLC systems.
6. Pore size: generally between 8-12 nm in diameter for molecules <3000da in size, the greater the number, the better the retentive qualities of the particles and hence the column. For samples above 10,000 Da, including peptides and proteins, a 30 nm pore size provides the best efficiency and peak shape.
7. Carbon chain length and particle sub groups: all play an important role in retentive properties and specific interactions for more chemically challenging compounds.
8. Temperature: if increased (between 35-55^{o}C), the chromatographic separation process becomes faster and, in general, more efficient.
9. Flow rate: if increased, will reduce retention times but increase pressure.

Reverse phase HPLC, is the most commonly used, however, there are other types of chromatography that are worth mentioning.

1.12.3 Normal phase chromatography:

Opposite to reverse phase in that the column is more polar than the mobile phase and therefore the more polar compounds being separated will have a greater affinity to the stationary phase. When the solvent(s) are passed through the column, less polar compounds elute faster than the more polar ones. The stationary phase usually consists of non-bonded or polar-bonded silica and mobile phases tended to be solvents such as hexane, heptane, chloroform and back in the old days, benzene or toluene which is why its popularity waned when the lesser toxic reverse phase HPLC was introduced.

1.12.4 Hydrophilic interaction chromatography (HILIC)

HILIC may be viewed as a variant of normal-phase chromatography. In normal-phase chromatography, the mobile phase is 100% organic and polar analytes bind strongly to the polar stationary phase. But by adding 10-20% water to an organic mobile phase like acetonitrile (which is aprotic) facilitates

separation and elution of the strongly retained polar compounds, i.e. those compounds that would be weakly retained in revere phase HPLC, making water the strong solvent in this case. Analytes are eluted in order of increasing hydrophilicity.

1.12.5 Ion-exchange chromatography

With the chromatographic methods mentioned so far, the rule is ‘like’ is attracted to ‘like’, however with ion-exchange chromatography the reverse is true. This method of separation is popular for steroid, protein and peptide analysis. The stationary phases in this instance contain either acidic or basic groups on their surfaces and so with cation exchange, the process is used to retain and separate positively charged ions on a negative surface. The reverse is true for anion exchange chromatography. This method is very dependent on the pH of the mobile phases and the ability of the ions to be retained or released from the stationary phase and can often fall foul of subtle changes in buffer constitution or ignorance of the pKa. This is the pH at which 50% of the functional group(s) are ionized and 50% are neutral. Therefore, to assure a fully neutral, or a fully charged, analyte or particle surface, the pH must be adjusted to a value at least 2 units beyond the pKa, as appropriate. LC-MS/MS has removed the complexity in measurement of compounds, once popular with this method of analysis.

1.12.6 Affinity chromatography

Another type of chromatography used predominantly for protein analysis involves binding a ligand to the analyte molecules of interest in a sample so that only molecules that have this ligand are retained in the column, and unbound analytes are passed through in the mobile phase. The stationary phase is usually agrose based or porous glass beads that are able to immobilize the bonded molecules. By changing the elution conditions such as the pH or ionic strength influences the binding ligand and determines the retention times of the molecules of interest.

1.12.7 Plate theory

No scientific method can ever escape the interference of mathematics in their principles, HPLC is no different. We must imagine the column is divided into hypothetical slices or plates where each analyte spends a finite time within, to achieve an equilibrium between the stationary and mobile phase, as it progresses through the column. In simple terms, it can be stated that a theoretical plate represents the distance that is needed for every adsorption-desorption step, with the greater the number of these plates, the more efficient the separation power of the column. In a similar way, the greater the number of pixels in a picture, the greater the overall clarity.

The number of plates (N) over the column length (L) is dependent on the height equivalent to a theoretical plate (HETP or H):

$N = L/H$

The theoretical plate number (N) usually is expressed by one of two equations below, and also shown graphically in figure 1.13:

$N = 16\ (t_R/W_b)^2$ or $N = 5.545\ (t_R/W_h)^2$

t_R refers to the retention time of the peak
W_b refers to the peak width at baseline
W_h refers to the peak width at half height

The higher the plate number N, the greater the efficiency of the column. Therefore, the narrower the peak (low W), the higher N, and thus the efficiency, whereas a less efficient column will contribute to peak broadening.

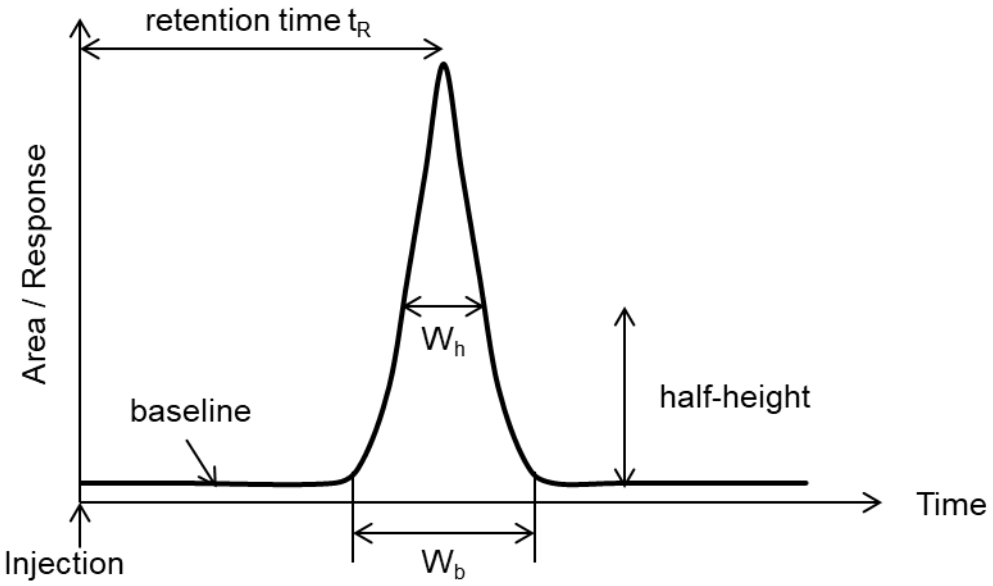


Figure 1.13: Explanation of the values used in the theoretical plate equation

The height equivalent to a theoretical plate (HETP) can be calculated when both N and the L are known:

HETP = L/N

Hence, the lower the HETP, the better the resolution and the more efficient the separation. Efficiency is optimized when N is maximized and HETP is minimized.

The other “popular” mathematical concept in HPLC is the van Deemter equation and plot (figure 1.14), devised to determine optimal mobile phase velocity based on column factors. The concept is and was a paper in itself (van Deemter et al., 1956) and requires a PhD to understand, but simplified states that:

- Smaller plate heights increase column efficiency
- Below the optimum flow rate the analysis time is too long and the quality of the separation suffers
- At high flow rates, separation quality pressure across the column become unacceptable
- Small particle size (< 5 µm) allow an increase in the flow rate to reduce the analysis time without significantly lowering resolution between the peaks of interest

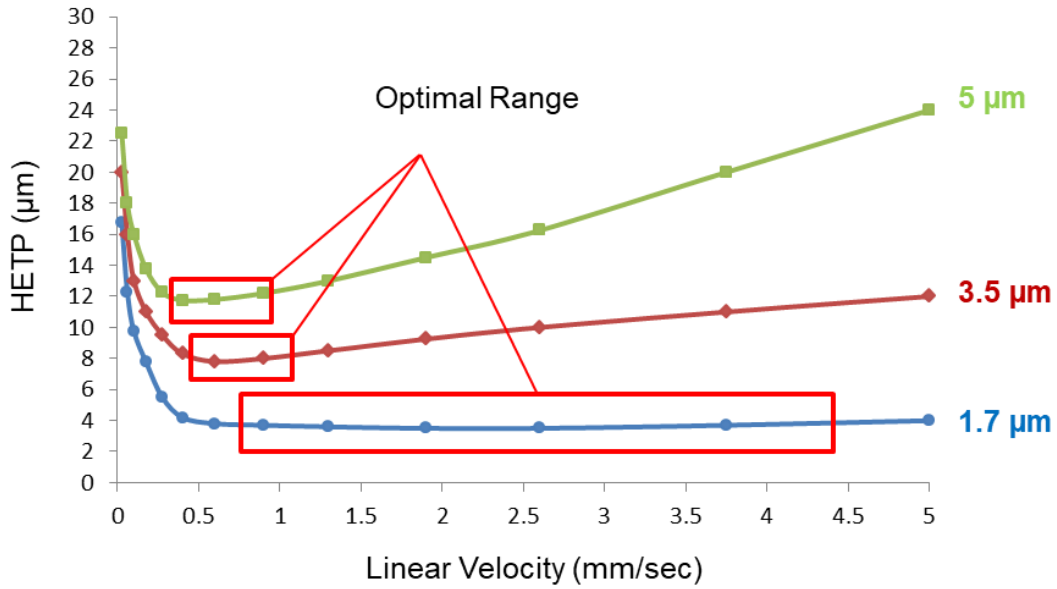


Figure 1.14: van Deemter plot depicting the optimal performance ranges for particular pore sizes vs mobile phase flow rates
(Waters, Primer on beginner's guide to UPLC)

Therefore, it can be stated with smaller particle size comes greater separation efficiency with the consequence of significantly higher pressures. However, this particular issue has been resolved in modern times with the higher capacity HPLC systems that can handle significant pressures (>10,000 psi). This is the realm of ultra performance liquid chromatography (UPLC).

1.13 Internal Standards

The concept of an IS is based on adding a known amount of the IS to every sample, i.e. calibrators, quality controls (QC) and unknowns, so instead of basing the calibration on the absolute response of the analyte, the calibration uses the ratio of response between the analyte and the IS. This is particularly important for assays that use any form of extraction from a matrix as any loss of efficacy to the sample mix will affect both compounds accordingly and as we are calculating the overall response, then the final concentration should not be affected.

In HPLC, the IS chosen should be a compound that behaves in a similar but not identical manner to the compounds of interest, as co-elution is counterproductive. However, in LC-MS/MS, due to the problems of ion suppression and enhancement the introduction of isotopically labelled IS proved to be a major step forward in eliminating error; in that both compounds elute at almost identical times but are different masses so fragment differently enough to be distinguished by the MS. A significant desirable characteristic of the IS is stability, and although there are many d-forms of IS on the market, they do over time tend to fall-off the molecule and in worst cases can be falsely recognised by the MS as the compound of interest. A solution to this issue is the introduction of a ^{13}C-isotope IS which eliminates the problem as the carbons are bound to the ring structure, see figure 1.15.

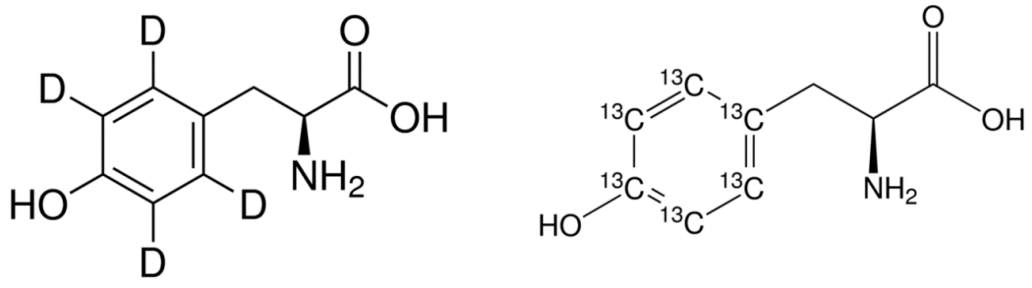


Figure 1.15: Examples of two forms of IS available. On the left the d_4-TYR and on the right the ^{13}C-$_6$ isotope.

1.14 Ion Suppression

A phenomenon where the presence of non-volatile or less volatile solutes, for example, phospholipids, salts, endogenous compounds, drugs or metabolites change the efficiency of droplet formation or droplet evaporation, which in turn affects the amount of charged ion in the gas phase that ultimately reaches the detector and was first described by Buhrmam et al., (1996). Molecules with higher mass will suppress the signal of smaller molecules and more polar analytes have a greater susceptibility to ion suppression (Annesley, 2003).

A clean extraction method such as protein precipitation with a strong organic solvent such as acetonitrile or an acid like perchloric, used in conjunction with a suitable IS, particularly a ^{13}C-$_6$ isotope, will minimise the detrimental effects of ion suppression. Validation guidelines set a percentage acceptable level of ion suppression (or enhancement) at ± 20%. If the percentage falls outside this parameter, even with normalising against the IS, then the gradient is usually the first parameter to adjust, as it is the most straightforward. The reason being that the peaks of interest could be eluting too soon in the chromatographic run where the intensity of ion suppression is usually at its worst. In cases of extreme ion suppression, then a re-evaluation of the method would usually be considered, perhaps introducing solid phase extraction (SPE) techniques for enhanced sample clean-up. These have their uses in assays of low throughput, but the procedure tends to be time consuming and labour intensive, and fortunately for our methods described, was not necessary.

The matrix factor experiments which determine the level of enhancement or suppression are probably the most involved and labour intensive in LC-MS/MS validation and involve spiking multiple (n=5) low and high concentrations of each analyte and their corresponding IS individually into water and pooled, crashed serum/plasma/whole blood matrix or urine. These are all analysed on the LC-MS/MS noting the areas of each metabolite, comparing against the water spike. The mean area of each compound is normalised against the IS area according to the equation below:

Mean analyte area in matrix / Mean analyte area in water = **X**
Mean IS area in matrix / Mean IS area in water = **Y**

Normalised **X/Y x 100**

This provides the percentage matrix suppression or enhancement and must be ± 20% for the assay to be acceptable.

1.15 Validation guidelines

With the optimisation of the MS, the choice and optimisation of the HPLC materials, and the acquisition of all the matrices required, one would expect plain sailing to have the new method implemented in no time at all. However, strict validation guidelines must now be adhered to otherwise any method would be rendered unfit for purpose. The concept of validation was first proposed by the Food and Drug Administration (FDA), in 1979 to improve the quality of pharmaceuticals. Refinements to these guidelines were conducted over the subsequent years until the European Medicines Agency (EMA) brought out their own set of guidelines in 2012. It was these guidelines which were adhered to for the initial development of the assays referred to in papers by Hughes et al., (2014 and 2015).

Then in 2014, the Clinical Laboratory Standards Institute devised a set of guidelines primarily aimed at LC-MS/MS method development named C62-A. These have also been incorporated in later validation. The main validation areas are summarised below as they are also referred to in more details in chapters 2, 3, 4 and 5.

1.15.1 Main points:

1. **Matrix effects:** low and high concentrations of analytes and their corresponding IS are spiked into water and extracted serum matrix or urine multiple times at each level. Analyte areas normalized against IS must be ± 20% (see section 1.14).
2. **Calibration:** a minimum of 6 levels plus a blank, to be run 20+ times to assign values.

3. **Recovery:** nominal value obtained from repeated analysis of low and high QC pools to determine inter- and intra-assay accuracy.
4. **Precision:** intra-: (n=6) and inter- (n=20+) CV <15%.
5. **Signal to Noise:** 20:1 preferable but not exacting as long as low calibrator and QC are reproducible.
6. **Low Level of Measuring Interval (LLMI):** not to be confused with limit of detection which could be lower but not definable.
7. **Upper Level of Measuring Interval (ULMI):** values exceeding this level must be diluted unless checked with QC that is reproducible.
8. **Selectivity/specificity:** extracted blank matrix processed to check for endogenous interferences at all masses. This should always be done first to ensure calibration and QC matrix are suitable.
8. **Carry-over:** top calibrator plus 5 blanks. Ensure areas in blanks are lower than lowest level of detection.
9. **Linearity:** beware of saturation. Dilute a sample/spiked sample of known concentration that is several times the upper calibrator level and test to see if concentrations are uniform and reproducible.
10. **IS concentration:** ensure that the working concentration and thus the area of the IS is 30-50% of top calibrator.
11. **Stability:** collecting a sample pool at low and high concentration, test freeze-thaw x3, room temperature for 24-48 hr, 4^{o}C up to one week and long-term when frozen at -20^{o}C or -80^{o}C.
12. **Dilution:** select a mid-range sample and dilute at x2, x5, x10, x20.
13. **Others:** tests for haemolysis, lipaemic, icteric or prominent drug interferences.

Therefore, the validation of any assay to acceptable standards is by no means a short or straightforward task. It usually takes several months and significant painstaking work. If the following points are considered then everything should run to plan:

- Develop method to meet a goal
- Cost, availability of reagents, IS, calibrators and QC

- Developing and validating it to a timeframe; understanding the chemistries and structures involved, literature searches; plan, tune, optimise
- Minimize analysis time for the greatest sample throughput
- Validate to a standard where the assay is reproducible and robust
- Above all: KEEP IT SIMPLE

1.16 Statistical analysis of data

Statistics are in place to test a theory or hypothesis, which is a proposed explanation for a phenomenon. Therefore, we are attempting to make rational decisions about the reality of observed effects, phenomena or experiments.

Probability is the measure of the likelihood that an event will occur and is quantified as a number between 0 and 1 (where 0 indicates impossibility and 1 indicates certainty).

The term **'null hypothesis' (H_0)** denotes that there is no difference between the population variables in question.

A **p value** is used in hypothesis testing to help support or reject the null hypothesis. The smaller the p-value, the stronger the evidence that you should reject the null hypothesis. P values are expressed as decimals for example, a p value of 0.0254 is 2.54%, meaning there is a 2.54% chance your results could be random (i.e. happened by chance). Conversely, a large p-value of 0.9 (90%) means your results have a 90% probability of being completely random and not due to anything in your experiment. Therefore, the smaller the p-value, the more important ("significant") your results. A common expression is if $p<0.01$ (1%), the result is highly significant and therefore we reject the null hypothesis, which in most cases we can do up to $p<0.05$ (5%).

1.16.1 Coefficient of Determination (R Squared)

The coefficient of determination, R2, is used to analyse how differences in one variable can be explained by a difference in a second variable. The range is 0 to 1 (i.e. 0% to 100%). The general equation of a straight line is y = mx + c, where m is the gradient, c is called the intercept on the y-axis. Again, this provides an insight as to whether 2 sets of data can be compared favourably.

Parametric tests are conducted when we infer a standard normal distribution where about 68.5% of the population are within 1 SD of the mean, 95.5% are within 2 SD's of the mean and 99.7% within 3 SD's of the mean. The most common types of parametric test include regression tests, comparison tests, and correlation tests.

Regression tests look for cause-and-effect relationships. They can be used to estimate the effect of one or more continuous variables on another variable. A regression line is the "best fit" straight line for your data. It's like an average of where all the points line up.

Comparison tests look for differences among group means. They can be used to test the effect of a categorical variable on the mean value of some other characteristic; for example **t-tests** are used when comparing the means of precisely two groups (e.g. the average heights of men and women). **ANOVA** and **MANOVA** tests are used when comparing the means of more than two groups (e.g. the average heights of children, teenagers, and adults). The larger the t score, the more difference there is between groups. The smaller the t score, the more similarity there is between groups. A t score of 3 means that the groups are three times as different from each other as they are within each other. When you run a t-test, the bigger the t-value, the more likely it is that the results are repeatable.

However, some distributions tend to be skewed to either extreme and therefore statistical analyses are in place to account for this:

Non parametric tests does not assume anything about the underlying distribution and suggests the population data may not have a normal distribution.

Wilcoxon signed rank test. With this test, you also estimate the population median and compare it to a reference/target value. However, the test assumes your data comes from a symmetric distribution

Mann-Whitney test: Used to test the null hypothesis that two samples have the same median or, alternatively, whether observations in one sample tend to be larger than observations in the other.

Kolmogorov-Smirnov (KS) test: Used to test whether two random samples are drawn from the same distribution. The null hypothesis of the KS test is that both distributions are identical.

Kruskal-Wallis test: Used to analyse if there is any difference in the median values of three or more independent samples.

Various statistical tests have been utilised throughout this thesis and additional explanation included where required.

Welch-correction: Used when two groups will have unequal standard deviations as a correction factor.

1.17 Aims of dissertation

This thesis originated with the drive to design, validate and implement a robust methodology incorporating key metabolites in the TYR pathway to:

- Support the diagnosis of AKU
- Quantitate urine and serum HGA in AKU patients
- Monitor the response biochemically to the use of NTBC
- Provide evidence of efficacy of NTBC in clinical trials

During the course of these studies, the methods have been refined and further developed as more evidence is revealed, including the development of a sensitive serum HGA method (chapter 5), monitoring of intermediates HPPA and HPLA (chapter 4), and creatinine measurements due to the interference of HGA in the routine enzymatic method (chapter 6).

With any long-term trials, sample stability is often not proven, but has been investigated in this thesis (chapter 10) to provide robustness to clinical finding.

Overall, this thesis has been a sojourn through method validation, application to clinical cohorts and refining analytical issues and has produced the biochemical metabolite data which was essential for the licensing of NTBC for use in AKU.

Chapter 2

Urine homogentisic acid and tyrosine: simultaneous analysis by liquid chromatography tandem mass spectrometry

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Preparation of calibration standards and quality controls, full method validation and data interpretation was all performed by Andrew T Hughes.

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2.1 Abstract

Alkaptonuria (AKU) is a rare debilitating autosomal recessive disorder of tyrosine (TYR) metabolism. Deficiency of homogentisate 1,2-dioxygenase results in increased homogentisic acid (HGA) which although excreted in gram quantities in the urine, is deposited as an ochronotic pigment in connective tissues, especially cartilage. Ochronosis leads to a severe, early-onset form of osteoarthritis, increased renal and prostatic stone formation and hardening of heart vessels. Treatment with the orphan drug, Nitisinone (NTBC), an inhibitor of the enzyme 4-hydroxyphenylpyruvate dioxygenase (HPPD) has been shown to reduce urinary excretion of HGA, resulting in accumulation of the upstream pre-cursor, TYR. Using reverse phase LC-MS/MS, a method has been developed to simultaneously quantify urinary HGA and TYR. Using matrix-matched calibration standards, two product ion transitions were identified for each compound and their appropriate isotopically labelled internal standards (IS). Validation was performed across the AKU and post-treatment concentrations expected. Intra-batch accuracy for acidified urine was 96-109% for TYR and 94-107% for HGA; inter-batch accuracy (n=20 across ten assays) was 95-110% for TYR and 91-109% for HGA. Precision, both intra- and inter-batch was <10% for TYR and <5% for HGA. Matrix effects observed with acidified urine (12% decrease, CV 5.6%) were normalised by the IS. TYR and HGA were proved stable under various storage conditions and no carry-over, was observed. Overall the method developed and validated shows good precision, accuracy and linearity appropriate for the monitoring of patients with AKU, pre and post-NTBC therapy.

Highlights:

- Developed LC-MS/MS method for HGA and TYR in human urine
- Validated in both acidified and non-acidified urine matrices
- Applicable for diagnosing patients with AKU
- Applicable for monitoring response to therapy in treatment of AKU
- Validated according to European Medicines Agency Guidelines

2.2 Introduction

Alkaptonuria (AKU) was the first inborn error of metabolism described by Garrod, in 1902 (Garrod, 1908) (OMIM # 203500). It is a rare autosomal recessive disorder, which results from a deficiency in homogentisate 1,2-dioxygenase activity in the liver (La Du et al., 1958, Gallagher et al., 2013) (figure 2.1). In the absence of the enzyme, homogentisic acid (HGA) is excreted in the urine in gram quantities (O'Brien et al., 1963, Phornphutkul et al., 2002, Introne et al., 2011) (equivalent to mmol/L concentrations), which turns black upon standing or alkalisation. HGA circulates at lower concentrations in plasma (µmol/L) and is oxidised to benzoquinones (BQA) which polymerise and bind to proteins, particularly in connective tissues including cartilage (O'Brien et al., 1963, Keller et al., 2005, Helliwell et al., 2008, Taylor et al., 2012). This process leads to ochronosis – a blue-black discolouration of the connective tissues especially cartilage. The mechanism of this remains unclear. Ochronotic pigmentation causes early onset degenerative arthritis of the spine and large weight bearing joints leading to increased pain and premature joint replacement. Aortic stenosis has been described as a cardiac complication of the ochronosis process (Hiroyoshi et al., 2013, Thakur et al., 2013) and in severe cases has led to aortic valve replacement. Additionally, an increased incidence of kidney stone formation is reported with an increase in prostate stones in males (O'Brien et al., 1963, Phornphutkul et al., 2002). The formation of BQA results in additional formation of reactive oxygen species and free radicals, which are suggested to play a significant role in the aetiology of AKU arthritis (Martin and Batkoff, 1987).

Although current therapy for AKU is palliative, there have been several reported trials of Nitisinone (2-(2-nitro-4-fluromethylbenzoyl)-1, 3-cyclohexanedione (NTBC) (Anikster et al., 1998, Phornphutkul et al., 2002, Suwannarat et al., 2005, Helliwell et al., 2008). NTBC is a potent inhibitor of the second enzyme in the tyrosine (TYR) pathway, p-hydroxyphenylpyruvic acid oxygenase which has been shown to lower circulating HGA and completely prevent pigmentation in AKU mice (Preston et al., 2014). NTBC is approved for treatment of hereditary tyrosinaemia type 1 (HT-1) (OMIM

#276700). It is currently being used at the National Alkaptonuria Centre (NAC) (Royal Liverpool University Hospital, UK), in a large multi-centre clinical trial (DevelopAKUre Clinical Trials – www.akusociety.org) to evaluate its effectiveness in the treatment of AKU. .

Currently published methods for quantitative and semi-quantitative analysis of HGA employ spectrophotometry, enzymatic spectrophotometry, gas chromatography mass spectrometry (GC-MS) and high performance liquid chromatography (HPLC) and integrated with mass spectrometry LC-MS/MS (Seegmiller et al., 1961, Lustberg et al., 1971, Akesson et al., 1987, Bory et al., 1989, Fernandez-Canon and Penalva, 1997, Wei-Yi et al., 2013, Preston et al., 2014). Urine TYR is generally measured as part of an amino acid screen with semi-quantitative reporting as part of a total screen, or quantitative analysis in select panels (Felitsyn et al., 2004).

The diagnosis of AKU relies on clinical presentation and evaluation and quantitative or semi-quantitative analysis of urine HGA. Due to the instability of HGA in an alkaline environment, the preferred sample collection criterion is acidified urine. The aim of this study was to develop a quantitative method for the simultaneous measurement of urinary TYR and HGA using LC-MS/MS. For validation and comparison purposes, the method has been validated in both acidified and non-acidified urine matrices.

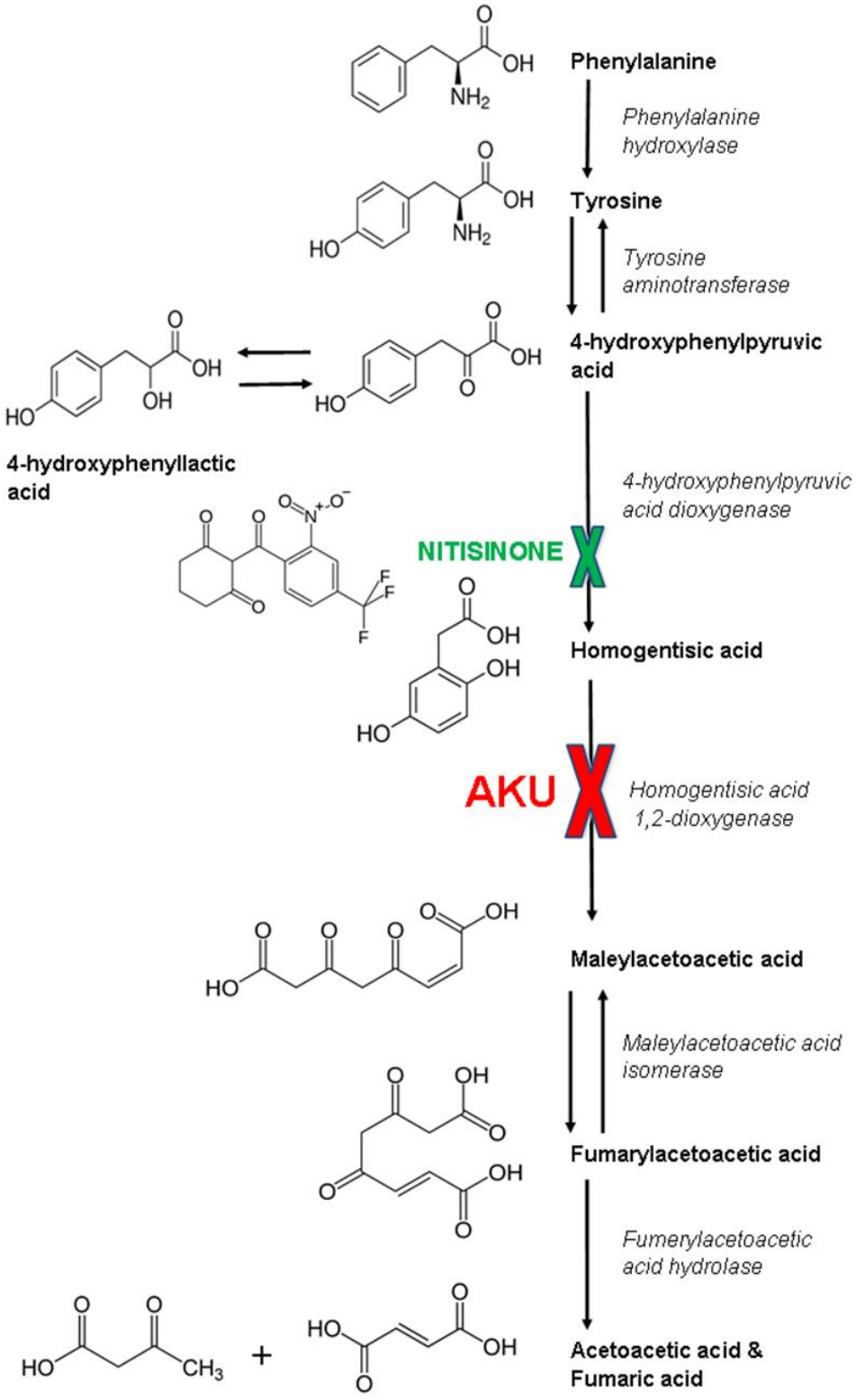

Figure 2.1: TYR degradation pathway showing the enzyme defect in AKU. NTBC blocks the enzyme prior to HGA.

2.3 Materials and methods

2.3.1 Chemicals and materials

TYR, TYR isotope-labelled IS (d_2-TYR) and HGA were obtained from Sigma-Aldrich UK. HGA isotope-labelled IS, $^{13}C_6$-HGA was obtained from Larodan Fine Chemicals (Sweden). LC-MS grade methanol and acetonitrile were obtained from Sigma Aldrich, UK. Formic acid was obtained from Biosolve. Water was purified in-house by DIRECT-Q 3UV Millipore water purification system. All dilutions and sample preparation was performed in glass. Oxygen free nitrogen was supplied by a Genius 2010 Peak nitrogen generator.

2.3.2 Instrumentation and operating conditions

All analysis were performed on an Agilent 6490 triple quadrupole mass spectrometer with Jet-Stream® electrospray ionisation (ESI-MS/MS) coupled with an Agilent 1290 infinity UHPLC pump and HTC autosampler. All data processing both qualitative and quantitative analysis was performed using Mass Hunter software package.

Chromatographic separation was achieved on an Atlantis C18 column (100 mm x 3.0 mm, 3 µm, Waters) maintained at 35°C. Initial conditions were 80:20 water:methanol with 0.1% formic acid (v/v) increasing linearly to 10:90 by 2.5 min. The mobile phase was maintained for 1.1 min, increased to 100% organic for 1 min and then returned to starting conditions 80:20. The flow rate was maintained at 0.4 ml/min throughout the run. The column was reconditioned for 2 min prior to the next injection. 2 µl of sample was injected with a total run-time of 7.0 min. Optimum operating ESI conditions were gas temperature 150°C, gas flow 17 L/min; nebulizer pressure 40 psi; sheath gas temperature 320°C and sheath gas flow 12 L/min. Capillary voltages were optimised to 3500V in positive mode and 2500V in negative mode with equal nozzle voltages (1500V) in both modes. The iFunnel parameters were optimised in both negative and positive mode as 60V for low pressure RF and 110V for high pressure RF.

2.3.3 Preparation of standard solutions

Super-stock standard solutions of TYR and HGA were prepared: TYR in 0.5N sulphuric acid (H_2SO_4) and HGA in deionised water at concentrations of 100 mmol/L and 400 mmol/L respectively. These were stored at -20°C.

IS were prepared: L-TYR-(phenyl-3,5-d_2) super-stock at 100 mmol/L in 0.1N H_2SO_4. An intermediate stock of 1 mmol/L was prepared by dilution in deionised water, and stored at -20°C. $^{13}C_6$-HGA was reconstituted at 1 mg/mL equivalent to 5.75 mmol/L, in deionised water with an intermediate 250 µmol/L stock in deionised water, stored at -20°C.

2.3.4 Preparation of calibrators and controls

To ensure matrix-matched calibration, super-stock aqueous standard solutions were added to either an acidified urine base pool (5N H_2SO_4, 1% v/v) or non-acidified urine base pool. The urine base was assayed prior to preparation to identify a low TYR pool suitable for standard addition. The super-stock standards were diluted to intermediate stocks at ten times the final required concentration. These were then added to urine pool in a ratio of 1:9, to create combined calibrators with final concentrations of TYR 20 µmol/L to 4 mmol/L and HGA 30 µmol/L to 52 mmol/L, whilst maintaining the integrity of the matrix.

A combined IS solution was used as the sample diluent, containing final concentrations of 0.4µmol/L $^{13}C_6$-HGA and 2 µmol/L d_2-TYR in 0.1% formic acid (v/v) in deionised water.

Quality controls (QC) were prepared in the same manner to the standards, in both acidified and non-acidified urine base pools at concentrations suitable to the dynamic range of the calibration curve. HGA QC values were 90-15,000 µmol/L and TYR QC values at 55-4000 µmol/L. All samples, calibrators and quality controls were assayed on a 1 in 1000 dilution with the internal standard solution. Calibrators and QC were spiked from separate stocks of TYR and HGA.

2.3.5 Assay Validation

The assay was validated using in-house protocols based on published guidance (EMA guidelines, 2011; Honour, 2011; FDA Bioanalytical Method Validation, 2018).

2.3.5.1 Linearity

Standard curves were fitted using linear regression with a 1/x weighting factor and a minimum of six calibration points plus urine blank (not a true blank for TYR due to endogenous levels) and curve fitting parameters excluded zero. Performance of fitted curves is presented as the coefficient of determination (R^2).

2.3.5.2 Accuracy

Accuracy was determined as closeness to the nominal spiked concentrations, this was determined both intra- and inter-assay with n=6 and n=20 respectively. Accuracy was calculated as: [measured concentration – nominal concentration] / [nominal concentration] x 100%.

2.3.5.3 Precision

Imprecision was determined both intra- (n=6) and inter-assay (n=20) using separately spiked urine pools and is expressed as co-efficient of variation (CV).

2.3.5.4 Matrix effects

The presence of ion suppression was evaluated for TYR and HGA and their respective IS. Deionised water, acidified and non-acidified urines (six individual donors) were spiked with a low, medium and high concentration of TYR or HGA or the equivalent of the final concentration of IS (prepared as in section 2.3.3). Matrix factor was determined by calculating the ratio of the peak area in the presence of matrix (spiked with analyte or IS) to the peak area in the absence of matrix (deionised water plus analyte or IS). Due to the endogenous levels of TYR in urine, the matrix spiked with an equivalent volume of water, as used for the spike was also measured. Using the matrix factors calculated, an IS normalised matrix factor can be determined (matrix

factor of analyte / matrix factor of IS multiplied by 100) (EMA Guidelines, 2011).

2.3.5.5 Dilution and Carry-over

Dilution integrity of urine HGA was assessed by pre-analytical dilution of five samples with high HGA in deionised water (due to endogenous TYR in urine) at factors of one in three, five and ten with recovery as a percentage of the base (n=6). Carry-over of both TYR and HGA and their respective IS was assessed by five separate water injections following injection of the top calibrator (approx. 4000 µmol/L for TYR and 16,000 µmol/L for HGA). Water was used due to the endogenous TYR in urine matrix base pool.

2.3.5.6 Stability

Stability of TYR and HGA in both acidified and non-acidified urine was assessed using three pools representing low, medium and high concentrations of TYR and HGA. Stability was determined following three freeze-thaw cycles (at -20°C), over 24hr at room temperature and over a 24hr period at 4°C (equivalent to the sample manager temperature, attached to the Agilent 6490). Results are expressed as a percentage of nominal values determined against a fresh calibration curve. Samples used for the on-board 24hr stability were also repeatedly analysed over the 24hr period for any deterioration which may limit batch and run-times.

2.3.6 Analysis of analytes in urine samples

HGA and TYR levels were determined in three AKU patients, who are on a daily dose of 2 mg of NTBC, as part of the National AKU Centre at Liverpool. Baseline, three, six and twelve month samples were analysed. 24hr acidified urine collections were analysed for creatinine (Jaffe assay) and samples were prepared at a 1 in 1000 dilution, for analysis.

2.4 Results

2.4.1 Method Validation

The mass spectrometer was operated in multiple reaction monitoring (MRM) mode. Two product ion transitions were determined for each precursor ion and the respective collision energies are detailed in table 2.1. For optimal sensitivity TYR and d_2-TYR were measured in positive ionisation mode while HGA and $^{13}C_6$-HGA were measured in negative ionisation mode. Additional parameters were optimised to ensure suitable operating conditions for method validation as detailed within Section 2.3.2.

	Ionisation mode	Product Ion 1 (Quantifier)	Collision energy	Product Ion 2 (Qualifier)	Collision energy
HGA	Negative	167>122	22	167>108	20
$^{13}C_6$-HGA	Negative	173>128	22	173>114	26
TYR	Positive	182>136	14	182>91	32
d_2-TYR	Positive	184>138	12	184>125	18

Table 2.1: Parameters for MS detection of HGA and TYR

A typical chromatogram is displayed in figure 2.2 for the primary product ion (quantifier) for each precursor, demonstrating the chromatographic separation of TYR from HGA in the urine matrix. No difference was observed in retention times of TYR and HGA upon comparison of acidified and non-acidified urine matrices. TYR eluted at 1.8 min and HGA at 2.5 min, with neither eluting in a region of ion suppression.

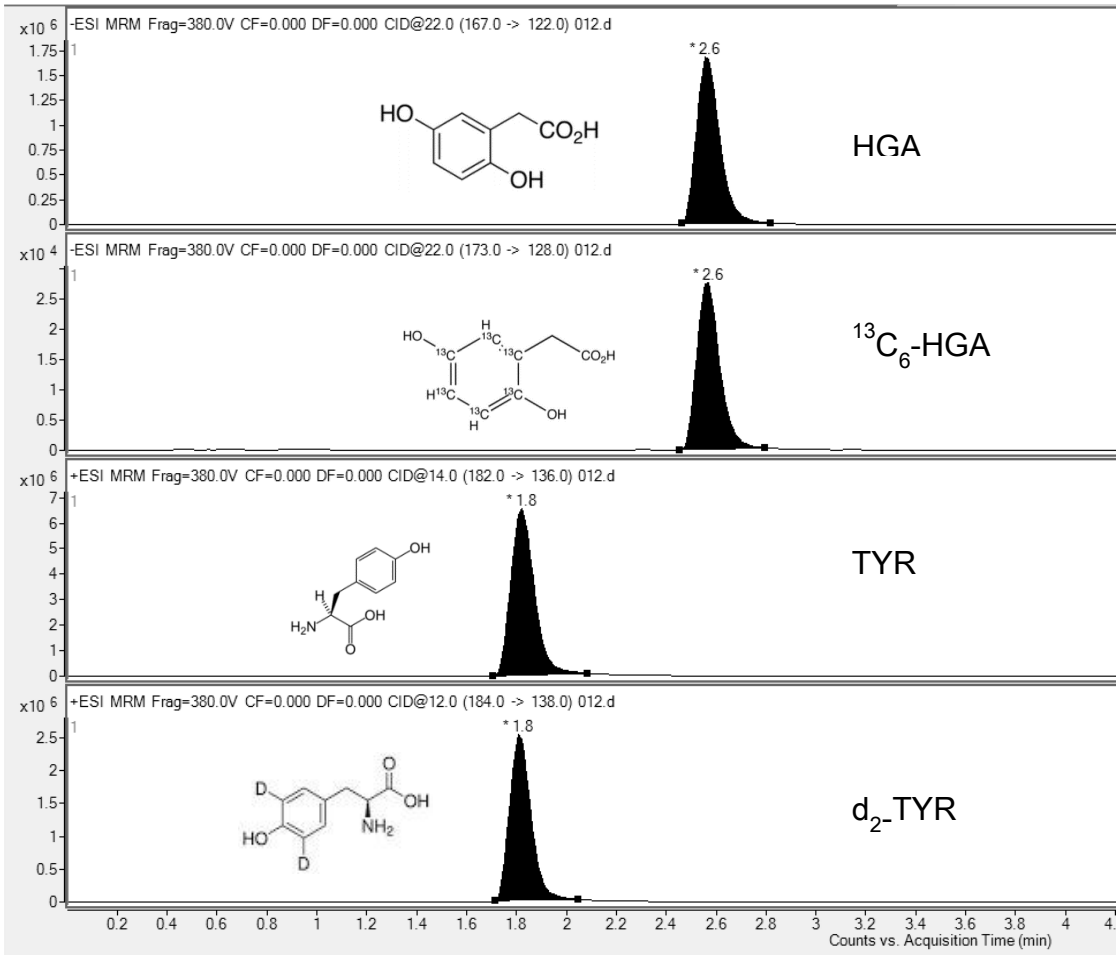

Figure 2.2: Chromatogram of TYR and HGA (1700 µmol/L and 4000 µmol/L respectively) with their respective IS. The horizontal axis is the time of elution and the vertical axis the counts.

2.4.2 Linearity

Calibration standard curves (seven points) exhibited a good fit over the range examined, with minimal inter-assay variability; TYR in acidified urine R^2=0.999, (n=8) and TYR in non-acidified urine R^2=0.997 (n=8) over a concentration range of 20-4000 µmol/L; HGA in acidified urine R^2= 0.999 (n=8) and HGA in non-acidified urine R^2=0.997 (n=8) over a concentration range 30-16,000 µmol/L.

2.4.3 Accuracy

Intra- and inter-assay accuracy was determined (table 2.2) in both acidified and non-acidified urine matrices. Results are represented as percentage recovery of a nominal amount of TYR or HGA spiked into matrix. Intra-batch accuracy was 96-109% for TYR in acidified urine and 97-108% in non-acidified urine. Inter-batch accuracy was 95-110% and 93-116% for TYR in acidified and non-acidified urine respectively. With regards to HGA, the intra-batch accuracy was 94-107% and 83-103% for acidified and non-acidified urine respectively; the inter-batch accuracy was 91-109% for acidified urine and 86-106% for non-acidified urine. Trimming of the intra-assay data for the low pool for non-acidified HGA reduced the CV to <15% (n=5).

TYR					HGA				
	Acidified Urine Matrix		Non-Acidified Urine Matrix			Acidified Urine Matrix		Non-Acidified Urine Matrix	
Expected Conc µmol/L	Intra-assay Mean±SD (%CV) n=6	Inter-assay Mean±SD (%CV) n=15	Intra-assay Mean±SD (%CV) n=6	Inter-assay Mean±SD (%CV) n=15	Expected Conc µmol/L	Intra-assay Mean±SD (%CV) n=6	Inter-assay Mean±SD (%CV) n=15	Intra-assay Mean±SD (%CV) n=6	Inter-assay Mean±SD (%CV) n=15
386	105.7±3.6 (3.4)	99.1±3.1 (3.1)	102.2±0.7 (0.7)	103.1±5.6 (5.4)	400	102.3±2.4 (2.3)	96.7±2.9 (3.0)	84.9±2.1 (2.5)	94.9±5.2 (5.5)
1586	98.5±3.2 (3.3)	101.3±4.0 (3.9)	103.4±4.1 (4.0)	102.8±2.6 (2.6)	5000	99.9±4.8 (4.8)	102.0±3.1 (3.0)	100.2±2.1 (2.1)	99.3±3.5 (3.5)
3086	103.3±2.5 (2.5)	104.4±2.7 (2.6)	100.0±3.7 (3.7)	99.9±3.6 (3.6)	12,000	103.1±2.6 (2.5)	104.7±2.8 (2.8)	101.1±1.5 (1.5)	100.3±3.7 (3.7)

Table 2.2: Intra- and Inter-batch accuracy in acidified and non-acidified urine. Data is as mean ± SD with percentage CV in parenthesis.

2.4.4 Imprecision

Imprecision (%CV), both intra- (n=6) and inter- (n=20) was determined in both acidified and non-acidified urine. In the acidified urine matrix, intra-assay precision was <5% for TYR (170-4000 µmol/L) and HGA (100-16,000 µmol/L). Inter-assay precision in acidified urine was <10% for TYR (70-4000 µmol/L) and <5% for HGA (100-16,000 µmol/L). Similar precision was seen in the non-acidified urine matrix for HGA with %CV <5% both intra- and inter-assay. Intra-assay precision for TYR in the non-acidified matrix was <12% (170-4000 µmol/L) and inter-assay precision was <10% (170-4000 µmol/L).

2.4.5 Limit of Quantification (LLOQ)

The LLOQ in validation protocols is defined as the lowest calibrator which satisfies a CV ≤ 20%. The LLOQ for HGA in urine was 30 µmol/L with an intra-assay CV of 3.7% (n=6) and an inter-assay CV of 6.7% (n=20). In addition, urine TYR demonstrated acceptable performance at a level of 20 µmol/L.

2.4.6 Matrix effect

The matrix effect of both acidified and non-acidified urine was assessed across the concentration range for TYR and HGA, with five individual urine matrices (both acidified and non-acidified). Results demonstrated a slight matrix effect for acidified urine (12% decrease, CV 5.6%) which was normalised by the IS. For both TYR and HGA, the %CV of the IS normalised matrix factor is <10% in both acidified and non-acidified urine matrices satisfying validation criteria (EMA guidelines, 2011; Honour, 2011; FDA Bioanalytical Method Validation, 2018).

2.4.7 Dilution integrity

Due to the range of urine HGA concentrations exhibited at the time of diagnosis with AKU (>12,000 µmoL/day) and the levels when patients are treated with NTBC (<1000 µmol/day) [unpublished data from the NAC, Liverpool, UK] the recovery of urine HGA, post-dilution was assessed. Acidified urine demonstrated recovery of 97.8 ± 7.1% at a 1 in 3 dilution; 97.3 ± 4.7% at a 1 in 5 dilution and 96.6 ± 6.6% at a 1in 10 dilution (n=6 for all).

Similar recoveries were measured for non-acidified urine with 97.8 ± 4.4%, 95.2 ± 5.4% and 96.5 ± 6.9% at a 1 in 3, 5 and 10 dilution respectively.

2.4.8 Stability

Stability following three freeze-thaw cycles demonstrated average recovery of 95.7 ± 1.8% and 101.4 ± 4.6% for TYR and 93.2 ± 3.1% and 97.4 ± 1.8% for HGA in acidified and non-acidified urine respectively (n=5 at each of three levels). Samples stored at room temperature demonstrated average recovery of 100.8 ± 5.1% and 101.7 ± 3.2 % for TYR and 100.1 ± 6.7 and 102.8 ± 3.2 for HGA in acidified and non-acidified urine respectively (n=5 at each of three levels). Repeated analysis of 3 urine pools over a 24hr period, stored at 4°C demonstrated average recovery at t=24hr of 97.4 ± 1.3% and 97.9 ± 4.8% for TYR and 95.1 ± 4.0% and 97.2 ± 2.1% for HGA in acidified and non-acidified urine respectively. There was no significant deterioration in recovery across a 24hr period for either TYR (%CV of 3.6% and 2.8%, n=21) or HGA (%CV 4.8% and 2.8%, n=21) in both acidified and non-acidified urine. In addition, no significant difference was observed between acidified and non-acidified (p=0.87 for TYR and p=0.07 for HGA).

2.4.9 Carry-over

Following injection of the top calibrator there was no clear visible peak in either TYR or HGA transition windows. Integration of background noise / chromatography gave a calculated percentage area of the LLOQ of <1.5% for both TYR and HGA in acidified and non-acidified urine. The IS were calculated as <0.7% for both d_2-TYR and $^{13}C_6$-HGA in both acidified and non-acidified urine matrices.

2.4.10 AKU urine samples

Three patients were analysed to demonstrate the application to AKU. The creatinine normalised HGA in the three samples pre and post treatment is displayed in table 2.3. Urine HGA suppressed in all three patients at three months with 90.5%, 99.0% and 92.2% respectively. There was a concurrent rise in urine TYR secretion, as anticipated with the use of NTBC. Urine TYR

was variable and there is an additional dietary influence as evident particularly at baseline.

Patient	Urinary HGA µmol/mmol creatinine			
	Baseline	3 months	6 months	1 year
AKU1	2,135	203	141	61.2
AKU2	2,868	27.3	7.9	13.5
AKU3	2,520	196	53.6	102
	Urinary TYR µmol/mmol creatinine			
	Baseline	3 months	6 months	1 year
AKU1	34.2	245	246	218
AKU2	6.9	93.3	73.1	97.1
AKU3	9.9	71.9	77.8	110

Table 2.3: Urinary concentration of HGA and TYR in AKU patients, pre and post-NTBC treatment.

2.5 Discussion

A method has been validated for the simultaneous quantitation of urine TYR and HGA. To date, this is the first published method for quantitation of TYR and HGA in urine. The method was validated with a simple sample preparation across a short, seven minute chromatographical separation. The method has been demonstrated as sensitive and specific with favourable accuracy and precision performance. The validation performance of the method satisfies that of key validation guidelines (EMA guidelines, 2011; Honour, 2011; FDA Bioanalytical Method Validation, 2018). Data from the National AKU Centre at Liverpool, UK (unpublished) and previously reported trial data (Introne et al., 2011) demonstrates that HGA is excreted in mmol/L concentration in AKU patients. Once they are commenced upon NTBC the levels drop to µmol/L, therefore the assay was evaluated over the entire HGA concentration range expected. The calibration curve consists of six standards covering this range and as demonstrated, exhibits an R^2>0.995 for both TYR and HGA with minimal inter-assay variability. In addition, recovery post-dilution proved that the linearity can be extended if required as dilution integrity was maintained up to a 1 in 10 dilution. Dilution greater than this was not examined as matrix integrity should be maintained. Data included on three patients with AKU demonstrates the suitability of the validated assay for analysing urine metabolites both pre- and post-NTBC treatment.

To maintain assay integrity, the calibration standards and QC pools have been matrix-matched, in either acidified or non-acidified urine. The variation of matrix was determined to be minimal ensuring reproducibility batch to batch with regards to the matrix. Although many methods utilise an aqueous matrix (phosphate buffered saline, water, organic solvents) as a base for in-house calibration standards, this does not contain other key constituents present in patient samples e.g. creatinine, urea, protein and electrolytes. The authors advocate using a matrix as similar to the sample base to be analysed and it is a requirement of several key method validation guidelines (EMA guidelines, 2011; Honour, 2011; FDA Bioanalytical Method Validation, 2018). In a clinical laboratory environment, with increasing requirements for robust validation of LC-MS/MS methods and in the UK, the adherence to the

ISO 15189 standard requires documented method validation in-line with published validation protocols. Determination of the IS-normalised matrix factor demonstrated that the slight signal suppression seen with a urine matrix was corrected by the respective IS. It cannot be assumed that IS will always correct for matrix effects, as is sometimes evident, especially with deuterated IS, a phenomenon called differential matrix effects (Bunch et al., 2014).

A characteristic of urine in patients with AKU is that the urine darkens upon standing or alkalinisation, therefore urine samples have been collected under acidified conditions. The assay described herein has been validated for analysis of both acidified and non-acidified samples. Stability of both HGA and TYR in a non-acidified urine has been demonstrated as equal to that of the acidified urine matrix, over the stability period examined with no significant under or over recovery of the analytes of interest. Therefore urine samples could be collected without acid preservative and acidified upon receipt within the laboratory, within a 24 hr period, or analysed as non-acidified random samples, again within a 24 hr period.

Previously published data on excreted levels of HGA in AKU have utilised the method of Lustberg et al., 1971, which involves ultra-filtration of acidified urine samples which are then either analysed on a 1 in 100 dilution pre-treatment or neat, post-treatment with NTBC. The spectrophotometric method indirectly measures urine HGA by measuring benzoquinone-2-acetic acid, an oxidised derivative. This then undergoes adduct formation with diethylenetriamine, the product of which is measured spectrophotometrically. More recently a study has been published which used LC-MS/MS for quantitation of HGA (Wei-Yi et al., 2013). Although the inter- and intra-assay variability was comparable, the method utilised aqueous calibration standards and required nine minutes for separation and an additional six for re-conditioning of the column, resulting in a 15 min analysis time. In addition, despite commercial availability there was no IS used. As demonstrated, there was a slight signal suppression with a urine matrix, but the isotope labelled IS corrected for this, emphasizing the usefulness of matrix-matching and IS.

With regards to TYR, published methods using LC-MS/MS are available in urine, serum and whole blood (Felitsyn et al., 2004; la Marca et al., 2012) which are integrated into panel assays for paediatric metabolic disorders and HT-1.

2.6 Conclusion

In conclusion, an assay has been developed to enable rapid biochemical diagnosis of AKU when high HGA levels and normal urine TYR levels are characteristic. In addition the assay is suitable for analysis of samples when patients are on NTBC therapy, where suppressed levels of urine HGA and increased urine TYR excretion are determined. An advantage is that the method involves a simple sample preparation and both pre- and post-treatment samples can be analysed on the same assay. This is due to the sensitivity of the LC-MS/MS, the lack of carry over, and the precision of the assay. This method will pave the way for analysis of clinical trial samples to determine the efficacy and response to NTBC in the treatment of AKU.

Chapter 2A - Addendum

Modification and additional validation of urine LC-MS/MS method for HGA and tyrosine

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Declaration and acknowledgments

All work undertaken by Andrew T Hughes

In preparation

2A.1 Introduction

The urine methodology was established as the first part of this thesis and subsequently the first publication to arise; with the primary aim to measure urine HGA both for screening patients to confirm the diagnosis of AKU and also to enable monitoring once commenced upon NTBC.

The benefit of the combined method which initially only including TYR in urine, enabled a one sample, one assay approach with a short analysis time suitable for scaling up for large clinical trials. Although clinical information was reported for urine HGA concentrations in AKU, there was less reported information on urine HGA concentrations post-treatment, largely as NTBC was still used in small cohort clinical trials as detailed in table 1.4. During the course of method development and upon commencing the clinical trials, additional knowledge on the concentration of urine metabolites pre- and post-NTBC treatment have been gained. This has led to several adaptions to the original urine method which was published in 2014; all of which have been undertaken during the course of this PhD, namely:

1. Calibration range optimisation
2. Change of IS
3. Verification of column choice
4. Ion selection modification
5. Addition and validation of hydroxyphenyllactate (HPLA), hydroxyphenylpyruvate (HPPA) and phenylalanine (PHE) (Chapter 4)

2A.2 Materials

All materials and LC-MS/MS conditions are as described in 2.3.1 and 2.3.2 with the addition of d_4-TYR IS which was obtained from Sigma-Aldrich UK. Calibration standard preparation principles are described in sections 2.3.3 and 2.3.4 and these procedures were followed throughout, with any changes in final concentrations, originating from the super-stock standard concentration, thereby maintaining the integrity of the matrix. Additional columns utilised in section 2A.3.3 were from the in-house column pool which are used for initial column selection prior to full method validation.

2A.3 Methodology and Results

2A.3.1 Calibration range optimisation

When the initial method was established, the calibration range focussed on the urine HGA ranges anticipated at the time of diagnosis, which were reported in the literature at mmol concentrations (Phornphutkul et al., 2002; Suwannarat et al., 2005; Introne et al., 2011). Urine TYR was known to increase post-NTBC, the magnitude wasn't clear and limited dose studies provided minimal additional information. In the original method, two calibration curves were established (two full sets of calibration standards) to cover pre-treatment AKU patients and AKU patients on NTBC (table 2A.1).

Urine calibration curve AKU pre-treatment								
µmol/L	Cal 0	Cal 1	Cal 2	Cal 3	Cal 4	Cal 5	Cal 6	Cal 7
HGA	0	200	1200	4000	12,000	24,000	52,000	n/a
TYR	0	10	100	200	300	400	500	n/a
Urine calibration curve AKU during-treatment								
µmol/L	Cal 0	Cal 1	Cal 2	Cal 3	Cal 4	Cal 5	Cal 6	Cal 7
HGA	0	2	20	100	500	1000	2000	4000
TYR	0	10	20	100	200	800	1500	2000

Table 2A.1 Initial calibration curves for measurement of HGA and TYR pre-, and post-treatment with NTBC

Ongoing studies with NAC patients, revealed that urinary HGA levels for a 24 hr collection had a mean concentration of 20,557 ± 5,445 µmol/L (Milan et al., 2017, chapter 7). In conjunction with previous data a combined calibration curve was created (top standard 4000 µmol/L) and a robust dilution protocol used for samples greater than the top standard on initial analysis. A further caveat to dealing with higher calibration concentrations will be discussed later in section 2A.3.4.

2A.3.2 Change of IS

As discussed in the introduction (section 1.13), IS are a necessary commodity in any method where there could be a potential loss of metabolite efficacy. The choices available range from deuterated (d), ^{13}C and ^{15}N forms, where ^{13}C are preferable as they are usually part of a ring structure, therefore potentially more stable. Unfortunately, the d-form does have the potential to suffer stability issues and 'fall-off' the compound and thus alter its mass; a point that was of particular relevance and noted by Davison et al., (2013) when investigating stability issues with an in-house 5-HIAA assay. Owen et al., (2012) had also compared deuterated and ^{13}C IS in a testosterone assay, highlighting the potential issues with d-forms. The problem being that an altered mass can mimic the compound of interest and be recognised (incorrectly) by the MS, and significantly affect the concentrations throughout the analysis.

With this in mind, the choice was made to switch to the d_4 form from the d_2 IS as a four mass unit difference, should pose less of an issue should a 'd' fall off the structure. A $^{13}C_6$ form of TYR IS was not available at start of the clinical trial and method development and although they are now more commonplace, the change to a d_4 had already occurred. Changing from the initially evaluated d_2-TYR IS to a d_4- TYR IS was subsequently validated and verified. This was achieved, post tuning, by evaluating a set of calibration curves, QC's and known patient samples using the two different IS and comparing the concentrations. The transitions used for d_4-TYR were a quantifier of 186>140 and a qualifier product ion of 186>127. The graph in figure 2A.1 shows a favourable R^2 value for the urine assay therefore assuring the new IS was acceptable to validate and subsequently introduce as a replacement.

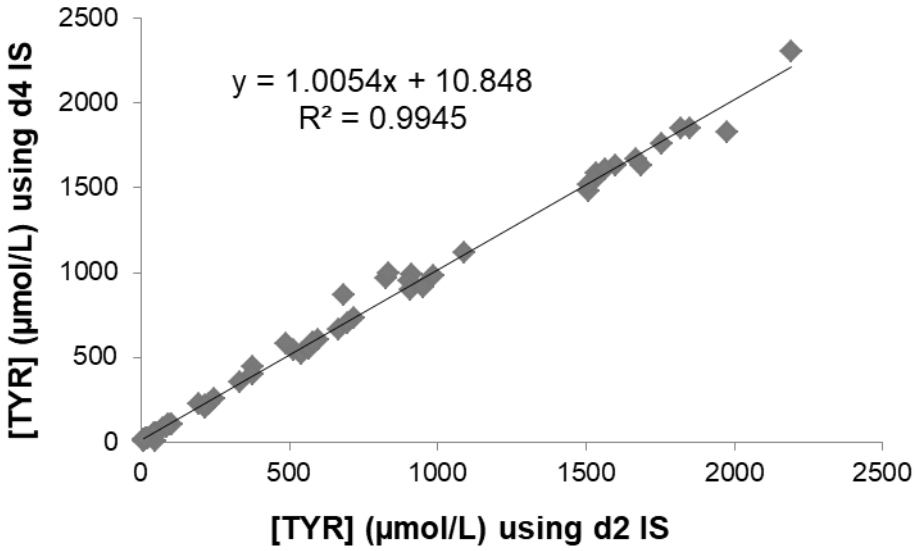

Figure 2A.1: Linear regression plot to determine the correlation of d_4 IS for the urine TYR assay

2A.3.3Verification of column choice

As previously mentioned, the choice of column is not an exact science but a sound knowledge of chemistry as well as column informatics is advantageous. The choice of the Waters Atlantis C18 100 mm x 3.0, 3µm was partly based on a previous experience with a similar chemical structure to the drug NTBC. That compound was the antiretroviral drug efavirenz. Both have a triple fluorinated group attached to a ring structure. Efavirenz did not separate very well using a standard C18 or functional group columns, with peak splitting, broadening or in some cases not being retained at all (unpublished data). Logic stated therefore that a column with more polar retentive functional groups would be the answer, which proved the case. With this information at hand, the Atlantis C18 was the column of choice for the work undertaken within this thesis.

Further enforcement of this column choice was verified by experiments injecting TYR, HGA, HPPA, HPLA, NTBC and corresponding IS down a selection of available columns to test, see figure 2A.2 and table 2A.2. There was significant variance in retention of metabolites with all the columns tested, with the majority showing poor retention and HILIC columns showing

poor resolution. The Atlantis C18 column was the only one that resolved HPPA to any satisfactory standard.

Even with acceptable degrees of metabolite separation, the poor retention of many columns dissuades from their use in that within the first one to two minutes of any run lies an area of significant ion suppression where up to 90% of a compound could be lost. This is of particular detriment to lower points on the calibration curve where sensitivity is of the utmost importance. It should be noted that the established gradient and run-time was maintained throughout therefore although NTBC isn't detected in runs where the compounds elute later, run-time wasn't expanded to monitor elution pattern.

Name	Stationary Phase	Separation Mode	Dimensions
Waters Atlantis	dC18 3 µm	Reversed phase	3.0 x 100mm
Waters Atlantis HILIC	Silica 3 µm	Hydrophilic liquid interaction	3.0 x 100mm
Waters X bridge HILIC	BEH 3.5 µm	Hydrophilic liquid interaction	3.0 x 100mm
Waters Phenyl-hexyl	CSH 1.7 µm	Reversed phase	3.0 x 50mm
Waters Sunfire	C18 3.5 µm	Reversed phase	2.1 x 50mm
Phenomenex Kinetex	C18 2.6 µm	Reversed phase	3.0 x 50mm
Phenomenex Bi-phenyl	Core shell 2.6 µm	Reversed phase	4.6 x 50mm
Phenomenex Phenyl-hexyl	Core shell 2.6µm	Reversed phase	4.6 x 100mm
Agilent Zorbax	C14 3.5 µm	Reversed phase	4.6 x 50mm
Agilent Zorbax extend	C18 3.5 µm	Reversed phase	4.6 x 50mm
Agilent Poroshell 120	Silica 2.7 µm	Reversed phase	4.6 x 50mm
Fortis C18	C18 5µm	Reversed phase	4.6 x 100mm
Fortis H_2O	C18 5 µm Polar endcapped	Reversed phase	4.6 x 100mm

Table 2A.2: Column characteristics used to assess separation of AKU metabolites

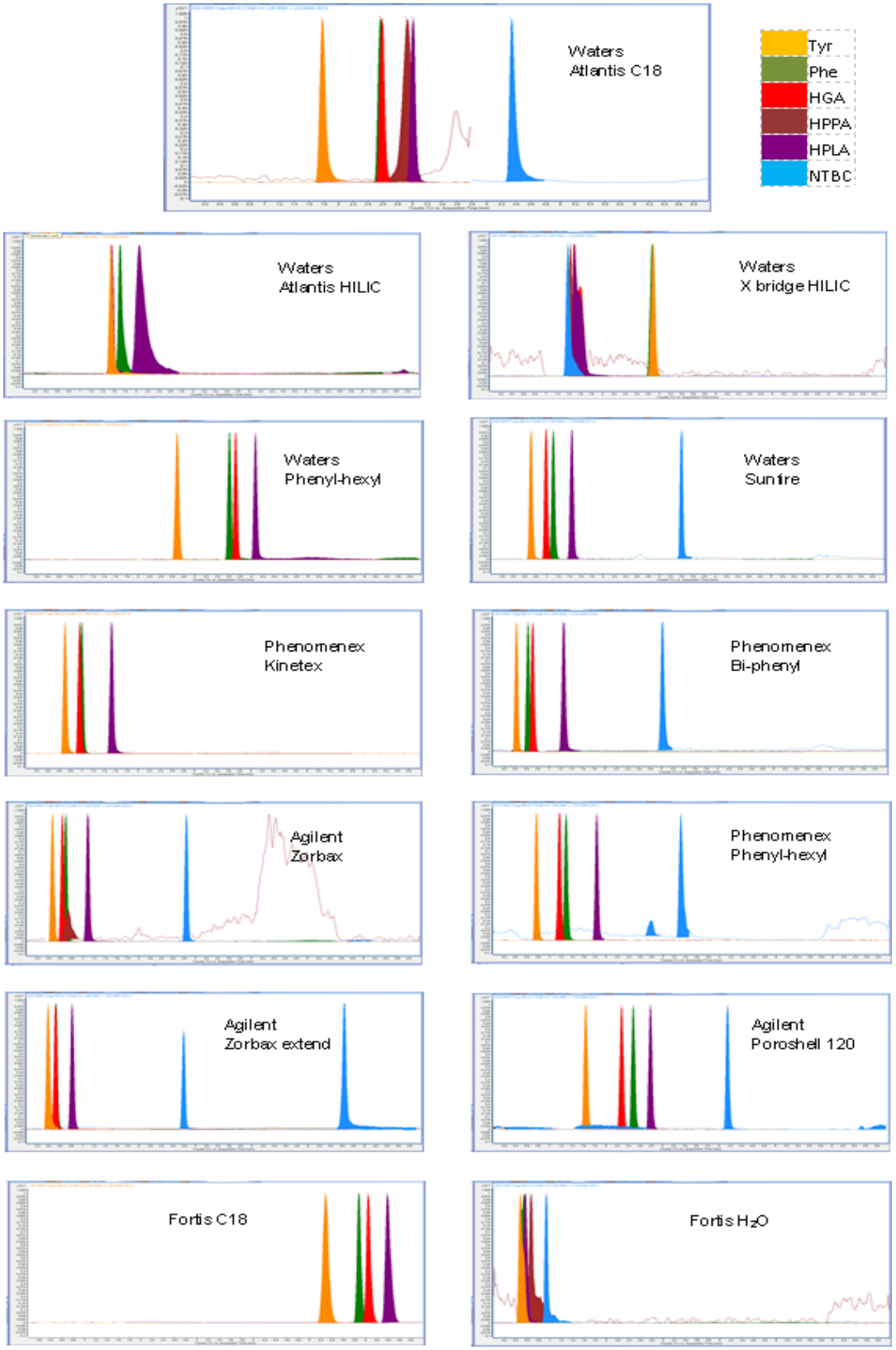

Figure 2A.2: AKU metabolites through selected columns. All analytes have been scaled to 100% to enable ease of comparison.

2A.3.4Ion selection modification

A question posed during mass spectrometry optimisation is should one always find the largest, most dominant product ion ?

The answer is - it depends. More is not always better, as was evidenced during the TYR studies, particularly post-NTBC when concentrations increased ten fold in urine on a 2 mg daily dose (Milan et al., 2017, Chapter 7). This compound ionises well and fragments into many product ions as demonstrated in table 2A.3.

Transition	Area	Collision Energy	Quant & Qual
182 > 107	2537283	38	
182 > 147	4234514	12	
182 > 95	6654825	30	
182 > 65	7677307	50	
182 > 77	7972744	48	
182 > 119	9174415	18	Qual (New)
182 > 123	11282325	16	Quant (New)
182 > 165	18669278	6	
182 > 91	22613255	32	Qual (Initial)
182 > 136	23568040	14	Quant (Initial)

Table 2A.3: Ion transitions and areas for top calibrator for urine TYR (3000 µmol/L).

The table shows the initial quantifier ion; 182>136 and qualifier ion; 182>91 with their corresponding areas for the urine TYR top cal. The calibration curves were linear and the assay was functioning within specification. During a routine PM (preventative maintenance service) parts were renewed, the quadropoles were cleaned, a new capillary fitted and coincidentally it was time for column replacement. The first assay post PM to check system suitability is displayed in figure 2A.3.

The areas significantly increased to result in a classic case of “saturation” where the MS receives a high influx of ions above what it can detect. This resulted in flattening of the curve at the upper end, poor R^2 values and failed performance (recovery) of the highest calibration standard. One solution without adjusting injection volumes or dilution ratio was to select less potent ions.

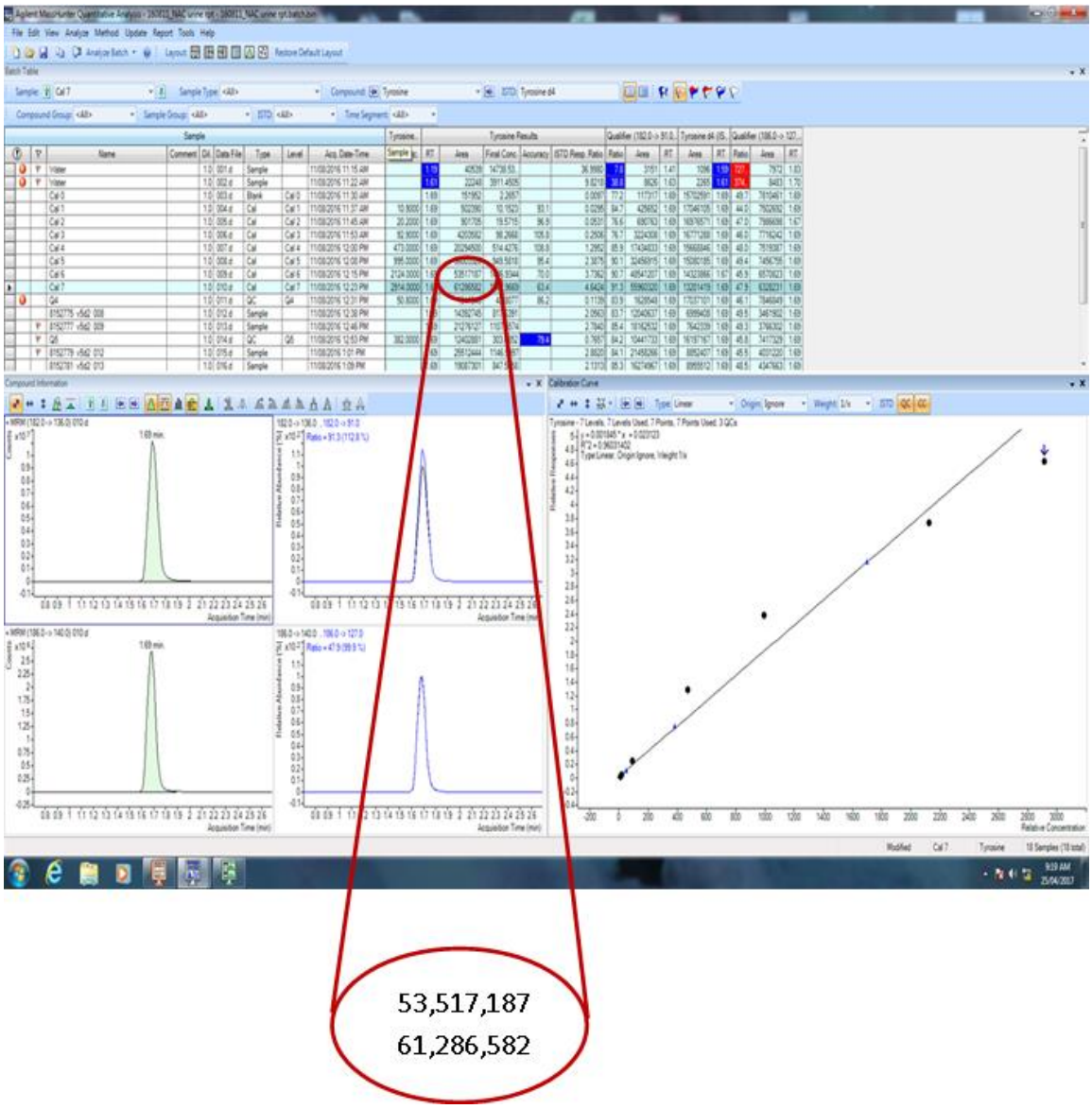

Figure 2A.3: Calibration curve for urine TYR post-PM on LC-MS/MS.
The areas in the circle demonstrate linearity has been lost with the top two calibration standards of 2200 µmol/L and 3000 µmol/L.

Table 2A.3 demonstrates acceptable areas for many product ions of the 182 molecular ion and therefore two 'lesser' ions were selected namely 182>123 for the quantitation ion and 182>119 for the qualifier ion. Reanalysis of the same samples resulted in a return to linear calibration curves. Of note this action of ion-deselection was not deemed necessary for the serum assay (Chapter 3) as the levels of TYR are lower, even post-NTBC and hence there was no saturation of the calibration curve observed.

Deselection of the TYR ions was carried out in conjunction with evaluating and replacing the d_2 IS with a d_4 IS. Figure 2A.4 shows the data comparison using the original d_2 IS against the new d_4 IS and alternative less abundant product ions. A good correlation is shown allowing us progress to figure 2A.5 which highlights the comparison between the old and new selected product ions using the new d_4 IS in full.

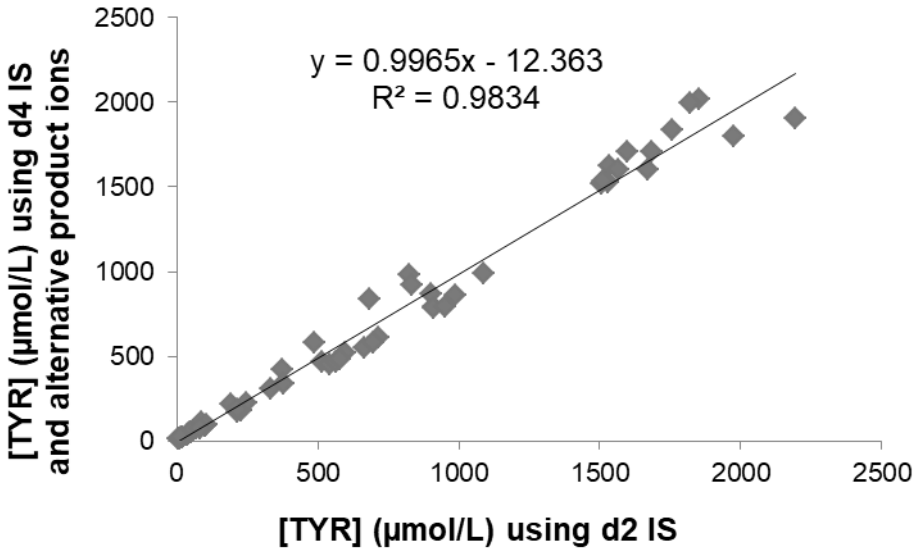

Figure 2A.4: Data plot to determine the acceptability of d_4 IS vs the d_2 IS and the introduction of alternative product ions for the urine TYR assay

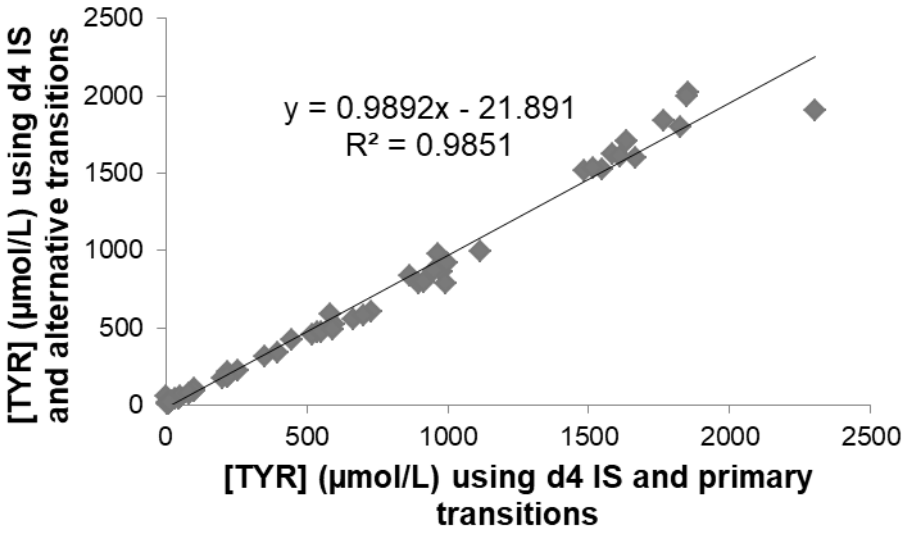

Figure 2A.5: Data plot to determine the acceptability of d_4 IS in full and the introduction of alternative product ions for urine TYR assay

2A.4 Concluding comments

It can be noted that there was a successful introduction and replacement to improve the existing assay with an IS that would be less prone to causing interference issues, as previously mentioned. However, only with the full introduction of the more reliable ^{13}C IS will problematic issues with deuterium loss be fully alleviated. It should be noted that most ^{13}C IS are $^{13}C_6$ on ring structures, however there are some with only 2 or 3 ^{13}C. These IS would suffer similar issues as d_2 IS in that there are naturally occurring ^{13}C forms of compounds and although approximately 1.1% in natural abundance, at the lower measuring range, this may reduce sensitivity.

The justification in column selection for this thesis was emphasised with the comparison experiments that were carried out, however with the hundreds of columns available on the market for dedicated chemical analyses, it would take a monumental study to compare and contrast to find the ideal. What was selected for this thesis proved to be more than capable for the analytes required and the linearity and resolution required.

Finally, the work carried out to select alternative ‘lesser’ product ions was deemed necessary due to the saturation seen for the TYR urine calibration curve, therefore emphasising the fact that more is not always better, even in the world of LC-MS/MS. In conclusion, the original assay from the 2014 publication has been improved and justified to provide an even more solid baseplate for AKU patient analysis.

Chapter 3

Serum markers in alkaptonuria: Simultaneous analysis of homogentisic acid, tyrosine and nitisinone by liquid chromatography tandem mass spectrometry

Running Title: Serum markers of alkaptonuria by LC-MS/MS

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Declaration and acknowledgments

Preparation of calibration standards and quality controls, full method validation and data interpretation was all performed by Andrew T Hughes.

Jean Devine and Jeannette Usher are acknowledged for patient sample collection.

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3.1 Abstract:

Background: Alkaptonuria (AKU) is a rare debilitating autosomal recessive disorder of tyrosine (TYR) metabolism, where deficiency of homogentisate 1,2-dioxygenase results in increased homogentisic acid (HGA). HGA is deposited as an ochronotic pigment in connective tissues, especially cartilage leading to a severe, early-onset form of osteoarthritis, increased renal and prostatic stone formation and hardening of heart vessels. Treatment with the orphan drug, nitisinone (NTBC), an inhibitor of 4-hydroxyphenylpyruvate dioxygenase (HPPD) has been shown to reduce urinary excretion of HGA.

Method: A reverse phase liquid chromatography tandem mass spectrometry (LC-MS/MS) method has been developed to simultaneously analyse serum HGA, TYR and NTBC. Using matrix-matched calibration standards, two product ion transitions were identified for each compound (HGA, TYR, NTBC) and their respective isotopically labelled internal standards (IS) ($^{13}C_6$-HGA, d_2-TYR, $^{13}C_6$-NTBC).

Results: Intra-batch accuracy was 94-108% for HGA; 95-109 % for TYR and 89-106% for NTBC; inter-batch accuracy (n=20) was 88-108% for HGA; 91-104% for TYR and 88-103% for NTBC. Precision, both intra- and inter-batch was <12% for HGA and TYR; <10% for NTBC. Matrix effects observed with acidified serum were normalised by the IS (<10% CV). HGA, TYR and NTBC proved stable after 24hr at room temp, three freeze-thaw cycles and 24hr at 4°C. The assay was linear to 500 µmol/L HGA, 2000 µmol/L TYR and 10 µmol/L NTBC; increased range was not required for clinical samples and no carry-over was observed.

Conclusions: The method developed and validated shows good precision, accuracy and linearity appropriate for the monitoring of AKU patients, pre and post-NTBC therapy.

3.2 Introduction

Alkaptonuria (AKU) is a rare autosomal recessive disorder (OMIM # 203500) of the tyrosine (TYR) degradation pathway (Figure 3.1). Deficiency of the third enzyme in the catabolic pathway, homogentisate 1,2-dioxygenase, results in accumulation of homogentisic acid (HGA). HGA is excreted in gram quantities in the urine, equivalent to mmol/L concentrations, and circulates at lower concentrations in the serum (µmol/L). Ancillary pathways of HGA metabolism result in oxidation of HGA to benzoquinones (BQA), which polymerise and bind to connective tissue proteins, in particular cartilage (O'Brien et al., 1963; Keller et al., 2005; Helliwell et al., 2008; Taylor et al., 2012; Ranganath et al., 2013). This process leads to ochronosis – a blue-black discolouration of the connective tissues, the mechanism of which remains unclear. Ochronotic pigmentation causes early onset degenerative arthritis of the spine and large weight bearing joints, leading to increased pain and premature joint replacement (Phornphutkul et al., 2002; Taylor et al., 2012; Ranganath et al., 2013). Aortic stenosis has been described as a cardiac complication of the ochronosis process (Hiroyoshi et al., 2013; Thakur et al., 2013) and can lead to aortic valve replacement. Additionally, an increased incidence of kidney stone formation is reported and in males, an increase in prostate stones (O'Brien et al., 1963; Phornphutkul et al., 2002). The formation of BQA results in additional formation of reactive oxygen species and free radicals, which are suggested to play a significant role in the aetiology of AKU arthritis (Martin and Batkoff, 1987).

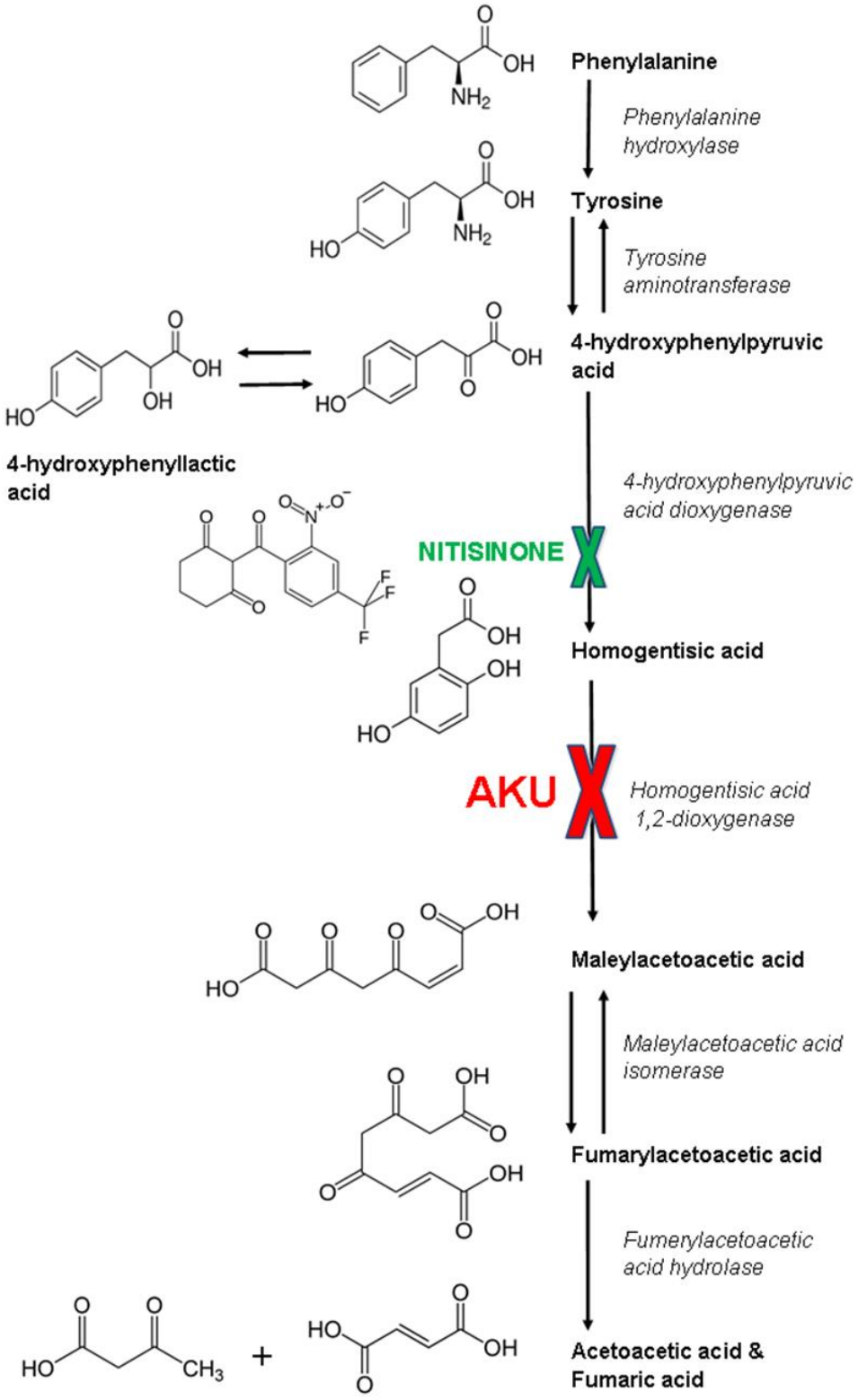

Figure 3.1: TYR degradation pathway

Current therapy for AKU is palliative, predominantly analgesia and arthroplasty. However there have been several reported trials of nitisinone 2-(2-nitro-4-flluromethylbenzoyl)-1, 3-cyclohexanedione (NTBC) (Phornphutkul et al., 2002; Suwannarat et al., 2005; Introne et al., 2011; Preston et al., 2014). NTBC is a potent inhibitor of the second enzyme in the TYR pathway, p-hydroxyphenylpyruvic acid dioxygenase which has been shown to lower circulating HGA and completely prevent pigmentation in AKU mice (Preston et al., 2014). NTBC is approved for treatment of hereditary tyrosinaemia 1 (HT-1) (OMIM #276700) and is now considered first-line treatment, replacing liver transplantation (McKiernan, 2006; Bartlett et al., 2014). With regards to AKU, NTBC is currently being used at the National Alkaptonuria Centre (NAC) (Royal Liverpool and Broadgreen University Hospital Trust, UK) as well as in a large multi-centre clinical trial (DevelopAKUre Clinical Trials – www.akusociety.org) to evaluate its effectiveness in the treatment of AKU.

Previous methods for the measurement of serum NTBC have used high performance liquid chromatography (HPLC) with photometric detection (Bielenstein et al., 1999), capillary electrophoresis (Cansever et al., 2010) and liquid chromatography tandem mass spectrometry (LC-MS/MS) (Herebian et al., 2009; Prieto et al., 2011; Davit-Spraul et al, 2012). Combined methods suitable for monitoring HT-1 have measured NTBC with several amino acids and metabolites in blood spots, suitable for paediatrics (la Marca et al., 2012; Hsu et al., 2013). To date there has been no published method for simultaneously monitoring NTBC, HGA and TYR concentrations in serum. The diagnosis of AKU relies on clinical presentation and evaluation and analysis of both urine and serum HGA.

Treatment with NTBC, whilst suppressing HGA production, circulation and secretion, results in a rapid increase in serum TYR concentrations, hence monitoring is essential for patient safety. Additionally circulating HGA is important pathophysiologically in AKU, due to the conversion to the damaging ochronotic pigment, therefore analysis and monitoring is novel and crucial for clinical management. A method for simultaneously monitoring urine HGA and TYR has previously been described (Hughes et al., 2014).

Herein, we describe development and validation of an LC-MS/MS method for the simultaneous measurement of serum HGA, TYR and NTBC concentrations.

3.3 Materials and methods

3.3.1 Chemicals and materials

TYR, TYR isotope-labelled internal standard (IS) (d_2-TYR) and HGA were obtained from Sigma-Aldrich UK. HGA isotope-labelled IS $^{13}C_6$-HGA was obtained from Larodan Fine Chemicals (Sweden). NTBC was generously provided by Swedish Orphan Biovitrum AB. NTBC isotope-labelled IS $^{13}C_6$-NTBC was obtained from Toronto Research Chemicals (Canada). LC-MS/MS grade methanol and acetonitrile were obtained from Sigma Aldrich, UK. Formic acid was obtained from Biosolve. Water was purified in-house by DIRECT-Q 3UV Millipore water purification system. All dilutions and sample preparation was performed in glass. Oxygen free nitrogen was supplied by a Genius 2010 Peak nitrogen generator.

3.3.2 LC-MS/MS Analysis

All analyses were performed on an Agilent 6490 triple quadropole LC-MS/MS with Jet-Stream® electrospray ionisation (ESI-MS/MS) equipped with an Agilent 1290 infinity pump and autosampler. All data processing both qualitative and quantitative analysis was performed using Mass Hunter software package.

Chromatographic separation was performed on an Atlantis C18 column (100 mm x 3.0 mm, 3 µm, Waters, UK) maintained at 35°C. Initial gradient conditions were 80:20 water:methanol with 0.1% formic acid (v/v) increasing linearly to 10:90 in 2.5 min. The mobile phase was maintained for a further 1.1 min, increased to 100% methanol for 1 min and then returned to starting conditions (flow rate 0.4 mL/min). The column was reconditioned for 2 min prior to the next injection. 2 µL of sample was injected with a total run-time of 7.0 min. Optimum operating ESI conditions were gas temperature 150°C (nitrogen), gas flow 17 L/min; nebulizer pressure 40 psi; sheath gas

temperature 320°C and sheath gas flow 12 L/min. Capillary voltages were optimised to 3500V in positive mode and 2500V in negative mode with equal nozzle voltages (1500V) in both modes. The iFunnel parameters were optimised in both negative and positive mode as 60V for low pressure RF and 110V for high pressure RF.

3.3.3 Preparation of standards, reagents and QC samples

Super-stock standard solutions of HGA and TYR were prepared in 0.5N sulphuric acid (H_2SO_4) and deionised water at concentrations of 100 and 400 mmol/L respectively. NTBC was stored at a concentration of 1 mmol/L in 50:50 acetonitrile:deionised water respectively. These were stored at -20°C.

L-TYR-(phenyl-3,5-d_2) super-stock was prepared at 100 mmol/L in 0.1N H_2SO_4. An intermediate stock of 1 mmol/L was prepared by dilution in deionised water, and stored at -20°C. $^{13}C_6$-HGA was reconstituted at 1 mg/mL (5.75 mmol/L), in deionised water with an intermediate 250 µmol/L stock in deionised water, stored at -20°C. An intermediate stock of 1 µmol/L $^{13}C_6$-NTBC was prepared by dilution in 50:50 acetonitrile deionised water, and stored at -20°C.

To ensure matrix-matched calibration, super-stock aqueous standard solutions were added to a serum matrix base pool (steroid depleted serum, BBI Solutions, SF236-7). The serum base was assayed prior to preparation to verify minimal endogenous TYR, suitable for standard addition (TYR assayed as ≈1.5 µmol/L). The super-stock standards were diluted to intermediate stocks at ten times the final required concentration.

Studies have identified that HGA is unstable in an alkaline environment, leading to BQA formation and subsequent ochronotic pigment; urine collections require acidification to stabilise the HGA and it has been suggested that serum samples may require deproteinisation (Bory et al., 1989). With this in mind, the serum assay described herein was developed to ensure stability of HGA, with serum acidification and deproteinisation (perchloric acid) and comparison with deproteinisation only (acetonitrile).

These were then added to serum pool in a ratio of 1:9 which was then treated with a) 60% perchloric acid (5.8N) (ratio 1:11) = acidified serum or b) acetonitrile 4:1, mix and centrifuge; 10 µl supernatant removed, dried and reconstituted in 2 mL combined IS solution (non-acidifed serum). Both preparation methods resulted in combined calibrators with final concentrations of TYR 10-2000 µmol/L, HGA 15-500 µmol/L and NTBC 0.5-10 µmol/L and dilution of 1:1000 sample in IS solution.

A combined IS solution was used as the sample diluent, containing final concentrations of 0.2 µmol/L $^{13}C_6$-HGA, 2 µmol/L d_2-TYR and 2 nmol/L $^{13}C_6$-NTBC in 0.1% formic acid (v/v). IS concentrations were titrated to ensure a response (area of analyte/area of IS) of approximately 1.0 at the mid-point of the calibration curve to ensure adequate sensitivity at the lower end of the calibration range whilst ensuring accuracy across the dynamic assay range. Quality controls (QC) were prepared independently, from separate stocks pools, in the same manner as the standards. All samples, calibrators and QC were assayed on a 1 in 1000 dilution with the IS solution. Calibrators and QC were spiked from separate stocks of HGA, TYR and NTBC.

3.3.4 Assay Validation

The assay was validated using in-house protocols based on published guidance (EMA guidelines, 2011; Honour, 2011; FDA Bioanalytical Method Validation, 2018). Method validation evaluated linearity, accuracy, imprecision, lower limit of quantification (LLOQ), matrix effect, carry-over and stability. Comparison of TYR was performed with a HPLC, post-column ninhydrin detection method in a local referral laboratory.

3.3.5 Patient sample analysis

Three patients with AKU were analysed pre- and post-NTBC therapy to demonstrate the suitability of the assay. The serum samples were collected and acidified with perchloric acid (as per the acidified assay protocol) and analysed with the appropriate calibration standards.

3.3.6 Statistical analysis

Data was compared using unpaired t-test, computed on Instat (GraphPad, version 3.0). Regression analysis was performed using Analyse-it package for Microsoft Office.

3.4 Results

3.4.1 Identification and separation of HGA, TYR and NTBC

Quantifier and qualifier product ion transitions and their respective collision energies were determined for each precursor ion (table 3.1). For optimal sensitivity, mass spectrometric detection was performed by multiple reaction monitoring (MRM) mode, monitoring in positive ionisation mode for TYR, TYR-d_2, NTBC and $^{13}C_6$-NTBC and negative ionisation mode for HGA and $^{13}C_6$-HGA. Additional parameters were optimised as stated in the LC-MS/MS method analytical section, to ensure suitable operating conditions for method validation.

Figure 3.2 shows a typical chromatogram for the primary product ion (quantifier) for each precursor, demonstrating the chromatographic separation of HGA, TYR and NTBC in the serum matrix. No difference was observed in retention times of HGA, TYR or NTBC upon comparison of acidified and non-acidified serum matrices.

	Ionisation mode	Product Ion 1 (Quantifier)	Collision Energy (V)	Product Ion 2 (Qualifier)	Collision Energy (V)
HGA	Negative	167>122	22	167>108	20
$^{13}C_6$-HGA	Negative	173>128	22	173>114	26
TYR	Positive	182>136	14	182>91	32
d_2-TYR	Positive	184>138	12	184>125	18
NTBC	Positive	330>218	14	330>126	32
$^{13}C_6$-NTBC	Positive	336>126	32	336>218	16

Table 3.1: Parameters for mass spectrometry detection of TYR, NTBC and HGA.

3.4.2 Method Validation

Calibration standard curves, six points plus serum blank, (not a true blank due to endogenous TYR) exhibited a good fit over the range examined with minimal inter-assay variability. Linear regression (1/x weighting factor) of the perchloric acid precipitated serum (acidified serum) HGA R^2=0.997 (n=9), TYR R^2=0.998 (n=9) and NTBC R^2=0.993 (n=9). In the non-acidified serum assay HGA R^2=0.997 (n=8), TYR R^2=0.998 (n=8) and NTBC R^2=0.997 (n=8). The concentration ranges covered were HGA 15-500 µmol/L, TYR 10-2000 µmol/L and NTBC 0.5-10 µmol/L. The gradient on the calibration line showed equal consistency.

Comparison of NTBC values obtained between non-acidified and the acidified assay were comparable (R^2=0.970; y=1.014*x*+0.003; 95% confidence interval (CI) for slope 0.94 to 1.09). This was verified by analysing 25 serum samples obtained from patients with AKU, on treatment, obtained both acidified and non-acidified. The correlation of results obtained on the appropriate standard curve was R^2=0.949 for HGA (y=1.005*x*-0.09; 95% CI for slope 0.91 to 1.10) and R^2=0.989 for TYR (y=0.970*x*+13.6; 95% CI for slope 0.93 to 1.01).

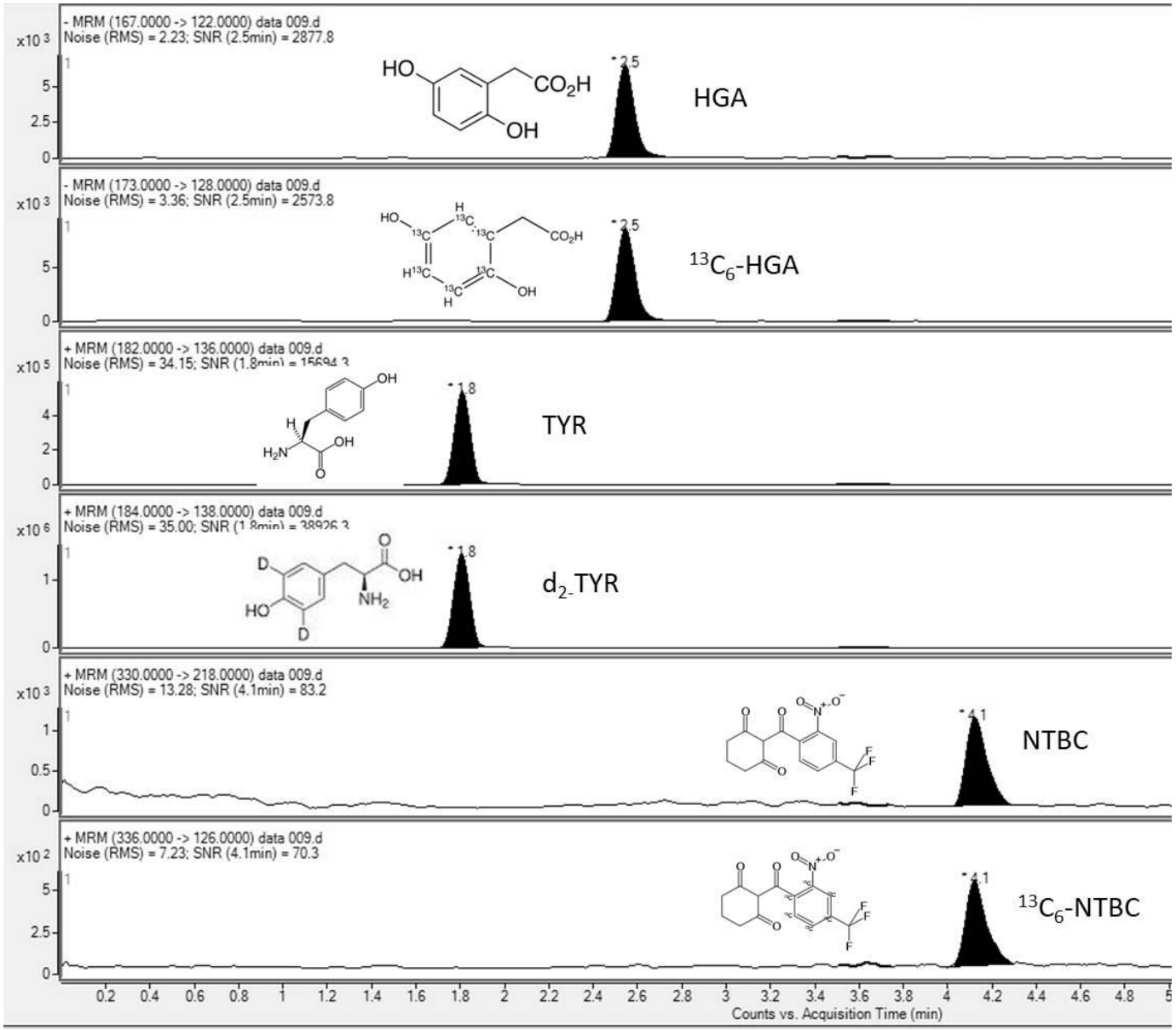

Figure 3.2: Chromatogram of HGA, TYR and NTBC respectively (200, 810 and 4 µmol/L) with their respective IS.

Intra- and inter-assay accuracy was determined (table 3.2) in both acidified (perchloric acid precipitated) and non-acidified serum matrices. Results are represented as percentage recovery of a nominal amount of HGA, TYR or NTBC spiked into the serum matrix. Samples were then analysed against their respective calibration curves.

In acidified (perchloric acid precipitated) serum, intra-assay accuracy was 94-108% for HGA (n=6), 95-109% for TYR (n=6) and 89-106% for NTBC (n=6) with inter-assay accuracy of 88-108% for HGA, 91-104% for TYR and 88-103% for NTBC (n=20). In the non-acidified serum the intra-assay accuracy (n=6) was 88-107% for HGA, 93-110% for TYR and 93-108% for NTBC, similarly inter-assay accuracy (n=20) was 86-110% for HGA, 98-114% for TYR and 84-114% for NTBC.

Imprecision (%CV), both intra- and inter-assay was determined in both acidified and non-acidified serum matrices across a broad concentration range, covering pre- and post-NTBC therapy concentrations. In the acidified serum matrix, intra-assay precision (n=6) was <5% for HGA across 15-500 µmol/L and 8.6% at 3 µmol/L; <5% for TYR (60-2000 µmol/L) and 9.4% at 10 µmol/L; <6% for NTBC (1.0-10 µmol/L) with 10.3% at 0.5 µmol/L. Inter-assay precision (n=21) in acidified serum was <8% for HGA (15-500 µmol/L) and 11.7% at 3 µmol/L; <5% for TYR (60-2000 µmol/L) and 11.7% at 10 µmol/L; <10% for NTBC across whole range 0.5-10 µmol/L.

HGA				
	Acidified Serum Matrix		**Non-Acidified Serum Matrix**	
Expected Conc µmol/L	Intra-assay	Inter-assay	Intra-assay	Inter-assay
30	102.7±3.2 (3.1)	102.4±4.9 (4.8)	98.8±6.2 (6.3)	102.2±5.4 (5.3)
200	100.8±4.1 (4.1)	92.9±3.0 (3.2)	94.3±4.7 (5.0)	97.9±5.9 (6.0)
400	97.8±3.2 (3.2)	97.1±4.8 (4.9)	96.7±4.3 (4.7)	100.8±2.8 (2.8)
TYR				
	Acidified Serum Matrix		**Non-Acidified Serum Matrix**	
Expected Conc µmol/L	Intra-assay	Inter-assay	Intra-assay	Inter-assay
260	98.8±1.4 (1.5)	98.4±2.5 (2.5)	102.3±6.0 (5.8)	101.7±3.0 (2.9)
810	100.3±3.5 (3.5)	99.8±1.9 (1.9)	101.0±5.5 (5.4)	105.0±5.6 (5.3)
1560	95.4±3.0 (3.1)	96.8±3.2 (3.3)	98.4±1.7 (1.7)	101.1±2.4 (2.3)
NTBC				
	Acidified Serum Matrix		**Non-Acidified Serum Matrix**	
Expected Conc µmol/L	Intra-assay	Inter-assay	Intra-assay	Inter-assay
1.5	95.9±4.2 (4.3)	93.4±6.2 (6.7)	105.2±2.8 (2.7)	105.3±3.2 (3.1)
4	100.7±4.6 (4.6)	96.4±6.5 (6.7)	97.8±5.2 (5.3)	97.3±7.2 (7.4)
8	101.4±3.1 (3.0)	99.8±5.5 (5.5)	98.1±3.2 (3.3)	99.4±2.7 (2.7)

Table 3.2: Intra- (n=6) and Inter-batch (n=21) accuracy in acidified and non-acidified urine. Results are expressed as mean ± SD (percentage coefficient of variation).

Similar precision was seen in the non-acidified serum matrix for HGA with %CV <8% both intra- and inter-assay for HGA. Serum TYR exhibited slightly higher intra- and inter-precision in the non-acidified but <10% across the whole concentration range examined. NTBC was <7% for intra-assay and inter-assay precision across the concentration range 0.5-10 µmol/L.

The LLOQ, defined as the lowest calibrator which satisfies a CV ≤ 20%, (EMA guidelines, 2011; Honour, 2011; FDA Bioanalytical Method Validation, 2018) for HGA in both acidified and non-acidified serum was 3 µmol/L, for TYR 10 µmol/L and NTBC 0.2 µmol/L. Signal:noise at these concentrations was >100:1, so additional sensitivity is possible especially considering the small injection volume, but was not necessary from a clinical perspective for the assay described.

The matrix effect of both acidified and non-acidified serum was assessed across the concentration range for HGA, TYR and NTBC, with six individual serum matrices (both acidified and non-acidified). Results demonstrated a greater matrix effect in acidified serum for HGA and TYR (average 50% suppression), compared with non-acidified serum (HGA 25% and TYR 3% suppression). No matrix effect was seen for NTBC in either acidified or non-acidified serum matrices. The IS demonstrated similar suppression resulting in all analytes exhibiting a %CV of <10% for the IS normalised matrix factor, satisfying validation criteria (EMA guidelines, 2011; Honour, 2011; FDA Bioanalytical Method Validation, 2018).

Following injection of the top calibrator (500 µmol/L HGA, 2000 µmol/L TYR and 10 µmol/L NTBC) there was no clear visible peak at the retention time of HGA, TYR or NTBC, on five subsequent water injections. Integration of background noise / chromatography gave a calculated percentage area of the LLOQ of <1.5% for all analytes in both acidified and non-acidified serum. The IS were calculated as <0.5% for $^{13}C_6$-HGA, d_2-TYR and $^{13}C_6$-NTBC in both acidified and non-acidified serum matrices.

Stability was assessed following three freeze-thaw cycles, 24hr at room temperature and 24hr on-board stability (equivalent to 4°C) (table 3.3). There was no statistical significance between the acidified and non-acidified serum preparation on analyte stability over the period assessed (24hr) ($p > 0.05$). Repeat analysis of serum pools (n=3) over a 24hr period, stored at 4°C, demonstrated no significant deterioration in recovery across a 24hr period ($p > 0.05$).

Comparison of 50 serum samples (acidified upon collection) for TYR was performed against a referral laboratory assay (cation exchange chromatography using Biochrom amino acid analyser with post-column nihhydrin detection at 570 nm). The values ranged from 25 to 1200 µmol/L, representing pre and post-NTBC treatment. The line of best fit was $y=0.96x – 7.13$, $R^2=0.979$. EQA performance (ERNDIM, European Research Network for evaluation and improvement of screening, Diagnosis and treatment of Inherited disorders of Metabolism serum amino acid scheme) demonstrated satisfactory performance for serum TYR over a six month period (within ± 2SD of target and method mean).

Three freeze-thaw cycles			
	HGA	TYR	NTBC
Acidifed serum Matrix	100.6 ± 6.2	101.0 ± 0.97	96.9 ± 2.9
Non-acidifed serum matrix	102.7 ± 6.5	99.4 ± 4.0	98.9 ± 3.6
Room temperature (24hr)			
Acidifed serum Matrix	101.9 ± 5.6	101.2 ± 0.97	105.1 ± 3.9
Non-acidifed serum matrix	101.4 ± 2.5	94.8 ± 5.8	104.1 ± 7.4
4°C for 24hr			
Acidifed serum Matrix	96.6 ± 5.1	101.2 ± 1.6	94.6 ± 2.2
Non-acidifed serum matrix	98.6 ± 5.8	99.5 ± 2.1	99.3 ± 3.5

Table 3.3: Summary of stability data for HGA, TYR and NTBC in serum matrices. Results expressed as average recovery of nominal values over low, medium and high concentrations (n=15, 5 at each level).

Three patients with AKU were assayed, both pre- and post-NTBC to assess the suitability of the assay. Data is presented in table 3.4 demonstrating the rapid rise in serum TYR post-NTBC treatment. This was only done with acidified serum as this was the chosen mode of sample preservation. The data clearly shows a significant rise in serum TYR post-treatment with a concurrent suppression of serum HGA. The change in serum HGA is mirrored in urine HGA levels, with approximately 95% reduction in levels.

Patient	**Serum HGA µmol/L**		
	Baseline	3 months	6 months
AKU1	75.2	9.1	7.9
AKU2	30.2	8.7	9.4
AKU3	42.6	8.5	7.9
	Serum TYR µmol/L		
	Baseline	3 months	6 months
AKU1	82	705	465
AKU2	31	315	608
AKU3	41	541	497
	Serum NTBC µmol/L		
	Baseline	3 months	6 months
AKU1	<0.2	1.5	2.8
AKU2	<0.2	1.3	1.6
AKU3	<0.2	2.3	3.6

Table 3.4: Serum concentrations of HGA, TYR and NTBC in AKU patients, pre and post-NTBC treatment.

3.5 Discussion

A LC-MS/MS method has been validated for the simultaneous quantitation of serum HGA, TYR and NTBC. To date, this is the first published method for quantitation of all three analytes in a single method, in serum. A method with simple sample preparation and a seven minute chromatographical separation has been validated, which is sensitive and specific with favourable accuracy and precision performance.

To maintain assay integrity, the calibration standards and QC pools have been matrix-matched, in either acidified or non-acidified serum. The variation of matrix was determined to be minimal ensuring reproducibility batch to batch with regards to the matrix. Although many methods utilise an aqueous matrix (phosphate buffered saline, water, organic solvents) as a base for in-house calibration standards, this does not contain other key constituents present in patient samples e.g. proteins, electrolytes, urea and creatinine and as such may not mimic the performance seen by the patient sample matrix. The authors advocate using a matrix as similar to the sample base to be analysed and it is a requirement of several key method validation guidelines (EMA guidelines, 2011; Honour, 2011; FDA Bioanalytical Method Validation, 2018).

Determination of the matrix factor, demonstrated signal suppression with a serum matrix for HGA and TYR and a difference upon serum acidification. However, the isotopically labelled IS corrected for this suppression as evidenced by the IS normalised matrix factor. It cannot be assumed that IS will always correct for matrix effects, as is sometimes evident, especially with deuterated IS: a phenomenon called differential matrix effects (Bunch et al., 2014).

Validation of the assay was performed in both an acidified serum matrix and a non-acidified serum matrix (precipitated with acetonitrile). Previous studies with mice have suggested that HGA is unstable in alkaline urine and in unpreserved serum samples (Bory et al., 1989), hence the validation of an

assay for acidified serum. Stability has been assessed short-term, representative of routine sample collection and sample analysis time, but long-term stability is currently being assessed. Stability of HGA, TYR and NTBC were shown to have no significant difference over the time periods presented. Although little difference was observed, due to the previous evidence for long-term stability, acidified serum is the current sample of choice, especially considering long-term storage of trial samples and transport of sample to the NAC for analysis.

Previous methods for NTBC are for HT-1 monitoring which has a suggested therapeutic range of 20-120 µmol/L NTBC (Davit-Spraul et al., 2012); although there is no therapeutic target for AKU, patients on 2 mg daily have NTBC levels 2-4 µmol/L at steady state (un-published data from the National AKU Centre, 2mg NTBC post-six months treatment, n=38). The assay described herein is more sensitive than previously published methods, with a LLOQ of <0.2 µmol/L (CV<10%) in comparison with 0.75 µmol/L (intra-assay CV 12%, inter-assay CV 20%) (Davit-Spraul et al., 2012). Dried-blood spot analysis of NTBC by LC-MS/MS demonstrated similar performance characteristics albeit with a LLOQ of 0.3 µmol/L; however, precision was only examined at concentrations of >5 µmol/L (La Marca et al., 2012).

Data from the NAC in Liverpool, UK (unpublished[1]), and previously reported trial data (Introne et al., 2011) demonstrates that HGA is excreted in mmol/L concentration in the urine of patients with AKU. Once they are commenced upon NTBC the concentration drops to µmol/L. The effect of suppressing HGA is a concurrent rise in serum TYR. Table 3.4 demonstrates a typical biochemical response to NTBC with a snapshot of the study data. Serum TYR rises >10 fold by 3 months of NTBC therapy. Although data is not included, this rise occurs within the first week of NTBC therapy. The rapid and significant rise in TYR requires monitoring to ensure the safety and efficacy of NTBC therapy. In addition to metabolite measurement, all patients

[1] Note at the time this paper was published, this data was considered unpublished. This is now published (Milan et al., 2017) and is included and expanded upon in Chapter 8.

attending the National AKU Centre in Liverpool are treated and monitored with a low protein diet.

NTBC has been used in the treatment of HT-1, albeit at significantly higher doses up to 2 mg/kg compared with 2 mg once daily in AKU (McKiernan, 2006). Non-compliance with protein restriction in HT-1 has led to several reported ocular symptoms, including corneal crystals and opacities (Holme et al., 1998; Ahmad et al., 2002; Gissen et al., 2003). Similar corneal opacities have been reported in AKU (Introne et al., 2011; Stewart et al., 2014). In all cases, the corneal opacities resolved upon cessation of NTBC and normalisation of TYR levels. In addition, by measuring NTBC, the pharmacodynamics can be monitored.

NTBC is metabolised by the cytochrome P450 system and in using low doses for treatment, monitoring of concentrations can aid in understanding metabolism and in titration of therapy if required. Monitoring of urine HGA is a primary outcome measure in determining whether NTBC has adequately suppressed HGA as there is both renal clearance and renal secretion of HGA. Serum HGA falls as expected and this is mirrored in urine excretion levels. Monitoring of serum HGA is paramount in clinical management of AKU as circulating levels are key in the pathological development of the disorder when alternative pathways metabolise HGA to BQA, ultimately resulting in ochronosis.

In conclusion, an assay has been developed and validated which enables monitoring of the safety and efficacy of NTBC therapy in the treatment of AKU, applicable to both pre- and post-metabolite levels. In addition, although NTBC concentrations are significantly higher, in HT-1, the assay, with additional validation would lend itself to monitoring this and other metabolic disorders of the TYR degradation pathway.

Chapter 3A - Addendum

Additional validation of serum LC-MS/MS method for TYR, HGA and NTBC

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Declaration and acknowledgments

All work undertaken by Andrew T Hughes.

In preparation

3A.1 Introduction

At the very start of this assay development it was postulated that un-precipitated serum could be used rather than the need to protein crash in perchloric acid. However, as an LC-MS/MS purist and even though the dilution involved was 1 in 1000, there was still protein present with significant sample to sample variability to warrant not pursuing the plain serum option. In addition, no long-term evidence was available for serum HGA which was a key analyte in all the clinical trials; therefore to maintain procedures throughout, sample preparation was maintained as described with a perchloric acid crash (section 3.3.3). Although storing at -80^{o}C should preserve sample integrity for the longest, the stability was almost approached blind as long-term data would only be possible once the clinical trials were actually running.

Due to the concentration range of the main analytes initially validated in the serum assay (namely TYR, HGA and NTBC), the issues of saturation and calibration ranges did not require adjustment for the serum assay as previously described for the urine assay (Chapter 2 addendum).

Development of the combined serum assay enabled a snapshot of the metabolites in the pathway and their response to NTBC to be easily monitored with one sample. As previously discussed in Chapter 3, published assays for NTBC have been designed for monitoring hereditary tyrosinaemia type 1 (HT-1) patients where the therapeutic NTBC concentrations range from 20-120 µmol/L (Davit-Spraul et al., 2012) whereas initial data from the NAC (Milan et al., 2017, Chapter 7) demonstrated mean concentrations of 2.11 ± 0.64 µmol/L at 6 months and 1.05 ± 0.43 µmol/L at two years. Therefore, the assay validated was more sensitive than published methods for HT-1.

As with all assays with in-house calibration standards, careful monitoring of stability is required, especially long-term stability. Although the calibration standards were prepared in a matrix (steroid depleted serum), initial stability was carefully monitored by regular repeat analysis of samples and QC

performance. Calibration standards were also replaced and run-in using a standard in-house protocol as discussed below in section 3A.3.3.

With this knowledge in mind and to ensure the assay was operating optimally, the following reviews and adaptations have been undertaken during the course of this thesis:

1. Review of TYR transitions and saturation
2. Substitution of IS for TYR
3. Monitoring of NTBC calibration standards
4. One year stability of TYR and HGA
5. Addition of HPLA, HPPA and PHE into the combined serum assay (chapter 4)

3A.2 Materials

All materials are described in 3.3.1 with the addition of the d_4-TYR IS which was obtained from Sigma-Aldrich UK. Calibration standards are described in section 3.3.3 and those for the 'acidfied' assay were continued throughout the course of this thesis. Fresh super-stocks were prepared at 12 months and 24 months to determine initial stability and enable comparison across longer time windows for trial data.

3A.3 Methodology and Results

3A.3.1 Review of TYR transitions and saturation

Serum TYR concentrations ranged from within the reference range (21-87 µmol/L, Davison et al., 2015) to 594 ± 184 µmol/L at two years in the NAC patient cohort (Milan et al., 2017). The quantifier product ion (182>136) and qualifier product ion (182>91) were the same as initially used for the urine assay. As reported in 2A.3.4 this required modification for the urine assay as saturation was observed for the two highest urine standards. The serum assay calibration range for TYR initially validated to 2000 µmol/L was reduced to 1500 µmol/L in later sets of standards. No saturation has been

observed however the qualifier product ion was changed to 182>119 to support monitoring of quantifier:qualifier ratios with a meaningful number.

Transition	Area	Collision Energy	Quant & Qual
182 > 107	451267	38	
182 > 147	789887	12	
182 > 95	1280422	30	
182 > 77	1392816	48	
182 > 65	1422660	50	
182 > 119	1665265	18	Qual (New)
182 > 123	1991676	16	
182 > 165	3242416	6	
182 > 91	3729642	32	Qual (initial)
182 > 136	4153459	14	Quant

Table 3A.1: Ion transitions and areas for top serum calibrator for TYR (1500 µmol/L).

3A.3.2 Substitution of d_2-TYR IS with d_4-TYR IS

For reasons discussed in 2A.3.2. it was important to ensure the most appropriate IS was utilised for the assay. Commercially these are constantly evolving and improving, and choice is available. Performance, availability and cost are among many reasons why certain IS are utilised and although there is now a $^{13}C_6$-TYR IS available, it is still an order of magnitude more expensive (approximately £700 per 1g d_4-TYR and £8000 for 1g $^{13}C_6$-TYR). In methods with numerous IS this can significantly increase the cost of running the assay and has to be a consideration.

However, d_4-TYR was implemented with a quantifier of 186>140 and a qualifier product ion of 186>127. Samples were analysed using both IS (added separately) and data is displayed in figure 3A.1.

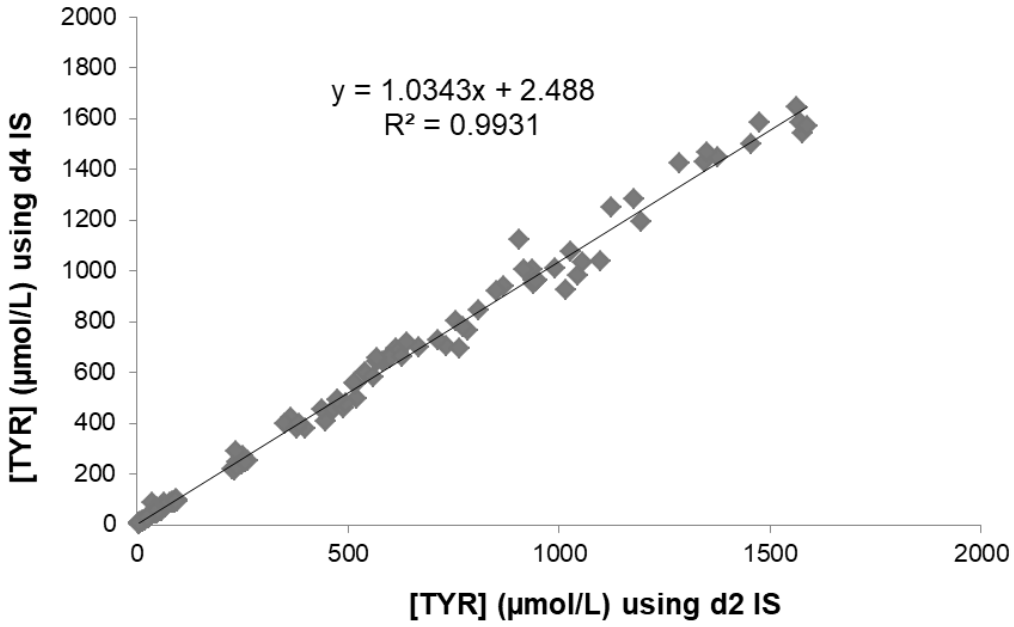

Figure 3A.1 Data plot to determine the acceptability of d_4 IS for serum TYR assay

With the change of IS as described the calibration curves showed no detriment of efficacy for the serum assay and patient comparison demonstrated no significant change.

3A.3.3 Calibrator verification and subsequent monitoring of NTBC

Preparation of calibration standards is described (section 3.3.3), however to verify their performance prior to implementation, they were analysed in duplicate on ten different assays and recovery against the nominal spike value was determined. Due to the lack of commercial standards and certified reference standards for the majority of analytes in this assay, confirmation of their assigned concentrations has to be undertaken robustly and in a standardised way. Various guidelines (EMA and FDA) provide information of the criteria and more recent publications namely the CLSI C62-A states more than one approach should be undertaken (Lynch, 2016). The suggestion is that one or more of the following should be utilised:

- ≥40 patient comparison to a reference method
- Analysis of commutable reference materials
- Spike and recovery analysis

Throughout the method development of this thesis, the spike and recovery analysis approach has been utilised. Then a subsequent patient comparison undertaken (using recent samples) to confirm. The nature of a bespoke, in-house assay is that adherence where possible to guidelines is a priority but a robust verification provides confidence and in fact has enabled both the serum and urine assays to be UKAS accredited.

Once values were assigned and recovery against nominal concentrations was within 15% (20% at LLOQ) as defined by EMA guidelines (2011), sample comparison was undertaken and for TYR, reanalysis of serum EQA samples.

	TYR (5-1500 µmol/L)	**HGA (3.1-250 µmol/L)**	**NTBC (0.2-12 µmol/L**
Cal 1	104.0 ± 10.0	101.1 ± 8.2	104.9 ± 9.1
Cal 2	94.1 ± 6.6	93.6 ± 10.3	96.8 ± 6.3
Cal 3	94.7 ± 7.8	91.9 ± 8.9	94.6 ± 7.4
Cal 4	96.6 ± 8.4	96.9 ± 8.1	99.4 ± 8.3
Cal 5	96.9 ± 8.1	103.2 ± 6.5	100.6 ± 7.9
Cal 6	100.3 ± 8.0	102.3 ± 6.1	101.7 ± 6.5
Cal 7	103.0 ± 7.6	101.9 ± 5.1	102.7 ± 7.3

Table 3A.2 – Percentage recovery of serum standards - Lot 310414

To confirm the assigned values, a patient comparison was performed. No issues were determined with serum TYR and serum HGA with R^2= 0.9932 and 0.9917 respectively. However NTBC demonstrated a 25% positive bias with the new calibration standards. To confirm this, a repeat set of NTBC only standards was prepared and demonstrated a similar finding. Fresh NTBC powder was supplied by Swedish Orphan Biovitrum AB which was tested confirming the original calibration standards had deteriorated for NTBC (at this stage they had been in use for 24 months). Reanalysis of patient samples were undertaken over a longer period of 12 months to corroborate this finding, with the older samples comparing favourably, whilst those over the previous two months demonstrating the 25% bias (which included the initial run of SONIA-2, visits 1 to 3). No trial data had been

released as only the initial visits had been assessed and only serum and urine HGA were the primary endpoint markers.

3A.3.4 One year stability of TYR, HGA and NTBC

Within the initial method development, short-term stability was assessed and published. Long-term stability of both serum and urine analytes has been comprehensively reported in Chapter 10, however additional data reported below is available from all the samples available for one year stability, stored at -80°C. In total 120 samples were analysed, one year post sample preparation and storage, for serum TYR and HGA. Due to the issue with the NTBC calibration standards, insufficient data was available at the reanalysis time. Figure 3A.2 demonstrates the comparison for serum TYR and figure 3A.3 for serum HGA.

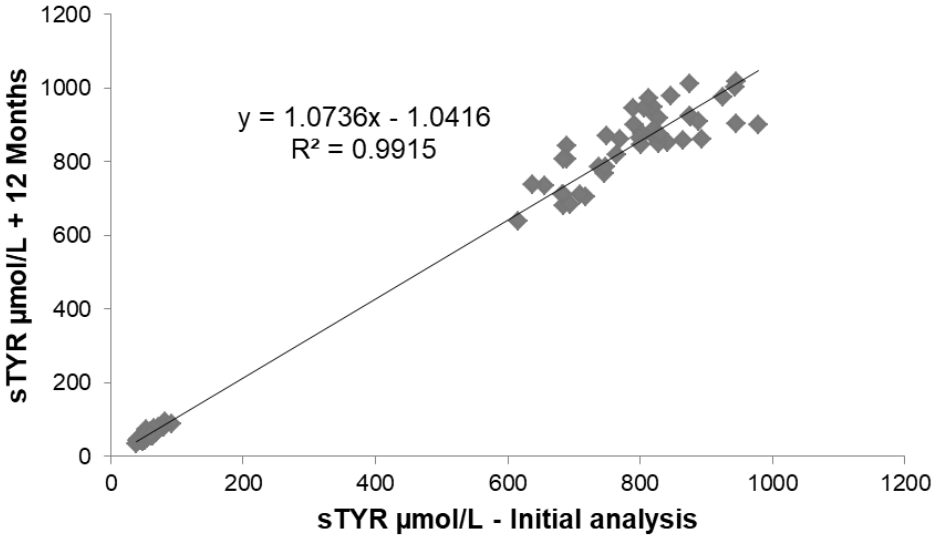

Figure 3A.2: One year stability at -80°C for serum TYR.

The average percentage bias at 12 months was +5.1% (± 8.6 %) suggesting no obvious deterioration of serum TYR over this period.

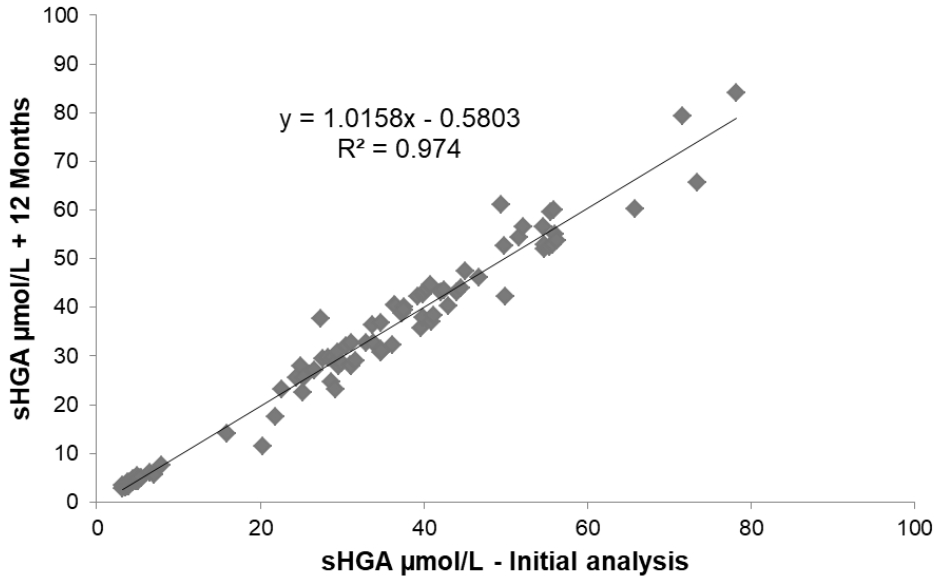

Figure 3A.3: One year stability at -80°C for serum HGA.

The average percentage bias at 12 months was -2.2% (± 10.4%) again indicating no obvious deterioration of sample integrity.

3A.4 Discussion

Additional assay modification and verification steps have ensured that the original published serum assay for HGA, TYR and NTBC remains consistent and enables longitudinal patient comparisons. Although saturation was not observed in the serum assay, the transitions were reviewed and the qualifier ion was changed to enable more manageable ion ratios to be used as a visual check on the analyser. Change of IS from a d_2-TYR to a d_4-TYR ensured that any potential for cross talk was completely reduced, which was important considering the ease of ionisation of TYR and the subsequent large areas of the native compound at higher concentrations.

As with all in-house assays, monitoring of calibration standards and QC performance is vital, and in the early stages, until stability and range was assessed, a fresh set of standards was made to verify the current running batch. This identified an issue with NTBC at 2 years storage at -80°C. Initially assumed to be deterioration of the NTBC stock powder, it was identified that solubility was the issue as when fresh solutions were compared with the

super-stock stored, they were not comparable with a 25% positive bias for new preparations. Therefore several levels of checking have been implemented to prevent this re-occurring and the NTBC super-stock has been assigned an 18 month stability. For all assays now as part of the day to day validation protocols, the area of the top calibrator and the response (with respective IS) for all analytes is recorded and monitored. In addition, as no EQA scheme is available for NTBC, a patient sample is also analysed on every serum assay. The acceptance of the batch is determined by QC performance (three levels) and the patient sample QC.

The issue with the NTBC calibrators prevented the initial one year stability check, however long-term stability has been reported in Chapter 10. Short-term stability for TYR and HGA was undertaken to evidence sample storage as part of the SONIA-2 clinical trial as samples were analysed in batches, however long-term stability was required to enable confidence in reporting analyte changes observed over the course of the trial. There was no significant difference in serum TYR and serum HGA after 12 months storage at -80°C which additionally provided confidence that perchloric acid precipitation of the serum samples, stabilised HGA and preventing long-term degradation.

The issue with assigning calibrator values for the AKU assays is slightly different from routine clinical assays, whereby there may be certified reference materials or commercial calibrator sets. In addition, with the exception of serum TYR there are no commercial EQA schemes. Calibration standards cannot be assigned values based upon the current ‘in-use’ standards as if they have deteriorated, then lower values will potentially be assigned. Therefore in-line with CLSI C62-A (Lynch, 2016) a spike and recovery approach with subsequent patient comparison is utilised. All the steps described have resulted in a robust assay which has gained international reputation for AKU diagnosis and for monitoring response to treatment with NTBC.

Chapter 4

Method Development and Validation for analysis of phenylalanine, 4-hydroxyphenyllactic acid and 4-hydroxyphenylpyruvic acid in serum and urine

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Declaration and acknowledgments

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4.1 Abstract

Alkaptonuria (AKU) is a rare debilitating autosomal recessive disorder of tyrosine (TYR) metabolism which results in a deficiency of the enzyme homogentisate 1,2-dioxygenase activity. Several studies have reported the metabolic changes in homogentisic acid (HGA) concentrations and subsequent deposition of an ochronotic pigment in connective tissues, especially cartilage. Treatment with nitisinone (NTBC), reduces urinary and circulating HGA, but its mode of action results in hypertyrosinaemia. The effect of NTBC on other metabolites in the TYR pathway has not been reported.

Modification of the current reverse phase liquid chromatography tandem mass spectrometry (LC-MS/MS) methods for serum and urine (Hughes et al., 2014, 2015) to include phenylalanine (PHE), hydroxyphenyllactate (HPLA) and hydroxyphenylpyruvate (HPPA) have been validated. HPPA and HPLA (negative ionisation) eluted at 2.8 and 2.9 min respectively on an Atlantis C18 column with PHE (positive ionisation) eluting earlier at 2.4 min.

Intra- and inter-assay accuracy was between 96.3 – 100.3% for PHE; 96.6-110.5% for HPLA and 95.0-107.8% for HPPA in both urine and serum. Precision, both inter- and intra-assay was <10% for all analytes in both serum and urine. No significant issues with carry-over, stability or matrix interferences were seen in either the urine or serum assays. Measurement of serum and urine from an AKU patient has demonstrated a robust, fully validated assay, appropriate for monitoring of patients with AKU and for demonstrating metabolite changes, following NTBC therapy.

4.2 Introduction

The tyrosine (TYR) catabolic pathway along with its primary metabolites and enzymes has been well described (Hughes et al., 2014; 2015; Ranganath et al., 2016; Davison et al., 2020) as displayed in figure 4.1 and consists of a series of enzymatic reactions which yield acetoacetate and fumarate. Deficiency or severely reduced activity of these enzymes results in the autosomal recessive disorders commonly referred to as hereditary tyrosinameia (HT) types 1, 2 and 3 and Alkaptonuria (AKU) (figure 4.1). HT-1, HT-2 and HT-3 are characterised by hypertyrosinameia, however in AKU, normal plasma TYR concentrations are observed (Phornphutkul et al., 2002; Introne et al., 2011; Ranganath et al., 2016; Milan et al., 2017). HT-2 and HT-3 are largely managed by a diet low in TYR and phenylalanine (PHE) to maintain TYR below a target of 500 µmol/L; whereas for HT-1, nitisinone (NTBC) is an approved first-line treatment (Lindstedt et al., 1992; Lock et al., 1998; McKiernan, 2013). NTBC inhibits p-hydroxyphenylpyruvate dioxgenase, the enzyme which leads to the formation of homogentisic acid (HGA), the compound that is the characteristic biochemical abnormality used in the diagnosis of AKU. It is also pathologically linked to the formation of the ochronotic pigment seen in AKU, although the exact mechanisms have not been fully elucidated.

Whilst NTBC has recently been approved for treatment of AKU in 2020 (Ranganath et al., 2020a), prior to this there was no approved disease modifying treatment for this disorder; analgesia and joint replacement being the main-stays in the management of the musculoskeletal pain associated with AKU (Davison et al., 2020). Clinical trials into the use of NTBC for AKU have been on-going and demonstrate the efficacy in reducing serum and urine HGA concentrations (Phornphutkul et al., 2002; Introne et al., 2011; Ranganath et al., 2016; Milan et al., 2017; Ranganath et al., 2020a). The consequence, as in HT-1, is hypertyrosinaemia (Phornphutkul et al., 2002; Suwannarat et al., 2005; Introne et al., 2011; Ranganath et al., 2016; Milan et al., 2017; Ranganath et al., 2020a).

Quantitative metabolite studies have largely focussed on measuring HGA and TYR, however, interest in the metabolites upstream have become interesting with regards to flux changes during treatment (Milan et al., 2019). The detection and measurement of hydroxyphenyllactate (HPLA) and hydroxyphenylpyruvate (HPPA) in the TYR pathway has been a challenge to analysts over the years largely due to their proposed instability and the reported undetectable levels in normal individuals. Previous methods for HPPA and HPLA have used paper chromatography (Sass-Kortsak et al., 1967), gas chromatography mass spectrometry with an extensive ether extraction protocol followed by sialylation (Crawhall et al., 1971), enrichment and isothermal capillary gas chromatography (Spaapen et al., 1987), HPLC with chemiluminescence (Nakahara et al., 1990) and isotope dilution gas chromatography (Deutsch, 1997).

This paper describes a validated liquid chromatography tandem mass spectrometry (LC-MS/MS) method for HPLA and HPPA with other key metabolites in the TYR pathway, namely PHE, HGA and TYR, in conjunction with serum NTBC (previously published Hughes et al., 2014; 2015). In addition, this method is applied to an AKU patient to determine and demonstrate the changes in these metabolites in both serum and urine, with NTBC treatment.

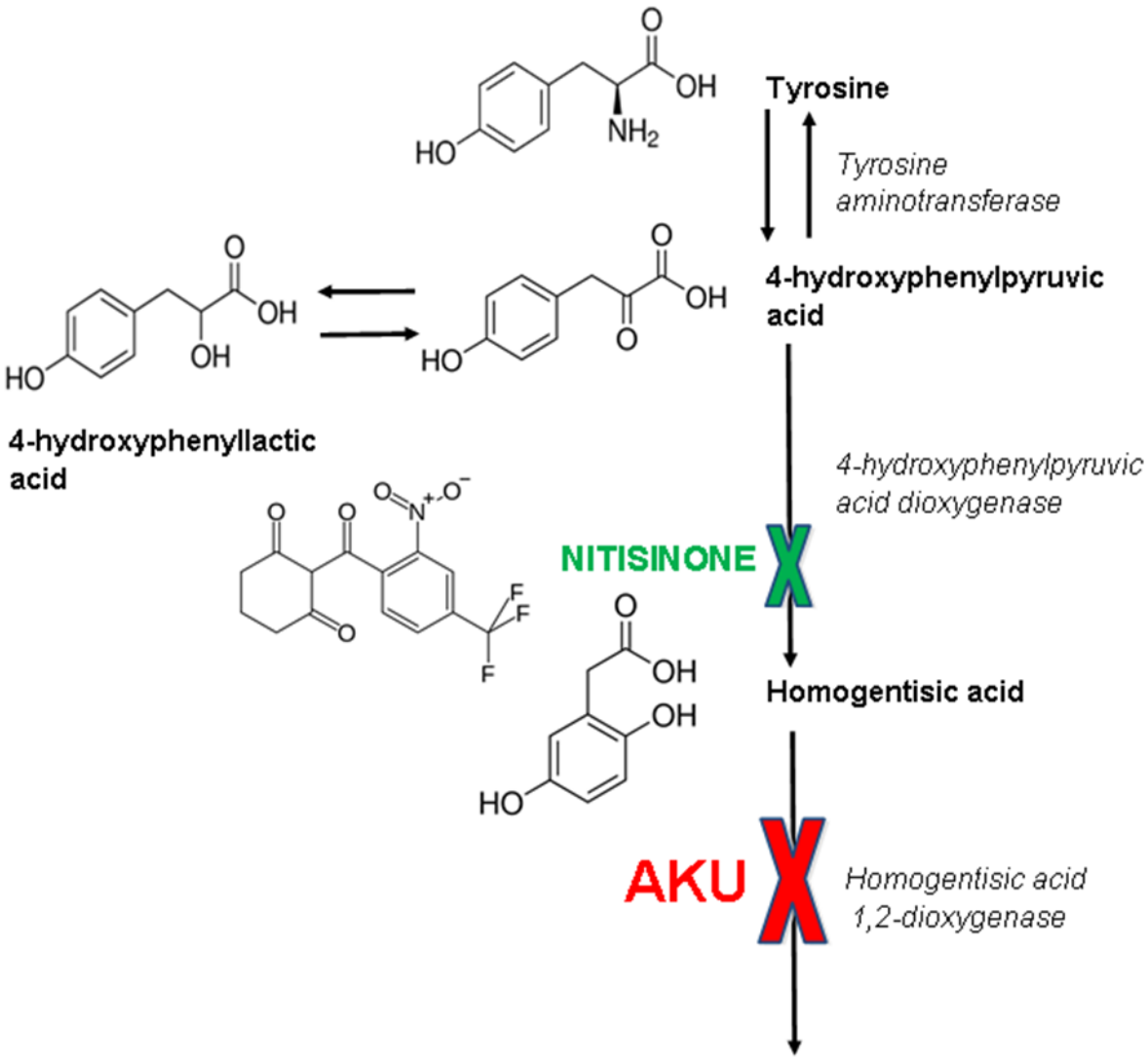

Figure 4.1 The TYR pathway depicting the enzyme deficiency for AKU and the enzyme blocking effect of NTBC.

4.3 Materials and Methods

4.3.1 Chemicals and materials

The previously described methods for urine and serum TYR, HGA and NTBC (serum only) were modified to include a d_4-TYR internal standard (IS) (Hughes et al., 2014, 2015). TYR, PHE with their corresponding IS (d_4 and d_5 respectively), HGA, HPLA and HPPA were purchased from Sigma–Aldrich, UK. HGA isotope-labelled IS $^{13}C_6$-HGA was obtained from Larodon Fine Chemicals (Sweden). LC–MS/MS grade methanol, acetonitrile (Honeywell, UK) and formic acid (Biosolve) were used throughout. Water was purified in-house by the Elix Essential with a DIRECT-Q 3UV Millipore water purification system. All dilutions and sample preparation were performed in glass. Oxygen-free nitrogen was supplied by a Genius 3010 nitrogen generator from Peak Scientific.

4.3.2 Instrumentation and operating conditions

Analysis was performed on an Agilent 6490 Triple Quadrupole mass spectrometer with Jet-Stream® electrospray ionisation (ESI–MS/MS) coupled with an Agilent 1290 infinity UHPLC pump and 1290 multi-sampler. All data processing both qualitative and quantitative analysis was performed using Mass Hunter soft-ware package (version B.06.00).

Chromatographic separation was achieved on an Atlantis C18 column (100 mm × 3.0 mm, 3 µm, Waters) maintained at 35^{o}C. Conditions were 20% organic (methanol, 0.1% formic acid) for 30 seconds, increasing linearly to 90% organic by 2.5 min, held for 1 min followed by 1.4 min at 100% organic and re-equilibration to starting conditions for 2 min. Flow rate was 0.4 mL/min, and MS conditions were: Gas temp 150°C, gas flow 17 L/min, sheath gas temperature 320°C and sheath gas flow 12 L/min.

4.3.3 Preparation of standard solutions

Super-stock standard solutions of HPPA and HPLA were made to a concentration of 400 mmol/L in deionised water (20-50 µL 40% sodium hydroxide for dissolution). PHE was dissolved at 10 mmol/L in deionised water (100 µL 5N sulphuric acid for dissolution). All super-stock solutions

were sonicated prior to preparation of standards. Information regarding TYR, HGA and NTBC has been previously published (Hughes et al., 2014, 2015).

4.3.4 Preparation of calibrators and controls urine and serum assay

To ensure a matrix-matched calibration for the urine assay, super-stock aqueous standard solutions were added to an acidified urine base pool (1% v/v 5N sulphuric acid) which was assayed prior to preparation to verify minimal endogenous metabolites (due to stability of HGA all urine collections for AKU patients are collected into containers with 5N sulphuric acid). Initially the super-stock standards were diluted to intermediate stocks at five times the final required concentration. These were then added to the urine pool in a ratio of 1:4 (x5 dilution) to create combined calibrators with final concentrations ranging from 10 µmol/L to 20,000 µmol/L for HPLA and HPPA and 5 µmol/L to 250 µmol/L for PHE. In-house quality controls (QC) were made in a similar manner independently covering the dynamic range of the calibration curve.

For the serum assay to ensure matrix-matched calibration, super-stock aqueous standard solutions were added to a serum matrix base pool (steroid depleted serum, BBI Solutions, SF236-7) and again the base serum was assayed prior to preparation to verify minimal endogenous metabolites (TYR and PHE). The super-stock standards were diluted to intermediate stocks at 10 times the final required concentration. These were then added to the steroid depleted serum matrix in a ratio of 1:9 (x10 dilution) to create combined calibrators with final concentrations ranging from 5 µmol/L to 500 µmol/L for HPLA, 20 µmol/L to 500 µmol/L for HPPA and 2 µmol/L to 250 µmol/L for PHE. In-house QC were made independently to cover the calibration range. TYR, HGA and NTBC were included at concentrations previously described (Hughes et al., 2014, 2015).

4.3.5 Assay Principle:

A combined IS solution was used as the assay diluent, containing final concentrations of 500 nmol d_4-TYR, 12.5 nmol/L d_5-PHE and 1 µmoL $^{13}C_6$-HGA per 500 mL deionised water with 0.1% formic acid for the urine

assay and 250 nmol d_4-TYR, 12.5 nmol/L d_5-PHE and 100 nmoL $^{13}C_6$-HGA and 1 nmoL$^{13}C_6$-NTBC per 500 mL deionised water with 0.1% formic acid for the serum assay. All samples, calibrators and QC were assayed on a 1 in 1000 dilution with the above IS solutions.

4.3.6 Assay validation

The assay was validated using in-house protocols based on published guidance (EMA guidelines, 2011; Honour, 2011; FDA Bioanalytical method validation, 2018). Only details pertaining to the validation of HPLA, HPPA and PHE are contained within this manuscript; for validation of TYR, HGA and NTBC readers are referenced to previous method publications (Hughes et al., 2014, 2015).

4.3.6.1 Linearity

Standard curves were fitted using linear regression with a 1/x weighting factor and a minimum of six calibration points plus matrix blanks (assayed first to check for endogenous levels) and curve fitting parameters excluded zero. Performance of fitted curves is presented as the coefficient of determination (R^2).

4.3.6.2 Accuracy

Accuracy was determined as closeness to the nominal spiked concentrations, both intra- and inter-assay with n = 6 and n = 20 respectively. Accuracy was calculated as: [measured concentration – nominal concentration] ***/*** [nominal concentration] × 100%. No external quality assurance schemes exist for HPPA or HPLA. However for serum PHE, participation in ERNDIM (European Research Network for evaluation and improvement of screening, Diagnosis and treatment of Inherited disorders of Metabolism) was undertaken.

4.3.6.3 Precision

Imprecision was determined both intra- (n = 6) and inter-assay (n = 20) using separately spiked urine and serum pools and is expressed as coefficient of variation (%CV).

4.3.6.4 Matrix effects

The presence of ion suppression was evaluated for HPPA, HPLA, PHE and their respective IS in both urine and serum matrices. Deionised water, acidified urine (three individual donors) were spiked with low, medium and high concentrations of HPPA, HPLA and PHE and each matrix was also spiked with the equivalent of the final concentration of IS. Similar was performed for deproteinised serum matrices. All spikes were individual analyte only.

Matrix factor was determined by calculating the ratio of the peak area in the presence of matrix (spiked with analyte or IS) to the peak area in the absence of matrix (deionised water plus analyte or IS). Using the matrix factors calculated, an IS normalised matrix factor can be determined (matrix factor of analyte ***/*** matrix factor of IS multiplied by 100) (EMA Guidelines, 2011). Noting in this case $^{13}C_6$-HGA is also used as an IS for HPPA and HPLA (at the time of method development, no isotopic IS were available for HPLA and HPPA). More recently isotopic IS for HPLA have been produced but swapping IS during the clinical trial and the cost were two reasons for not implementing this into current practice.

4.3.6.5 Dilution and carry-over

Dilution integrity of urine and serum HPPA, HPLA and PHE was assessed by pre-analytical dilution of five samples with high concentrations in deionised water at factors of one in three, five and ten with recovery as a percentage of the base sample analysed three times. Carry-over of urine and serum HPPA, HPLA and PHE was assessed by five separate water injections following injection of the top calibrator.

4.3.6.6 Stability

Stability of urine and serum HPPA, HPLA and PHE was assessed using three pools representing low, medium and high concentrations. Stability was determined following three freeze-thaw cycles (at −20^{o}C), over 24hr at room temperature and over 24hr at 4^{o}C (equivalent to the sample manager

temperature, attached to the Agilent 6490). Results are expressed as a percentage of nominal values determined against a fresh calibration curve. Samples used for the on-board 24hr stability were also repeatedly analysed over the 24hr period for any deterioration which may limit batch and run-times.

4.3.7 Analysis of analytes in urine and serum samples

To confirm the utility of the assay, three samples of urine and serum were assayed from a known AKU subject who attends the National AKU centre at Liverpool. Patients are administered a daily 2 mg dose of NTBC and samples were analysed at baseline (pre-NTBC), 6 months and 1 year. Analysis of these samples are covered under the NAC survey approved by the Institutional Audit Committee (Audit No: AC03836).

4.3.8 Analysis of analytes in healthy volunteer samples

Control samples were obtained following an amendment to the ethical application for the Natural History Study of AKU (NRES No 07/H1002/111). Participants (n=22) were recruited from the Royal Liverpool and Broadgreen University Hospitals Trust and the University of Liverpool after obtaining informed written consent. The age range was 25-61 in females (n=11) and 32-63 years in males (n=11). None of the volunteers had AKU and all medications were documented.

4.4 Results

4.4.1 Method Validation

The mass spectrometer was operated in multiple reaction monitoring (MRM) mode. Two product ion transitions were determined for each precursor ion and the respective collision energies are detailed in table 4.1. For optimal sensitivity PHE and its IS were measured in positive ionisation mode while the acids HPPA, HPLA and $^{13}C_6$-HGA IS were measured in negative ionisation mode.

Compound	Ionisation	Product ion (Quant)	Product Ion (Qual)
PHE	Positive	166>91	166>77
HPLA	Negative	181>163	181>135
HPPA	Negative	179>134	179>107
d_5-PHE	Positive	171>125	171>106
$^{13}C_6$-HGA	Negative	173>128	173>114

Table 4.1 Parameters for MS detection of HPPA, HPLA and PHE.

A typical chromatogram is displayed in figure 4.2 showing the primary product ions (quantifiers) for metabolites HPPA, HPLA and PHE with IS; and figure 4.3 showing full chromatographic separation of all measurable TYR pathway analytes – AKU related compounds in a urine matrix. Chromatograms are identical for serum matrix (namely retention times) except for the addition of the drug NTBC eluting near the end of the run (retention time = 4.3 min).

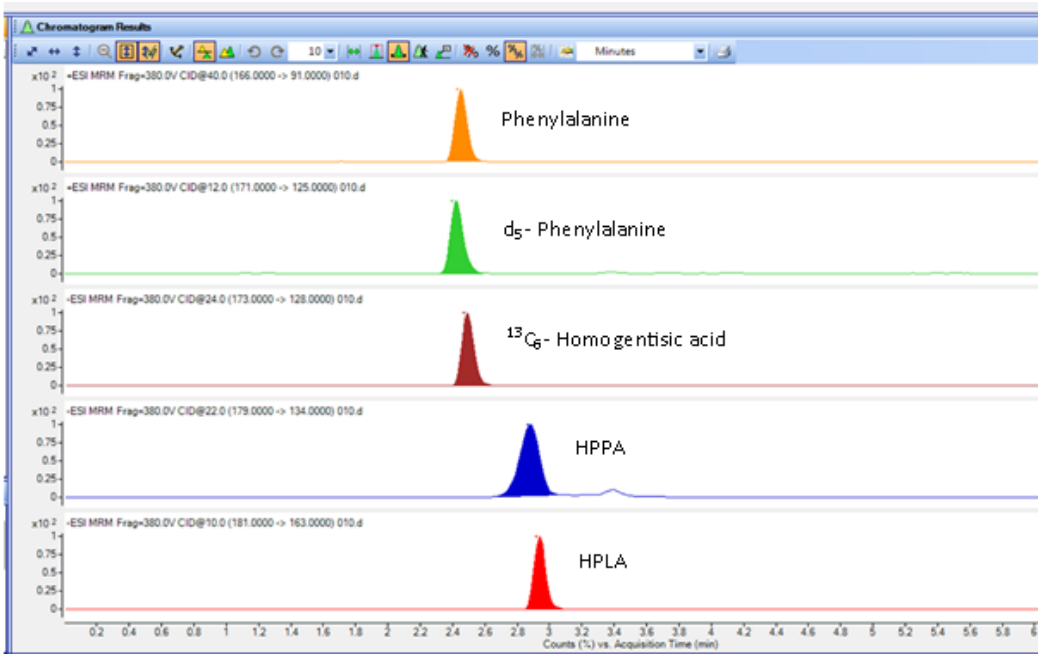

Figure 4.2 Chromatogram showing metabolites HPPA, HPLA, PHE and their respective IS.

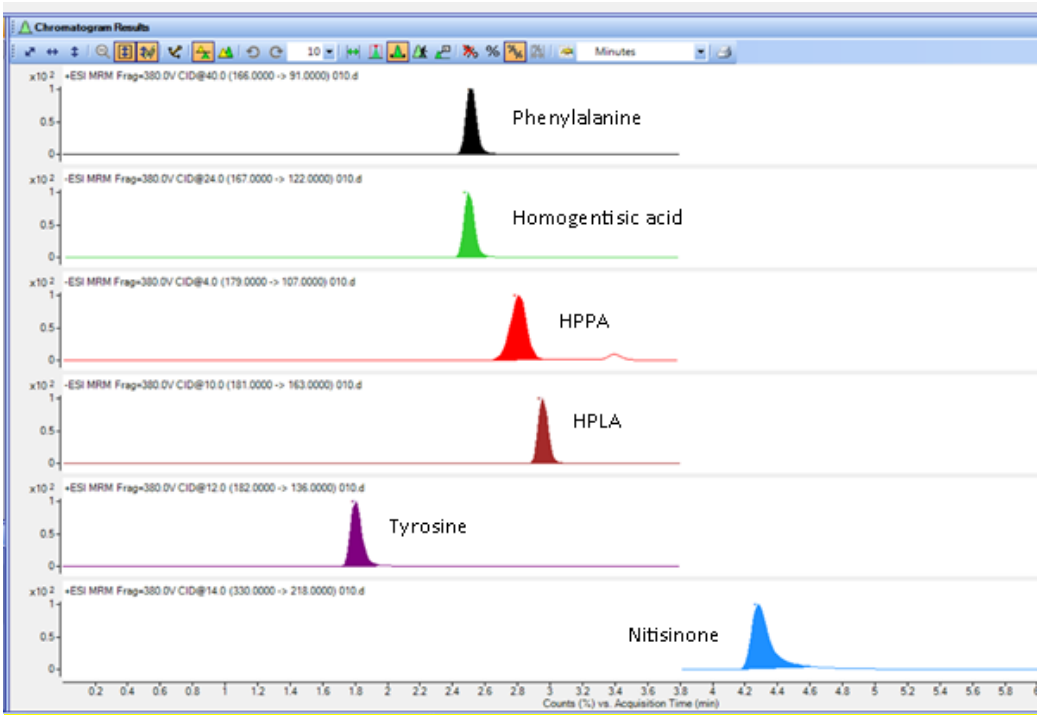

Figure 4.3 Chromatogram showing all metabolites related to AKU in the TYR pathway (no IS included in view)

4.4.2 Linearity

Previous knowledge regarding the high urine HGA concentrations at baseline, in patients with AKU, and preliminary experiments on HPLA and HPPA, post NTBC, led to the understanding that urine concentrations would be in the same range as baseline HGA. Therefore, the measuring range validated in urine was considerably larger than that in serum. Although the assay is linear, the range of calibration standards chosen determined the measuring range stated in both urine and serum samples. For serum these are: PHE 10 – 525 µmol/L, HPLA 5-500 µmol/L, HPPA 10-540 µmol/L and urine: PHE 10-520 µmol/L, HPLA 20-20,000 µmol/L and HPPA 50 – 22,000 µmol/L.

Standard calibration curves (seven points including blank) exhibited a good fit over the range examined, with minimal inter-assay variability over the concentration ranges described with $R^2 = 0.999$ for all analytes in both serum and urine (n=8). For all assays the calibration curve software was set at 'exclude zero' to ensure any contamination or baseline drift was detected.

4.4.3 Accuracy

Intra- and inter-assay accuracy was determined in both urine (table 4.2) and serum (table 4.3) matrices. Results are represented as percentage recovery of a nominal amount of HPPA, HPLA or PHE spiked into matrix. Intra- and inter- batch accuracy was shown to be almost entirely within ±10% of the nominal expected value in both matrices.

Participation in serum PHE EQA with ERNDIM has demonstrated acceptable performance for all samples (n=8 per year) for the last fours years to date.

Table 4.2 Intra- and inter-assay accuracy for the urine metabolite assay

Expected Conc µmol/L	Urine PHE		Expected Conc µmol/L	Urine HPLA		Expected Conc µmol/L	Urine HPPA	
	Intra	Inter		Intra	Inter		Intra	Inter
30	97.5 ± 3.4	97.7 ± 3.3	150	107.1 ± 4.5	109.4 ± 5.6	150	101.1 ± 4.1	103.6 ± 5.9
150	97.0 ± 3.1	97.7 ± 3.0	3,000	110.3 ± 2.6	110.5 ± 4.1	3,000	96.5 ± 5.8	95.0 ± 7.3
450	99.0 ± 3.7	99.4 ± 3.3	16,000	97.4 ± 3.1	96.6 ± 4.9	16,000	107.8 ± 4.2	101.7 ± 9.9

Table 4.3 Intra- and inter-assay accuracy for the serum metabolite assay

Expected Conc µmol/L	Serum PHE		Expected Conc µmol/L	Serum HPLA		Expected Conc µmol/L	Serum HPPA	
	Intra	Inter		Intra	Inter		Intra	Inter
20	96.3 ± 5.8	98.6 ± 5.6	25	108.5 ± 4.5	109 ± 5.1	70	100.9 ± 7.1	102.6 ± 9.5
150	99.7 ± 2.7	100.3 ± 3.0	150	100.5 ± 6.0	101.3 ± 5.0	150	101.9 ± 6.0	105.1 ± 7.5
450	97.4 ± 6.1	97.7 ± 5.3	450	103.7 ± 5.4	99.7 ± 6.4	450	103.7 ± 2.6	102.2 ± 4.6

4.4.4 Intra- and Inter- assay precision

For intra-assay, ten aliquots of each analyte pool were analysed in a single batch with a calibration curve at the front and QC at beginning, middle and end of the run. For inter-assay precision, the pools were assayed on 20 different runs across a two week period. Assay validation requirements are for precision to be within a CV of 15% except for the LLOQ which should not exceed ± 20%. All analytes performed within these criteria for both the urine and serum assay. For the urine assay: PHE <6% intra-assay and <8.1% inter-assay (10-520 µmol/L); HPLA <5% intra- and <9% inter-assay (30-19,000 µmol/L) and HPPA <7.2% intra- and <10% inter-assay precision (58-22,000 µmol/L).

For the serum assay: PHE <6.1 % intra- and <5.6% inter- (10- 520 µmol/L); HPLA <10.9% intra- and <10.6% inter- (6-506 µmol/L) and HPPA <11.0% intra- and <10% inter-assay (30-533 µmol/L). It should be noted that precision was <7.5% for serum HPLA and HPPA if the lowest concentration pool was excluded from the average precision profile. No data has been trimmed and without exception, all assays have intra- and inter-assay precision of acceptable performance.

4.4.5 Lower limit of quantification (LLOQ)

The LLOQ is defined as the lowest calibrator which satisfies a CV ≤ 20%. This is generally considered the lowest calibration standard. The signal should also be at least 5 times the signal of a blank sample. The LLOQ for HPLA in serum was 5 µmol/L and urine 20 µmol/L; for HPPA in serum it was 10 µmol/L and urine 50 µmol/L, and for PHE in serum it was 2 µmol/L and urine 5 µmol/L.

	Urine Assay			Serum Assay		
	PHE	**HPLA**	**HPPA**	**PHE**	**HPLA**	**HPPA**
LLOQ	5 µmol/L	20 µmol/L	50 µmol/L	2 µmol/L	5 µmol/L	10 µmol/L
% Deviation from nominal amount	1.3%	9.3%	9.8%	0.9%	6.8%	7.0%
% CV (n=10)	4.8%	7.5%	8.4%	5.2%	5.4%	11.3%

Table 4.4 LLOQ for metabolites PHE, HPLA and HPPA.

4.4.6 Matrix effect

The matrix effect of both serum and urine was assessed across the validated concentration range for HPPA, HPLA and PHE, with 3 individual urine and serum matrix pools. Results demonstrated that the IS counteracted any matrix suppression measured assuring acceptable levels of matrix effects normalized against the respective IS. For urine HPLA and HPPA there was a slight signal suppression (average -6.2% HPLA and -6.6% HPPA with CV <6.9% and <7.8% respectively), however the IS normalised the suppression, behaving similarly, justifying the use of HGA IS for these two compounds. Serum HPLA and HPPA demonstrated a similar response with IS showing a normalised matrix factor 0.96-1.03 (HPLA) and 0.95-1.01 (HPPA) with %CV <10% for both across the concentrations examined. PHE in both serum and urine matrices satisfied validation criteria with CV <10% and minimal ion suppression. The matrix suppression in serum was high but as proven in the serum paper (Hughes et al., 2015), the HGA IS behaves similarly leading to normalisation of the IS matrix factor.

4.4.7 Dilution integrity

Due to the large dynamic range of urine HPPA and HPLA concentrations exhibited post-NTBC treatment, urine samples were tested at x3, x5 and x10 dilution using deionised water, prior to assay preparation. Recovery

percentages (table 4.5) showed no effect on results when pre-diluting samples.

	Urine HPLA	Urine HPPA
1 in 3	92.8 ± 7.1	93.2 ± 8.4
1 in 5	94.5 ± 7.4	92.5 ± 7.4
1 in 10	96.6 ± 6.6	96.5 ± 5.6

Table 4.5 Recovery percentages of diluted urine samples for HPPA and HPLA

4.4.8 Stability

Stability following three freeze-thaw cycles demonstrated an average recovery of 97.3 ± 2.5% for urine HPLA, 96.8 ± 3.0% for urine HPPA and 98.1 ± 2.7% for urine PHE. For serum samples, similar recovery was demonstrated with 98.1 ± 3.4% for HPLA, 97.4 ± 3.3% for HPPA and 97.8 ± 2.1% for PHE. At room temperature, urine and serum samples showed no significant deterioration, although serum samples demonstrated a trend in decreasing concentration any samples kept at room temperature and not perchloric acid crashed within 12hr are not accepted for assay. Stability at 4°C demonstrated average recoveries 96.7 ± 4.5% for all analytes with repeat injections over a 24hr period.

4.4.9 Carry-over

Following injection of the top calibrator there was no clear discernible peak in either HPPA, HPLA or PHE transition windows in the subsequent injected blank samples.

4.4.10 Crosstalk

HPPA and HPLA are structurally and chemically very similar. Prior to evaluation it was essential to ensure that neither were detected within each others analytical window and no conversion happened during the sample preparation and analytical process. Solutions of either HPPA or HPLA were

injected periodically over a 24hr period and both HPPA and HPLA areas monitored. At no stage during this period was HPLA detected in the HPPA solution and vice versa. This period is unlikely to be exceeded during analysis time as a single assay batch can be assayed in less than 12hr.

4.4.11 Patient analysis

Three samples on an AKU patient treated with NTBC at the NAC were assayed using this validated assay. Routine measurement of TYR, HGA and NTBC (serum) was part of their scheduled review and the addition of HPPA, HPLA and PHE significantly enhances our understanding of the metabolic changes during treatment. At baseline, TYR and PHE are within reference range (Phornphutkul et al., 2002; Introne et al., 2011; Ranganath et al., 2016; Milan et al., 2017) and a characteristically high serum and urine HGA is evident. HPLA and HPPA are undetectable at baseline (below LLOQ). Once NTBC treatment begins, the characteristic rise in TYR is seen in serum. Suppression of serum and urine HGA is observed, although it does remain detectable (Ranganath et al., 2016; Milan et al., 2017), followed by a concurrent rise in HPLA and HPPA both in circulation and excreted through the kidneys.

4.4.12 Healthy subject analysis

Serum HPPA and HPLA in all subjects were below the LLOQ for the assay; <10 µmol/L and <5 µmol/L respectively. Serum PHE was detectable in all subjects with a mean of 64.1 µmol/L and a reference range of 41-80 µmol/L (95% percentile). Urine HPPA and HPLA were again undetectable in all urine samples, with concentrations below the LLOQ (<20 µmol/L HPLA and <50 µmol/L HPPA). Urine PHE results were reportable for 20 of the 22 subjects, with two having concentrations <5 µmol/L. Of the remaining subjects, the mean was 55.4 µmol/24hr (adjusted for 24hr urine volume) and 95% reference range 32.0 – 127 µmol/24hr.

AKU Patient SERUM	TYR µmol/L	PHE µmol/L	HGA µmol/L	HPLA µmol/L	HPPA µmol/L	NTBC µmol/L
Baseline	57	54	22.2	<5	<10	<0.2
3 Months	583	62	4.9	44	28	0.5
One Year	793	67	4.8	37	32	0.6
AKU Patient URINE	**TYR µmol/24hr**	**PHE µmol/24hr**	**HGA µmol/24hr**	**HPLA µmol/24hr**	**HPPA µmol/24hr**	
Baseline	45	29	21,063	<20	<50	
3 Months	675	37	284	12,073	12,355	
One Year	759	35	393	14,067	15,841	

Table 4.6 TYR pathway metabolites in AKU patient on NTBC (2mg daily).

4.5 Discussion

A method has been validated for the simultaneous quantitation of urine and serum PHE and the TYR metabolites HPPA and HPLA. To date, this is the first published method for quantitation of these by LC-MS/MS and certainly the first method that now encompasses the full range of compounds related to AKU diagnosis and treatment in the TYR pathway. These additional analytes have been incorporated into the existing published methods (Hughes et al., 2014, 2015) to enable a single sample to be utilised.

The method was validated with an uncomplicated sample preparation separating the compounds of interest across a short seven-minute chromatographical run-time. It has been demonstrated as sensitive and specific with favourable accuracy and precision performance satisfying key validation guidelines (EMA and FDA).

This all-encompassing method also has the advantage over previously cited GC methods (Crawhall et al., 1971) in that it avoids long laborious extraction techniques using volatile and toxic materials in favour of a liquid dilute and inject method for a fast and robust analysis of all the major compounds in the TYR pathway that are affected by AKU.

It is well understood that AKU patients have high circulating levels of HGA and once commenced on NTBC treatment, the levels decrease. Data from applying this method has demonstrated the significant consequential rise of metabolites HPPA and HPLA especially in urine (data in this paper, Milan et al., 2019). The calibration range was evaluated to account for this in the majority of patients on NTBC treatment. We have further enhanced an existing method for thorough analysis of clinical trial and routine samples to determine the efficacy and response to NTBC in the treatment of AKU.

Levels of HPLA and HPPA are largely undetectable in health, and in untreated AKU individuals as the pathway either goes to completion (in health) without any enzyme deficiencies or blocks, or HGA is the predominant metabolite (in AKU). Previously, Crawhall et al., (1971) detected

significant HPLA levels by GC in children with HT-1. Levels of HPPA proved more challenging possibly due to the ether extraction procedure involved or perhaps the alkalinity of the urine samples causing sample degradation and loss of efficacy. This method was further expanded, although by no means made any less labour intensive, by Deutsch (1997) using isotopically labelled IS and a derivatising agent to enable detection at levels found in normal individuals, followed by GC, obtaining cleaner chromatograms. This author also noted instability in these compounds of 10% per month for HPPA and 3% per month for HPLA over a 10-month period at -20^{o}C.

Due to stability issues and for sample preservation, assay validation within this report was only performed in acidified urine (1% 5N sulphuric acid) and perchloric acid precipitated serum samples (Hughes et al., 2014, 2015). This is standard throughout routine and trial sample preparation within our specialist AKU centre. Therefore the instability issues seen by Deutsch (1997) were also considerably reduced and further improved by storing samples at -80°C.

Deutsch (1997) stated that concentrations in health for HPPA (0.38 µmol/L) were similar to that found in a previous study (Nakahara et al., 1990), 0.34 µmol/L using HPLC with chemiluminescence detection. This is below the current LLOQ for the assay described so has not been confirmed. We have demonstrated that there are no measurable concentrations of HPPA or HPLA in healthy controls, in either serum or urine. Although the LLOQ are higher than the studies reporting HPPA in health, there is little clinical need within the AKU population to suggest further assay refinement at this concentration range.

In AKU patients treated with the drug NTBC we detect levels of these metabolites at significant concentrations especially in urine (mmol/24hr concentrations) and as methods have become more sensitive and sophisticated we are able to observe the behaviour of these metabolites in the disease and treated state with more accuracy. This becomes advantageous to the clinician in that they can build a picture of response to

treatment and any effects of metabolite accumulation has on the patients overall biochemistry. However the predominant target in AKU patients is to suppress circulating HGA and subsequently urine HGA; control of HPPA and HPLA prior to treatment is not a clinical issue.

The method described here provides an enhanced method for thorough analysis of clinical trial and routine samples to determine the efficacy and response to NTBC in the treatment of AKU and other disorders of the TYR pathway.

Chapter 5

Measurement and analysis of ultra-low levels of homogentisic acid in serum by liquid chromatography tandem mass spectrometry

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Declaration and acknowledgments

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In preparation

5.1 Abstract:

Background: Alkaptonuria (AKU) is a rare debilitating autosomal recessive disorder of tyrosine (TYR) metabolism, where deficiency of homogentisate 1,2-dioxygenase results in increased homogentisic acid (HGA). HGA is deposited as an ochronotic pigment in connective tissues, especially cartilage, leading to a severe early-onset form of osteoarthritis, increased renal and prostatic stone formation and hardening of heart vessels. Treatment with the orphan drug, nitisinone (NTBC), an inhibitor of 4-hydroxyphenylpyruvate dioxygenase (HPPD) has been shown to reduce urinary and circulating HGA. The existing in-house serum assay has a limit of quantification to 3.1 µmol/L. With increased dose and long-term use of NTBC a more sensitive assay was required as it is important for the clinician to be aware at what levels circulating HGA has been effectively suppressed to, by NTBC.

Method: Perchloric-acid crashed serum samples were diluted 1:10 in methanol containing 0.1% formic acid and 0.2 µmol $^{13}C_6$-HGA. Matrix-matched calibration standards, samples and quality controls (QC) were analysed by reverse phase liquid chromatography tandem mass spectrometry (LC-MS/MS) on a Waters Atlantis C18 column with increasing methanol (0.1% formic acid) gradient.

Results: Intra-assay accuracy was 101-109 % (n=10); inter-assay accuracy (n=20) was 90.4-111.8%. Precision, both intra- and inter-assay was <12% (0.1–9.5 µmol/L). HGA, proved stable after 24hr at room temp, three freeze-thaw cycles and 24 hr at 4°C. The assay was linear from 0.1 µmol/L to 10 µmol/L.

Conclusions: This assay developed and validated, demonstrates good precision, accuracy and linearity appropriate for the monitoring of ultra-low HGA concentrations in AKU patients on NTBC therapy. In addition, it enables measurement of serum HGA in healthy individuals.

5.2 Introduction

Alkaptonuria (AKU) is a rare autosomal recessive disorder (OMIM # 203500) of the tyrosine (TYR) degradation pathway (figure 5.1). Deficiency of the third enzyme in the catabolic pathway, homogentisate 1,2-dioxygenase, results in accumulation of homogentisic acid (HGA) which is excreted in gram quantities in the urine, equivalent to mmol/L concentrations, and circulates at lower concentrations in the serum (µmol/L).

The history and consequences of this disorder have been discussed in extensive detail (O'Brien et al., 1963; Helliwell et al., 2008; Phornphutkul et al., 2002; Ranganath et al., 2013). Whereas previous treatment and therapy has been palliative, recent trials using the drug nitisinone (2-(2-nitro-4-fluromethylbenzoyl)-1, 3-cyclohexanedione (NTBC), a potent inhibitor of the second enzyme in the TYR pathway, p-hydroxyphenylpyruvic acid oxygenase have been reported (Phornphutkul et al., 2002; Introne et al., 2011; Ranganath et al., 2016; Milan et al, 2017; Ranganath et al., 2020a). NTBC was originally licenced for treatment of hereditary tyrosinaemia type 1 (HT-1) (OMIM #276700) and is now front-line treatment (Spiekerkoetter et al., 2021; McKiernan 2006). Clinical trials and off-licence use in AKU have demonstrated positive outcomes (Suwannarat et al., 2005, Introne et al., 2011, Ranganath et al., 2016, Milan et al., 2017) and following the SONIA-2 (Suitability Of Nitisinone In Alkaptonuria-2) 4-year trial (Ranganath et al., 2020a) NTBC has successfully been licensed for use in AKU.

NTBC had been used at the National Alkaptonuria Centre (NAC, Royal Liverpool and Broadgreen University Hospital Trust, UK) since 2012, as well as in large multi-centre clinical trials (DevelopAKUre Clinical Trials – www.akusociety.org) to evaluate its effectiveness in the treatment of AKU. The primary target of treatment with NTBC was the normalisation of urine HGA which at baseline is mmol concentrations, however, it is the circulating concentrations of HGA which have gained more recent focus. The formation of an ochronotic pigment which contributes to the detrimental damage to connective tissues is an end product of HGA conversion to benzoquinones (BQA) and self-polymerisation to form ochronotic pigment (Braconi et al.,

2015; Roberts et al., 2015). Therefore, normalisation of serum HGA concentrations are also a focus of NTBC treatment and dosing regimens.

The mean serum circulating levels of HGA in AKU patients attending the NAC (n=58) was 30.0 ± 12.9 µmol/L with an excretable concentration measured significantly higher at 20,557 ± 5445 µmol/24hr (Milan et al., 2017).

At the NAC, patients are administered with 2 mg NTBC on alternate days for the first three months, increasing to 2 mg daily thereafter, with annual assessments to monitor response to therapy. The result after two years was a >94% reduction in urine HGA and an 83% reduction in serum HGA (Milan et al., 2017). Results from SONIA-1, which was a 4-week variable dose of NTBC resulted in 56% of patients being below the limit of assay quantification (<3.1 µmol/L) (Ranganath et al., 2016). In SONIA-1 the NTBC doses administered were 1, 2, 4 and 8 mg with a long-term agreed dose of 10 mg daily to be administered in the SONIA-2 trial. Therefore, there was a requirement for a more sensitive serum HGA assay to enable quantitation of HGA suppression at higher NTBC patient doses. This study describes the validation of an ultra-low serum HGA assay which has been applied to clinical trial patients as well as a group of healthy controls, with a view to determining the concentration of serum HGA which could be classified as ‘normalised’ or a suitable target for NTBC treatment in AKU.

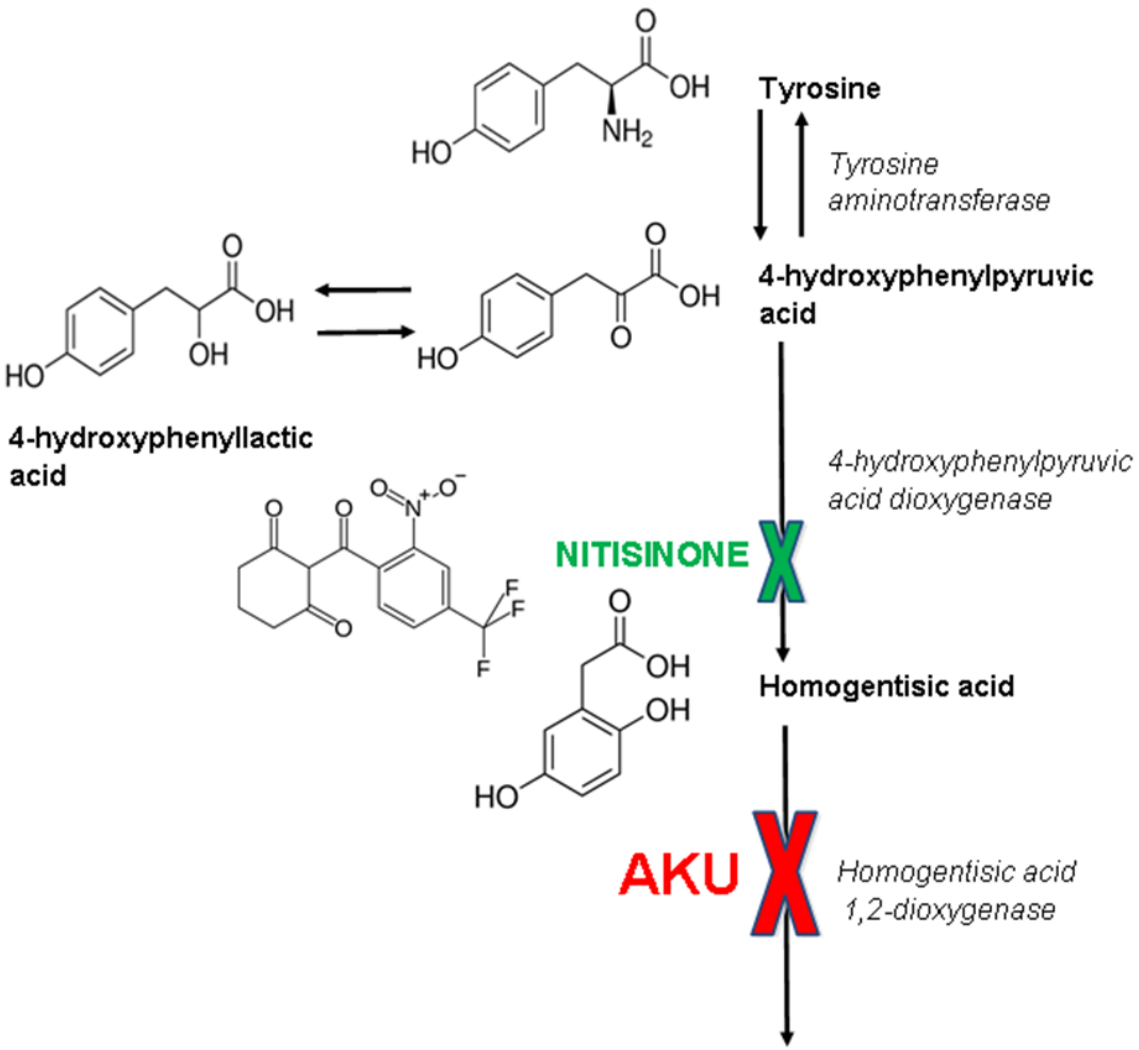

Figure 5.1: TYR degradation pathway

5.3 Materials and methods

5.3.1 Chemicals and materials

HGA was purchased from Sigma-Aldrich (UK) and its corresponding isotope-labelled internal standard (IS), $^{13}C_6$-HGA was obtained from Larodan Fine Chemicals (Sweden). LC-MS/MS grade methanol and acetonitrile were obtained from Honeywell, (UK) via the distributor company Metlab (UK). Formic acid was obtained from Biosolve/Greyhound HPLC, (UK). Water was purified in-house by Elix Essential 5UV with Synergy UV Millipore water purification system. All dilutions and sample preparation were performed in glass and 96 well plates supplied by Waters UK were also used. Oxygen free nitrogen was supplied by a Genius 2010 Peak nitrogen generator.

5.3.2 Preparation of calibration standards and quality controls

Super-stock standard solutions of HGA were dissolved and prepared in deionised water at concentrations of 100, 10 and 1 µmol/L respectively with 50 µL 5N sulphuric acid (H_2SO_4) added to aid dissolution of the solid and also providing a more stable environment for HGA. These were immediately used to make a set of standards which were diluted into steroid depleted serum (BBI International, UK) to produce a standard range from 0.1 µmol/L to 5 µmol/L. Standards were assigned a value based upon the nominal amount added as there are no traceable standards for HGA. C62-A guidelines require the assigned values to be within 15% of nominal concentration with the lowest standard value to be within 20%. Independent quality controls (QC) were prepared at three levels across the calibration range and QC values were confirmed once calibration values were established.

5.3.3 Sample preparation

Serum samples are transported frozen on dry ice with rapid separation. Perchloric acid (5.8M) is added to serum in a 1:11 (50 µL perchloric acid and 500 µL sample), mixed, centrifuged and the protein-crashed serum is then stored at -80 ^{o}C prior to analysis.

5.3.4 Internal standards (IS)

The IS $^{13}C_6$-HGA was reconstituted from the powder stock at 1 mg/mL (5.75 mmol/L), in deionised water. This was then diluted in deionised water 1 in 23 to give an intermediate concentration of 250 µmol and stored in 200 µL aliquots as previously described (Hughes et al., 2015). Modification of the working IS concentration was required to adapt to the lower concentration range of the assay, with dilution of the intermediate, 1 in 1000 in deionised water (20 µL to 20 mL deionised water) and 4 mL of this added to 500 mL deionised water with 0.1% formic acid. This is made fresh every assay.

5.3.5 Principle and method of the procedure used for analysis

Acidified serum samples are diluted with 0.1 % formic acid containing $^{13}C_6$-HGA IS at a 1 in 10 dilution depending on the volume of sample available. All samples are separated by reverse phase liquid chromatography. Mass detection is performed by electrospray ionization (ESI) tandem mass spectrometry in negative ion mode. Identification and quantification is based on multiple reaction monitoring (MRM) mode of HGA and $^{13}C_6$-HGA transitions.

5.3.6 LC-MS/MS Analysis

All analyses were performed on an Agilent 6490 triple quadrupole LC-MS/MS with Jet-Stream® electrospray ionisation (ESI-MS/MS) equipped with an Agilent 1290 infinity pump and autosampler. All data processing both qualitative and quantitative analysis were performed using Mass Hunter software package (Version B.07.00).

Chromatographic separation was performed on an Atlantis C18 column (100 mm x 3.0 mm, 3 µm, Waters, UK) maintained at 35°C. Initial gradient conditions were 80:20 water:methanol with 0.1% formic acid (v/v) increasing linearly to 10:90 in 2.5 min. The mobile phase was maintained for a further minute, increased to 100% methanol for 1.5 min and then returned to starting conditions (flow rate 0.4 mL/min). The column was reconditioned to initial gradient settings for 2 min prior to the next injection. A 20 µL sample was injected onto the column with a total run time of 7.0 min. Optimum operating

ESI conditions were: gas temperature 150°C (nitrogen), gas flow 17 L/min; nebulizer pressure 40 psi; sheath gas temperature 320°C and sheath gas flow 12 L/min. Capillary voltages were optimised to 3500V in positive mode and 2500V in negative mode with equal nozzle voltages (1500V) in both modes. The iFunnel parameters were optimised in both negative and positive mode as 60V for low pressure RF and 110V for high pressure RF.

5.3.7 Assay Validation

The pre-chromatographic sample preparation step has been modified with a new range of calibration standards and QCs. Extensive validation has not been required as the chromatography aspects such as ion suppression, matrix effects, and IS have all been previously validated (Hughes et al., 2015). Dilutional integrity was not required as only samples <3.1 µmol/L were analysed on the ultra-low serum assay. The difference between the standard assay and this is the volume of sample extracted; the standard HGA assay utilises 10 µL of sample in 10 mL working IS solution, the ultra-assay uses 100 µL of calibration standard / QC/ sample in 1 mL of working IS solution (which only contains $^{13}C_6$-HGA).

The assay was validated using in-house protocols based on previously used EMA guidleines and incorporated the C62-A published guidance (Clinical and laboratory standards institute, 2014; Lynch, 2016). Method validation evaluated linearity, accuracy, imprecision, lower limit of quantification (LLOQ), carry-over and stability.

5.3.8 Control samples

The control samples were obtained following an amendment to the ethical application for the Natural History Study of Alkaptonuria (NRES No 07/H1002/111). Participants (n=22) were recruited from the Royal Liverpool and Broadgreen University Hospitals Trust and the University of Liverpool after obtaining informed written consent. The age range was 25-61 in females (n=11) and 32-63 years in males (n=11). None of the volunteers had AKU and all medications were documented.

5.3.9 AKU Patients

Serum samples from the first two years at the NAC have been reported (Milan et al., 2017) prior to development of the ultra-low assay. Patients on 2 mg daily doses showed an 83% decrease in serum HGA from baseline, at 12 and 24 months. Within the data, there were two patients at 6 months, two at 12 months and eight patients at 24 months who were reported as <3.1 µmol/L and for the purpose of the data analysis a value of 3.0 µmol/L was used. These patient samples have been re-analysed using the ultra-low serum HGA assay.

5.4 Results

5.4.1 Ionisation and chromatography of HGA and IS

Quantifier and qualifier product ion transitions and their respective collision energies were determined for each precursor ion (table 5.1). For optimal sensitivity, mass spectrometric detection was performed by multiple reaction monitoring (MRM) mode in negative ionisation mode for HGA and $^{13}C_6$-HGA.

	Ionisation mode	Product Ion 1 (Quant)	Collision Energy (V)	Product Ion 2 (Qual)	Collision Energy (V)
HGA	Negative	167>122	22	167>108	20
$^{13}C_6$-HGA	Negative	173>128	22	173>114	26

Table 5.1: Quantifier and qualifier product ion transitions and their respective collision energies for each precursor ion

Separation was achieved with elution of HGA and $^{13}C_6$-HGA at 2.5 min (figure 5.2). This assay was designed to focus on the low concentrations of HGA in serum samples and therefore the concentration of the IS was calculated to generate a response of 1, between a third and halfway up the calibration curve, ensuring sensitivity and precision at the lower concentrations. A calibration curve is demonstrated in figure 5.3.

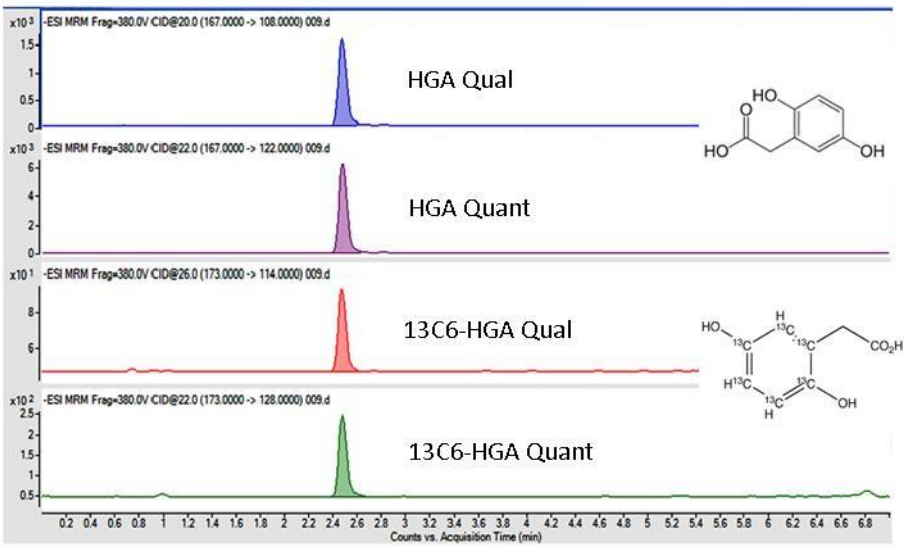

Figure 5.2: A typical chromatogram for the quantifier and qualifier product ions for HGA and $^{13}C_6$-HGA IS

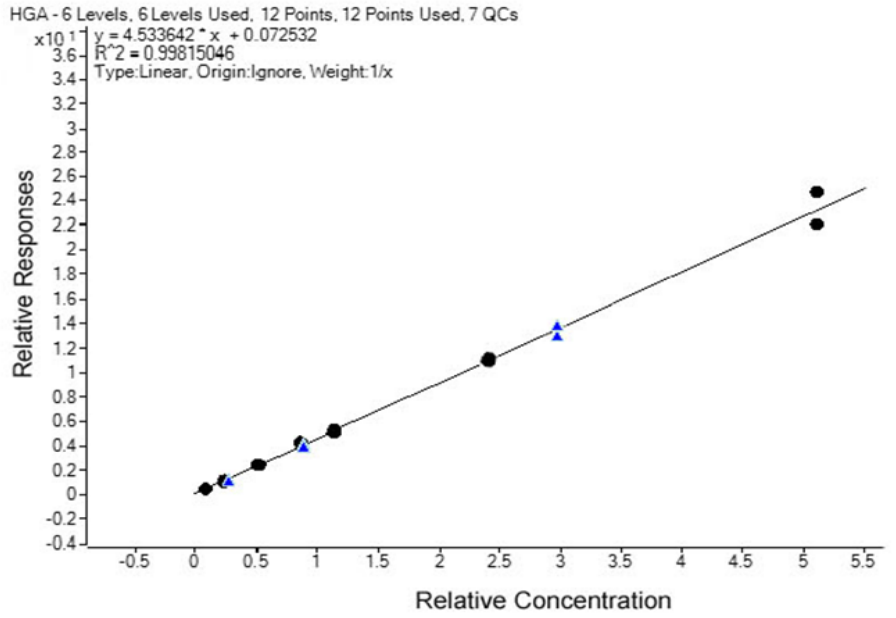

Figure 5.3: Typical calibration curve for ultra-low HGA assay.

(● = calibration standard, ▲= QC); x axis is HGA in µmol/L.

5.4.2 Method Validation

Calibration standard curves (figure 5.3), six points plus serum blank exhibited a good fit over the range examined with minimal inter-assay variability. Linear regression (1/x weighting factor) of HGA R^2=0.997 (n=9). The concentration range covered was 0.1-5 µmol/L.

Accuracy, both intra- and inter-assay was determined (table 5.2). Results are represented as percentage recovery of a nominal amount of HGA, spiked into the serum matrix. Samples were then analysed against the calibration curves and imprecision (%CV), both intra- and inter-assay was determined.

	Ultra-low HGA	
Expected concentration µmol/L	Intra-assay accuracy (n=10)	Inter-assay accuracy (n=20)
0.3	101.0 ± 8.60	90.4 ± 11.0
0.8	108.2 ± 10.7	108.8 ± 9.8
5.0	109.3 ± 10.4	111.8 ± 9.1

Table 5.2: Intra- and inter-accuracy for ultra-low HGA assay.
Results represented as recovery of spiked concentration (Mean ± SD)

Precision, both intra and inter-assay (table 5.3) was assessed by running three pools of spiked samples across the calibration range on a single batch, ten times and aliquots of the same three pools in duplicate across ten different days (n=20).

	Ultra-low HGA	
Pool	Intra-assay precision (n=10)	Inter-assay precision (n=20)
1	0.25 ± 0.03, 10.5%	0.28 ± 0.03, 10.7%
2	1.02 ± 0.06, 6.20%	0.98 ± 0.10, 10.2%
3	3.46 ± 0.15, 4.30%	3.48 ± 0.27, 7.80%

Table 5.3: Intra- and inter-precision for ultra-low HGA assay.
Results represented as mean [HGA] ± SD, %CV.

The LLOQ, defined as the lowest calibrator which satisfies a CV ≤20%, for HGA was 0.1 µmol/L, signal to noise at this concentration was >10:1 and %CV was 12.1% (n=20). Therefore, the assay was sensitive even with an injection volume of 20 µL. The matrix effect was not deemed necessary as it was previously described for this compound (Hughes et al., 2015), where the IS was demonstrated to counteract the ion suppression seen, leading to a normalised matrix factor. No carry-over was observed, following injection of the top calibrator (approximately 5 µmol/L HGA). There were no discernible peaks at the retention time of HGA (2.5 min) on five subsequent water injections ensuring no carry-over under the operating conditions of the assay.

Stability was assessed following 3 freeze-thaw cycles, 24hr at room temperature and 24hr on-board stability (equivalent to 4°C). There was no statistical significance on analyte stability following 3 freeze-thaw cycles, and over the other periods assessed (24hr; $p>0.05$), after repeat analysis of serum pools (n=3).

5.4.3 Analysis of control sample group

Previous study by our group has reported the serum HGA range in this control cohort as <3.1 µmol/L (Davison et al., 2015) as all controls were below the limit of quantification using the published AKU serum assay (Hughes et al., 2015). Reanalysis of the samples with the ultra-low HGA serum assay described within this study is able to report a new reference range for serum HGA in healthy controls. Three of the 22 controls had quantifiable serum HGA levels of 0.1 µmol/L. The remaining 19 controls were all below the new limit of quantitation and reported as <0.1 µmol/L. Further review of the three with levels of 0.1 µmol/L confirmed that if reported to 2 decimal places (assay routinely only reports to one decimal place) the values were 0.05, 0.05 and 0.06 µmol/L. Therefore, this assay has defined the healthy serum HGA level as <0.1 µmol/L.

5.4.4 Analysis of AKU patients on nitisinone

Initial data reported 12 AKU patients on a 2 mg daily dose of NTBC as <3.1 µmol/L. On reanalysis, they were all quantifiable. The two patients in the 6-month and 12-month cohorts were 2.5-3.0 µmol/L in all cases. The eight patients in the 24-month group were on average 2.3 ± 0.5 µmol/L and re-calculation of the percentage serum HGA reduction from baseline went from 83.3% to 84.1% at 24 months.

5.5 Discussion

An ultra-sensitive LC-MS/MS method has been validated for the quantitation of serum HGA for patients that are being treated with the drug NTBC for AKU. To date, this is the first published method for quantitation of HGA by LC-MS/MS to a level of sensitivity that can detect HGA in non-AKU individuals too and uses an adapted version of a previously described method by this author (Hughes et al., 2015).

To maintain assay integrity, the calibration standards and QCs have been matrix-matched, in a crashed acidified serum. This is a recommended guideline to ensure batch to batch reproducibility. Acidification is essential as HGA is unstable in alkaline, neutral or unacidified samples regardless of matrix. Although only short-term stability has been assessed which is representative of routine sample collection and analysis time, long-term stability is currently being assessed to indicate whether very low concentrations of HGA in samples are affected more or less significantly than levels many factors higher.

Monitoring of serum HGA is paramount in clinical management of AKU as circulating levels are key in the pathological development of the disorder when alternative pathways metabolise HGA to BQA, ultimately resulting in ochronosis. The treatment of these patients with 2 mg NTBC, at the NAC, Royal Liverpool University Hospital and subsequent monitoring of the drug levels and metabolites by previously described methods (Hughes et al., 2014, Hughes et al., 2015) provides the clinicians with short- and long-term as well as inter-patient effectiveness of treatment, not only as to suppression

of HGA levels but also as to continued effective suppression.

Although Deutsch and Santhosh-Kumar (1996) managed to measure HGA levels down to 5-10 ng/ml (0.03–0.06 µmol/L), only nine individuals were used in the study and the method involved a derivatisation initial step followed by several less routine-friendly steps accompanied by gas chromatography mass spectrometry (GC-MS), thus rendering the assay unfit for rapid use in a hospital laboratory. The LC-MS/MS method described by Hughes et al., (2015) and discussed regarding a reference range by Davison et al., (2015) for HGA at <3.1 µmol/L can now be further expanded upon and demonstrate the finding that healthy volunteers have a serum HGA ≤0.1 µmol/L, providing a therapeutic target for patients on NTBC, with a view to normalising circulating HGA concentrations.

This validated assay also provides an additional tool to monitor serum HGA concentrations in higher NTBC doses. NAC data has been re-analysed and especially after long-term NTBC, more patients had a serum HGA <3.1 µmol/L. With additional reporting ability, we can now confidently report serum HGA levels to 0.1 µmol/L benefiting patient management and supporting a therapeutic target. In the NAC cohort, although there was an additional reduction at 24 months in serum HGA suppression from 83.3% to 84.1%, there is still circulating HGA.

In conclusion, this ultra-sensitive user-friendly serum assay has been developed and validated which enables a rapid, robust and effective monitoring of the efficacy of NTBC therapy in the treatment of AKU, applicable to determining very low HGA levels and therefore the continued long-term monitoring and effectiveness of treatment.

Chapter 6

A simple method for the determination of creatinine by LC-MS/MS: Quantitation of HGA interference

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Declaration and acknowledgments

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In preparation

6.1 Abstract

Introduction

Although there are long-established routine, high-throughput automated methods within hospital laboratories globally, there are still sample interference obstacles that potentially pose analytical headaches.

Methods:

We have established and validated an assay for the measurement of creatinine in serum/plasma and urine by LC-MS/MS. AKU patient samples have been analysed to determine the influence of HGA upon creatinine assay performance.

Results:

Both validated serum and urine creatinine assays performed within defined criteria and demonstrate a bias compared with Roche automated platform assays. Analysis of urine AKU patients demonstrated complete intereference in enzymatic assay (y=23.033x - 2.933) but improved performance using the kinetic Jaffe assay (y=1.2542x – 0.8284), compared with LC-MS/MS.

Conclusion:

LC-MS/MS methods are less likely to succumb to sample issues and the described easy to prepare method, enables small volume trial samples to be analysed for creatinine without issues from common interferences.

6.2 Introduction

There have been numerous publications citing and investigating serum creatinine as an indicator of renal function and predominantly they revolve around analysis of creatinine via automated chemistry assays. Although there are limitations of the methods on the market (Delanaye et al., 2017), namely various interferences, it is still the most commonly used test to assess renal function. Creatinine is a by-product of muscle creatine and phosphocreatine breakdown and it is largely excreted unchanged by the kidneys with little or no reabsorption. Therefore, if there is renal impairment, levels of creatinine will rise in plasma. However, only conjoined with urine creatinine to assess the true glomerular filtration rate (GFR) can the clinician assess the 'ideal' functioning capacity of the kidneys. Ultimately, this requires a precisely timed 24hr urine collection paired with a blood sample, which for routine monitoring isn't viable. Therefore a serum creatinine is used to estimate GFR, with various formulas used in routine clinical laboratories to provide an eGFR.

Routine serum creatinine assays evolved from the reaction, described by Max Jaffe in 1886 (Jaffe, 1886) which has been translated and explained alongwith his work, by Delanghe and Speckaert (2011). Despite various modifications to improve specificity it wasn't until the assay was changed to an enzymatic reaction with sample blanking, that some interferences were reduced, such as protein and acetoacetate (Peake and Whiting, 2006). The interference of bilirubin, is the most routine interference remaining for the colourimetric kinetic Jaffe reaction (Peake and Whiting, 2006). Experiments with creatininase and creatinase to try and eliminate the interferences led to the currently used enzymatic creatinine assay. In a current UK NEQAS report (distribution 1113, October 2021), 66% of users (374/570) report serum creatinine using an enzymatic method with the remaining 34% using the compensated kinetic jaffe.

Myers et al., (2006) published an extensive paper discussing the creatinine methods available and the introduction of an international standardisation to

align laboratories and therefore reduce discrepancies. External quality control (EQA) schemes further assist laboratories in their pursuit of the 'ideal', however no method is perfect and they are both open to interferences from exogenous factors ranging from the quality of the sample to medication the patient may be administered. Although they are widely regarded as the 'gold standard' in the measurement of small molecules, liquid chromatography mass spectrometry (LC-MS/MS) has limitations in that they are not designed as high-throughput analysers and could never cope with the daily-weekly-monthly numbers of samples demanding creatinine analysis, both for serum and urine, which is why we have automated analysers for that purpose.

The gold standard method for creatinine is isotope dilution gas chromatography mass spectrometry (ID-GC/MS) and although methods claim to be traceable to the reference method, there is still room for improvements (Hoste et al., 2015). There were significant discrepancies at low creatinine concentrations which have the potential to misclassify children and those with muscle wasting conditions.

Currently there are two methods used by the clinical laboratory at the Royal Liverpool University Hospital in the measurement of creatinine. The colourimetric enzymatic Jaffe reaction which involves creatinine reacting with picric acid in alkaline conditions to produce an orange-red coloured compound. The rate of formation of the complex is measured spectrophotometrically and is directly proportional to the concentration of creatinine in the sample (figure 6.1). The Roche analyser uses "rate-blanking" to minimize interference by bilirubin and to correct for non-specific reactions caused by interfering serum/plasma pseudo-creatinine chromogens, such as proteins and ketones; the results for serum or plasma are corrected by -26 µmol/L (-0.3mg/dL). Other disadvantages to this method is that the reaction is non-specific for creatinine and potential interferences such as ascorbic acid, acetone, or cephalosporin antibiotics and bilirubin (Daugherty et al., 1978) may erroneously produce an orange-red colour as they react with the picric acid (Salazar 2014).

$$Creatinine + Picric\ Acid \xrightarrow{Alkaline\ pH} Creatinine - Picric\ acid\ complex$$

Figure 6.1: Jaffe reaction for measurement of creatinine

This assay is routinely used for urine creatinine analysis, and serum creatinine analysis in AKU patients, for the reason that elevated levels of homogentisic acid (HGA) interfere in the enzymatic assay (figure 6.2).

The second method, referred to as the enzymatic assay is used for the majority of serum creatinine measurements on the Roche analyser and utilises a method based on the conversion of creatinine with the aid of creatininase, creatinase and sarcosine oxidase to glycine, formaldehyde and hydrogen peroxide (H_2O_2). Catalysed by peroxidase, the liberated H_2O_2 reacts with 4-aminophenazone and 2,4,6-triiodo-3-hydroxybenzoic acid (HTIB) to form a quinone- imine-chromogen. The colour intensity of the quinone-imine-chromogen is directly proportional to the creatinine concentration in the reaction mixture (figure 6.2).

$$Creatinine + H_2O \xrightarrow{Creatininase} Creatine$$

$$Creatine + H_2O \xrightarrow{Creatinase} Sarcosine + Urea$$

$$Sarcosine + O_2 + H_2O \xrightarrow{SOD} Glycine + HCHO + H_2O_2$$

$$H_2O_2 + 4 - aminophenazone + HTIB \xrightarrow{POD} quinone\ imine\ chromagen + H_2O + HI$$

Figure 6.2: The enzymatic assay for measurement of creatinine.

HTIB - 2,4,6-triiodo-3-hydroxybenzoic acid, SOD – sarcosine oxidase, POD - peroxidase

Loken et al., (2010) postulated that HGA interferes in the enzymatic assay by competing with HTIB and 4-aminophenazone in the reaction forming the chromophore due to affinity for the peroxidase. By adding increasing concentrations of HGA to a normal urine sample and measuring creatinine levels by both the Jaffe reaction and the enzymatic method results showed increasing concentrations of HGA caused no decrease in creatinine levels

with the Jaffe reaction, but significant suppression in the enzymatic method with -13% at HGA concentrations of 2 mmol/L and -47% at 10 mmol/L.

The conclusions were also supported in later papers (Pauwels et al., 2012; Curtis et al., 2014) who demonstrated a -30% suppression of creatinine at a HGA concentration of 100 µmol/L and >-50% at 400 µmol/L. This paper also emphasised the significant interference by HGA on assays where the production of H_2O_2 is a key component, for example urate (using urate oxidase) and for various fractions of cholesterol (using cholesterol oxidase). A further expansion on this work demonstrated no interference by the other major metabolites, tyrosine (TYR), hydroxyphenyllactate (HPLA) and hydroxyphenylpyruvate (HPPA) on any serology assays, however urine dip strip testing should be avoided (Curtis et al., 2019). Of note, the Roche kit inserts for both the Jaffe reaction and the enzymatic assay states “high HGA concentration in urine samples leads to false results” (Roche 2011-04, V5; Roche 2014-09, V7).

With all the interference possibilities that could potentially cause issues of false positives or negatives within trial studies and certainly our AKU routine service we have developed a simple LC-MS/MS method to measure creatinine in serum and urine. We also investigated sample stability, as this is also an important factor when conducting trial analyses over a long-term.

6.3 Materials and Methods

6.3.1 Chemicals and materials

Isotope-labelled internal standard (IS) (d3-creatinine) was purchased from CDN Isotopes, UK. LC–MS grade methanol and acetonitrile were obtained from Honeywell, UK. Formic acid was obtained from Biosolve. Water was purified in-house by the Elix Essential 5UV with Synergy UV Millipore water purification system. PBS (Fisher Scientific) one tablet was dissolved in 100 mL deionised water. All dilutions and sample preparation were performed in glass, and samples transferred into 96 well plates (Waters). Oxygen-free nitrogen was supplied by a Genius 2010 Peak nitrogen generator.

6.3.2 Instrumentation and operating conditions

Analysis was performed on an Agilent 6490 Triple Quadrupole mass spectrometer with Jet-Stream® electrospray ionisation (ESI–MS/MS) coupled with an Agilent 1290 infinity UHPLC pump and autosampler. All data processing both qualitative and quantitative analysis was performed using Mass Hunter software package (version B.07.00). Chromatographic separation was achieved on an Atlantis C18 column (100 mm × 3.0 mm, 3 µm, Waters) maintained at 35^{o}C. A 2 µl sample was injected onto the column over a 3 min run-time which was deemed sufficient with a gradient starting at 95% water (with 0.1% formic acid) which changed to 90% organic (methanol with 0.1% formic acid) at 1.5 min. Gradient was held for 30 sec and returned to starting conditions for re-equilibration and the flow rate was 0.4 mL/min throughout. Optimum operating ESI conditions were: gas temperature 150°C (nitrogen), gas flow 17 L/min; nebulizer pressure 40 psi; sheath gas temperature 320^{o}C and sheath gas flow 12 L/min. Capillary voltages were optimised to 3500V in positive mode and 2500V in negative mode with equal nozzle voltages (1500V) in both modes. The iFunnel parameters were optimised in both negative and positive mode as 60V for low pressure RF and 110V for high pressure RF.

6.3.3 Preparation of standard solutions, calibrators and controls

A super-stock standard solution of creatinine was made at a concentration of 100 mmol (100,000 µmol) and was diluted accordingly in phosphate buffered saline (PBS) to make a urine calibration range from 1 mmol to 20 mmol and a serum range from 10 µmol to 1000 µmol. PBS was used for this assay due to the ubiquitous nature of creatinine and so matrix matching was not possible. Quality controls (QC) were purchased from Technopath for serum (levels 1 and 3) and urine (levels 1 and 2), and are routinely used by our Jaffe and enzymatic assays on the Roche analyser.

IS d3-creatinine was provided as a certified reference material (Merck) at 100 µg/mL. An intermediate was made by adding 10 µL stock to 20 mL deionised water. A working serum IS was made by taking 600 µL of

intermediate and diluting with 500 mL deionised water plus 0.1% formic acid (giving a concentration of 0.504 nmol/L). A working urine IS was made by taking 3 mL of intermediate and diluting with 500 mL deionised water plus 0.1% formic acid (equivalent to a final working concentration of 2.25 nmol/L). All samples, calibrators and QC were assayed on a 1 in 1000 dilution with the working internal standard solution pertinent to the assay.

6.3.4 Assay validation

The assay was validated using in-house protocols based on C62-A published guidance (Clinical and laboratory standards institute, 2014; Lynch, 2016). Method validation evaluated linearity, accuracy, imprecision, matrix factor, lower limit of quantification (LLOQ), carry-over and stability.

6.3.4.1 Linearity

Standard curves were fitted using linear regression with a 1/x weighting factor and a minimum of six calibration points plus urine and serum blanks and curve fitting parameters excluded zero. Performance of fitted curves was presented as the coefficient of determination (R^2).

6.3.4.2 Accuracy

Accuracy was determined as closeness to the nominal spiked concentrations, both intra- and inter-assay with n = 6 and n = 20 respectively. Accuracy was calculated as: [measured concentration – nominal concentration] ***/*** [nominal concentration] × 100 (%). In addition, EQA samples were used for both serum and urine as part of the NEQAS EQA schemes.

6.3.4.3 Precision

Imprecision was determined both intra- (n = 6) and inter- assay (n = 20) using commercial QC material (Technopath QC for urine and for serum).

6.3.4.4 Matrix effects

Matrix factor experiments were determined for three serum and urine matrices at two different concentrations. Due to the endogenous presence of creatinine, standards were prepared in PBS, however, commercial QCs are

in a matrix, and all samples are matrix based, therefore the experiments were deemed necessary to determine any ion suppression effects. Deionised water and three urine samples (acidified) were spiked with two concentrations of creatinine or the equivalent of the final concentration of IS (as per section 6.3.3). For serum a similar process was undertaken using steroid depleted serum (SDS), serum or plasma samples. Due to endogenous creatinine concentrations, all matrices had an equivalent volume of water as that used for the creatinine additions (ratio of 9:1 matrix:water maintained throughout). Using the matrix factors calculated, an IS normalised matrix factor can be determined (matrix factor of analyte / matrix factor of IS multiplied by 100) (EMA Guidelines, 2011).

6.3.4.5 Dilution and carry-over

Dilution integrity of urine and serum creatinine was assessed by pre-analytical dilution of five samples with high creatinine concentrations in deionised water. This was assessed at a dilution of 1:1 for both serum and urine due to the measuring range of the calibration standards (n = 6). Carry-over of urine and serum creatinine and d3-IS was assessed by five separate water injections following injection of the top calibrator.

6.3.4.6 Stability

Stability was assessed for both serum and urine, using three pools representing their low, medium and high concentrations, analysed three times. Stability was determined following three freeze-thaw cycles (at −80°C), over 24hr at room temperature and at 4°C for 7 days, equivalent of cold lab storage. Results are expressed as a percentage of nominal values determined against a fresh calibration curve. Prepared samples were also reanalysed after 24hr to determine any issues with re-injection of samples, stored on the analyser (approximately 10°C).

6.3.5 Analysis of analytes in urine and serum samples

This method uses a ‘dilute and shoot’ procedure. A 10 µL sample of serum or urine is diluted in 10 mL of the respected IS spiked diluent (1 in 1000). In the case of smaller samples, sample volume can decrease with adjustment of

diluent volume, maintaining the ratio at 1 in 1000. The mixed samples, calibrators and QCs are transferred to a 96 well plate which are analysed on the mass spectrometer over a 3 min run-time.

6.3.6 Comparison between automated chemistry methods

50 serum and urine samples were analysed on the LC-MS/MS method and compared with the Roche enzymatic creatinine method for serum, and kinetic Jaffe for urine. In addition, EQA samples were also compared with the same comparator method.

6.3.7 Analysis of AKU urine and serum samples

Samples from patients with AKU were analysed on both the automated Roche Jaffe and enzymatic assays and compared with the LC-MS/MS method, validated within this study. Current practise is that both serum and urine samples from AKU patients are analysed on the kinetic jaffe assay. The enzymatic creatinase assay is **not used** for AKU serum samples.

6.4 Results

6.4.1 Method Validation

The mass spectrometer was operated in multiple reaction monitoring (MRM) mode. Due to the relatively small molecular size of creatinine, only one ion transition was determined for the IS and the respective collision energies are detailed in table 6.1. For optimal sensitivity creatinine and its IS were measured in positive ionisation mode.

Compound	Ionisation	Transition	Collision energy (V)
Creatinine (Quant)	Positive	114 > 44	18
Creatinine (Qual)	Positive	114 > 86	8
d3-creatinine	Positive	117 > 47	20

Table 6.1: Parameters for MS detection of creatinine and its IS.

A typical chromatogram is displayed in figure 6.3 for the primary product ions (quantifiers) for creatinine and its IS. Chromatograms are identical for serum and urine matrix samples.

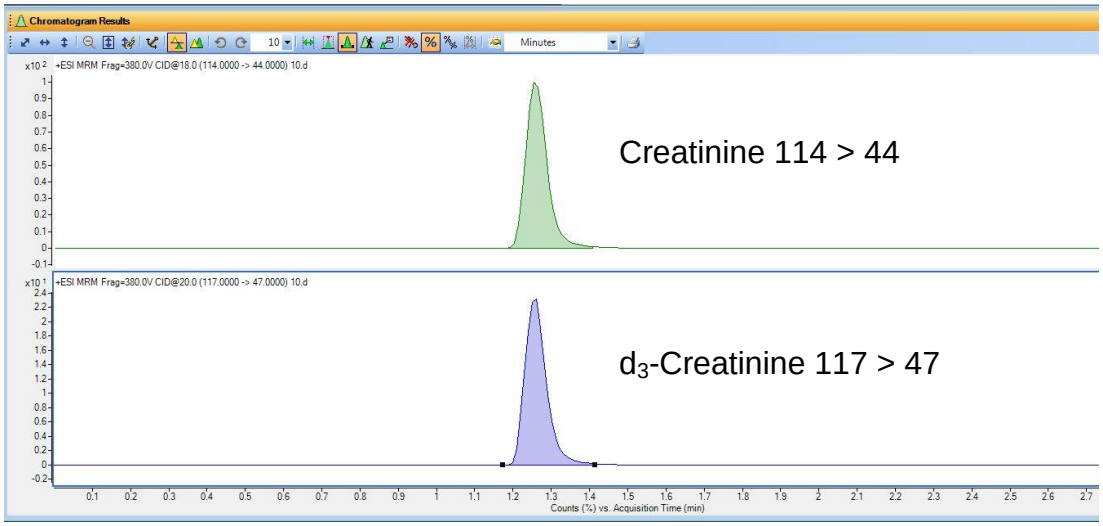

Figure 6.3: Chromatogram showing creatinine and its d3 IS

6.4.2 Linearity

Long-standing knowledge of the measuring ranges for creatinine ensured we set up our calibration range in urine considerably larger than that in serum. Although the assay is linear, the range of calibration standards chosen has determined the measuring range stated in both urine and serum samples. Serum calibration standards are 10–1000 µmol/L and urine are 1-20 mmol/L. Calibration standard curves (seven points including zero) exhibited a good fit over the range examined, with minimal inter-assay variability over the concentration ranges (figure 6.4).

Calibration standards are analysed at the front of the batch and when numbers exceed 50 samples, they are also prepared and analysed at the end to ensure that there is no significant drift. The calibration curve (calculated by the Agilent software) includes zero for both serum and urine.

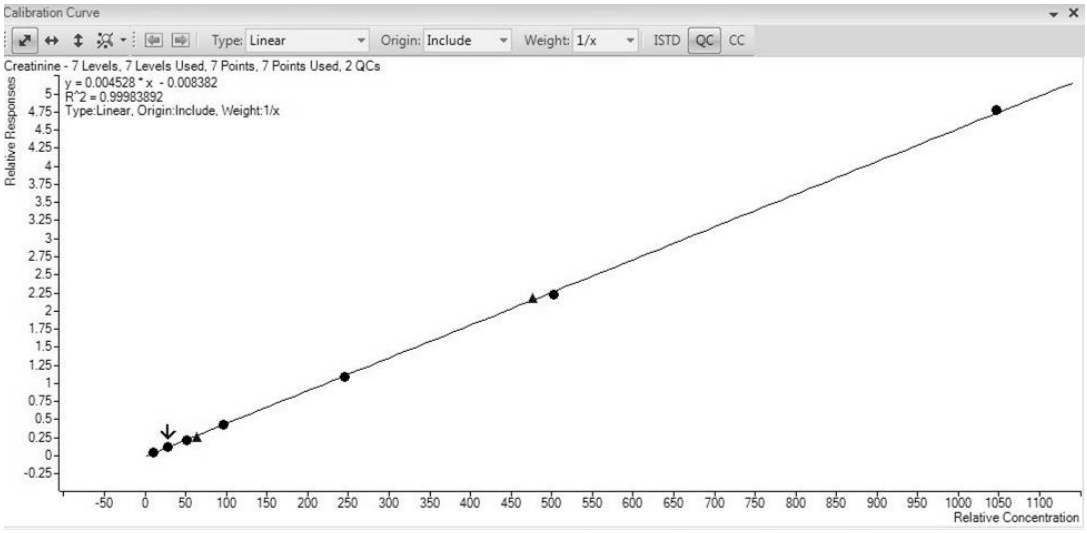

Figure 6.4: A typical calibration curve for serum creatinine (10-1000 µmol/L).

6.4.3 Accuracy

Intra- and inter-assay accuracy was determined (table 6.2) in both urine and serum matrices. Results are represented as percentage recovery of a nominal amount of creatinine spiked into PBS. Intra- and inter-batch accuracy was shown to be within ±10% of the nominal expected value in both matrices, satisfying assay validation criteria.

6.4.5 Precision

Precision (%CV), both intra- (n = 6) and inter- (n = 20) was determined in both matrices (table 6.3). Performance was within acceptable criteria with intra-assay precision <5% CV for both levels in serum and urine. Inter-assay precision was <6% CV for the serum assay and <8% for the urine assay, again demonstrating acceptable performance criteria.

Expected Conc mmol/L	Urine creatinine		Expected Conc µmol/L	Serum creatinine	
	Intra-assay	Inter-assay		Intra-assay	Inter-assay
2.5	92.7 ± 0.67	93.1 ± 0.60	25	96.4 ± 1.20	103.2 ± 7.80
7.5	97.3 ± 0.64	96.7 ± 2.11	100	93.2 ± 1.44	95.1 ± 3.80
15.0	101.3 ± 1.59	101.1 ± 1.19	250	95.2 ± 1.34	98.2 ± 1.14

Table 6.2: Intra- and inter-assay accuracy for urine and serum creatinine by LC-MS/MS

QC	Urine creatinine		QC	Serum creatinine	
	Intra-assay	Inter-assay		Intra-assay	Inter-assay
1	6.3 ± 0.22 (3.5%)	6.2 ± 0.25 (4.1%)	**1**	60.8 ± 2.1 (3.4%)	62.6 ± 3.4 (5.4%)
2	14.4 ± 0.50 (3.5%)	14.1 ± 1.1 (7.9%)	**2**	491 ± 6.6 (1.3%)	487 ± 11.1 (2.3%)

Table 6.3: Intra- and inter-assay precision for urine and serum creatinine assays

6.4.6 Matrix effects

Matrix effects are calculated as per EMA Guidelines (2011) whereby the IS normalised matrix factor is determined (section 6.3.4.4). Data is represented as the average IS normalised matrix factor, for each matrix at the two spiked concentrations. For acceptable performance, the CV has to be <10% and the IS normalised matrix factor 1.0 ± 0.2 (equivalent to ± 20% of 100% recovery). From the data presented in table 6.4 there was no significant ion suppression observed in either serum or urine matrices supporting the assay for quantitation of serum and urine samples against an aqueous calibration curve.

	50 µmol/L spike	**200 µmol/L spike**
Serum	1.10 ± 0.06 (5.6%)	0.96 ± 0.02 (2.3%)
Plasma	1.07 ± 0.05 (4.6%)	1.09 ± 0.04 (3.9%)
SDS	0.97 ± 0.04 (4.1%)	1.03 ± 0.04 (3.6%)
	10 mmol/L spike	**20 mmol/L spike**
Urine 1	0.96 ± 0.04 (4.6%)	1.08 ± 0.10 (8.7%)
Urine 2	1.05 ± 0.02 (2.0%)	0.91 ± 0.02 (2.3%)
Urine 3	0.98 ± 0.09 (9.1%)	1.04 ± 0.03 (2.9 %)

Table 6.4: Serum and urine IS normalised matrix factors.

6.4.7 Dilution and carry-over

Neat serum samples were assessed on a x2 dilution (prior to the standard 1 in 1000 assay dilution) as the top calibration standard was 20 mmol/L for urine and 1000 µmol/L for serum. Additional dilutions were unlikely to be required clinically due to the extensive calibration range of the LC-MS/MS assay. Serum samples from pre-dialysis patients were analysed and recovery was 99.7 ± 0.91 % (creatinine ranged from 1054-1862 µmol/L). Urine samples were more difficult to source (creatinine >20 mmol/L) and

therefore three were spiked. Recovery was 98.3 ± 1.25% (creatinine ranged from 20.3-26.5 mmol/L). Carry-over was determined for both serum and urine, with an average area of 0.06% for water samples following the top serum calibration standard and no observed peak following the urine top standard. The carry-over in the serum samples although able to integrate does not represent any quantifiable serum creatinine concentration. In addition, there was no cross-talk observed for creatinine or its respective internal standard.

6.4.8 Stability

Data displayed in table 6.5 demonstrates the robustness of creatinine in serum and urine samples. There was no stability issues post freeze-thaw cycles, storage at room temperature or storage in refrigerator conditions. This is consistent with manufacturer (Roche) guidance for the automated Jaffe and enzymatic assays. In addition, a plate of prepared samples can be reinjected without any influence on creatinine concentrations measured.

	Urine	**Serum**
Three freeze-thaw cycles	99.8 ± 1.2	101.1 ± 1.9
24hr room temperature	97.8 ± 1.8	98.1 ± 2.1
7 days at 4^{o}C	98.6 ± 2.1	97.5 ± 2.0
Re-injection	2.1 ± 1.9	1.8 ± 1.7

Table 6.5: Stability represented as percentage recovery, n=9 for all conditions.

Re-injections are percentage change in creatinine concentration compared with the initial injection.

6.4.9 Lowest Limit of Measuring Interval (LLMI)

The LLMI in validation as per C62-A guidance is defined as the lowest calibrator which satisfies a CV ≤ 20%. This is generally considered the lowest calibration standard. The LLMI for creatinine in urine was 1 mmol/L

and for serum 10 µmol/L and at both of these levels, the CV was <10% and a signal to noise of >20:1. However from a clinical view point, measurement at lower concentrations is not required, so whilst the LC-MS/MS has the potential to measure lower concetrations, it wasn't necessary to validate lower.

6.4.10 Comparison data between LC-MS/MS and Roche method

Comparison of urine creatinine between the Roche kinetic Jaffe assay and the LC-MS/MS assay provided a high degree of correlation, with an R^2=0.993 and equation of the line y=1.0218x – 0.2061 (figure 6.5). This was also evidenced with the NEQAS EQA scheme for urine chemistry, where comparison with the group mean demonstrated an R^2=0.998 and an equation y= 1.0298x + 0.4972 (figure 6.6).

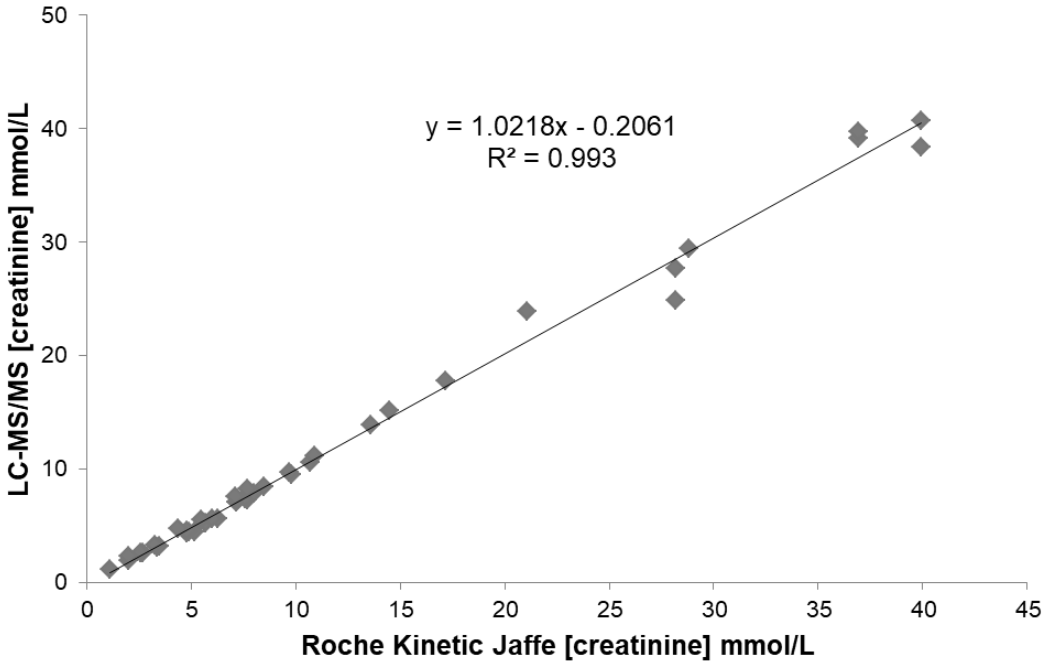

Figure 6.5: Comparison of in-house Roche Kinetic Jaffe with LC-MS/MS assay.

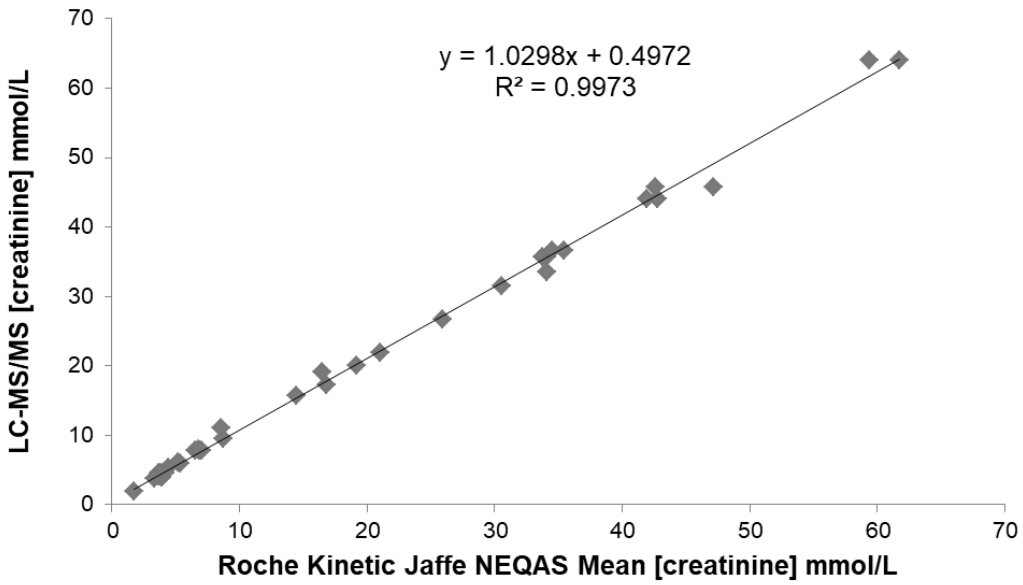

Figure 6.6: Comparison of NEQAS Urine Creatinine Roche kinetic Jaffe group with LC-MS/MS assay

Comparison of serum creatinine performed with the enzymatic creatinase assay (Roche) of 50 patient samples across a broad range, provided an overall correlation of R^2=0.9948 and an equation of fit, y= 0.9869x + 0.6675. However, when the data was examined it was clear that the higher concentrations tightened the correlation. Comparison of serum samples with [creatinine] <200 µmol/L demonstrated a significant bias (figure 6.7) with an equation of y=0.8755x + 10.975 and R^2=0.9208; the equation indicating both a bias and a blank issue. At concentrations >200 µmol/L, the correlation improves, however the blank baseline issue remains (figure 6.8) and corresponds with a negative value of 25.7 µmol/L.

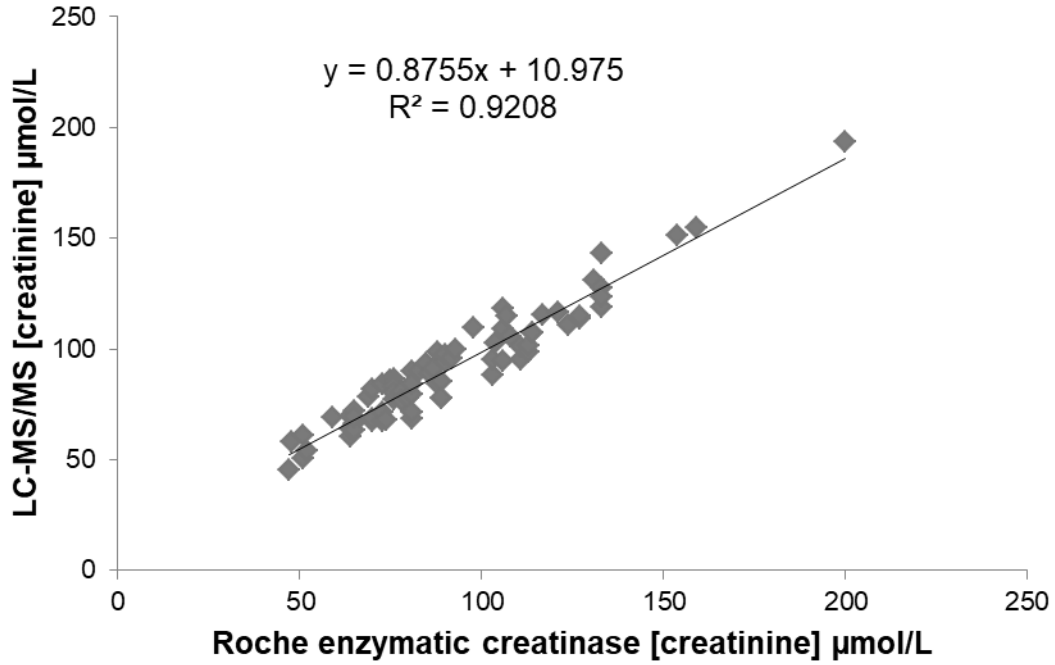

Figure 6.7: Comparison of serum creatinine results obtained on Roche enzymatic creatinase assay with LC-MS/MS (<200 µmol/L)

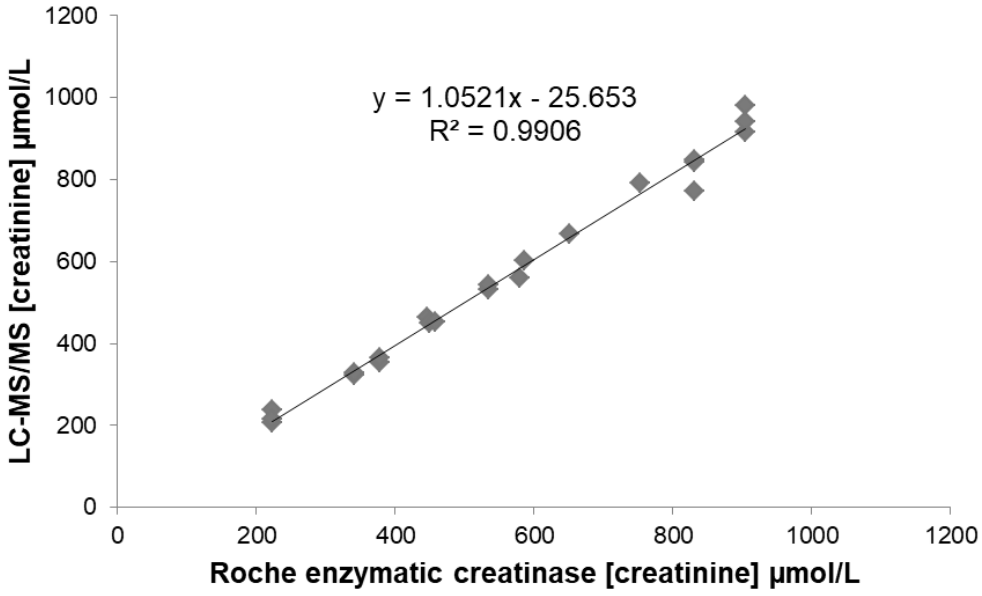

Figure 6.8: Comparison of serum creatinine results obtained on Roche enzymatic creatinase assay with LC-MS/MS (>200 µmol/L)

Similar comparison was seen with the NEQAS EQA scheme. However this method is the only LC-MS/MS method which returns EQA results to this scheme, so no comparison could be made within group (figure 6.9). We did note an average negative bias of 20% against the ALTM (all laboratory trimmed mean). Similar comparison was seen with the Roche enzymatic group mean with R^2=0.9944 and y=0.8457x – 4.8714.

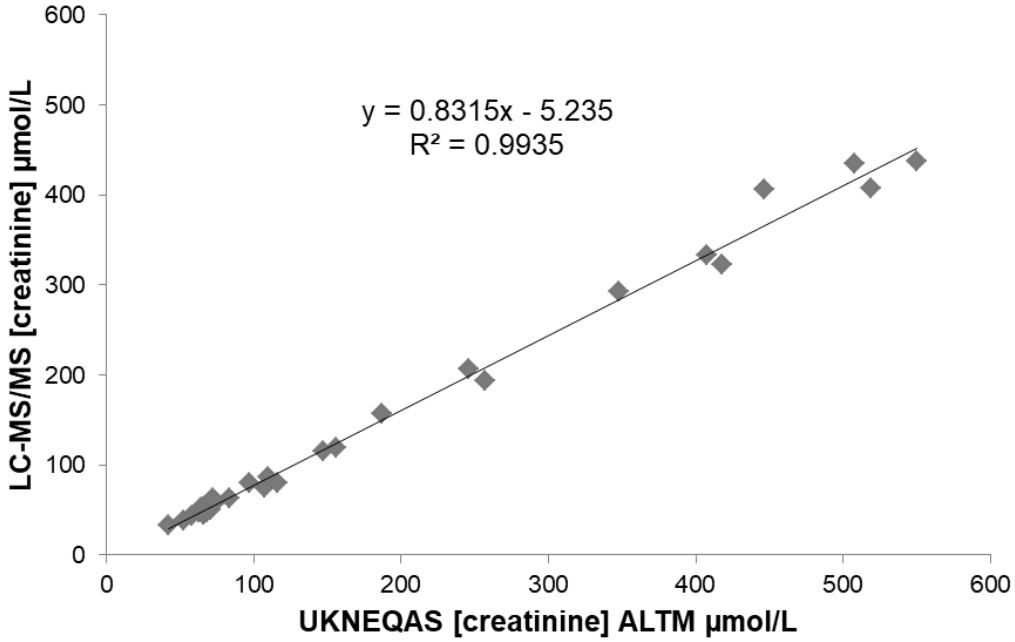

Figure 6.9: Comparison of LC-MS/MS creatinine performance with UK NEQAS ALTM.

6.4.11 Comparison of urine samples from AKU patients

Evaluation of urine samples analysed on all three methods demonstrated the influence of high HGA on creatinine methods. Comparison of all baseline SONIA-1 urine samples (u-HGA ranging from 4,052 to 33,784 µmol/L) revealed almost complete inhibition of the enzymatic reaction (figure 6.10) with a poor correlation (R^2=0.7866). Results ranged from 0.2 mmol/L to 0.7 mmol/L on the enzymatic assay with LC-MS/MS measuring the extremes at 1.8 to 18.5 mmol/L respectively.

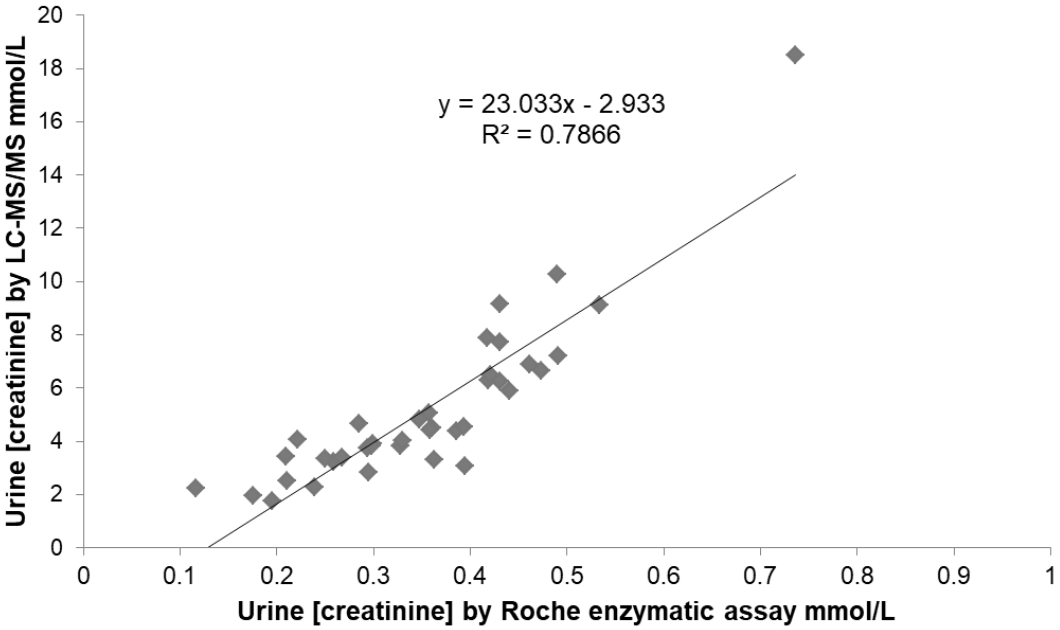

Figure 6.10: Correlation of Roche enzymatic creatinine assay with LC-MS/MS in urine samples from AKU patients (note difference in x axis scale to enable visual representation)

Correlation of Roche kinetic Jaffe and the LC-MS/MS method was more favourable (figure 6.11) with an R^2=0.9812. What is interesting is that the negative bias of the LC-MS/MS assay to the Jaffe assay seen with normal urine samples has shifted to a positive bias of approximately 6% (figure 6.12).

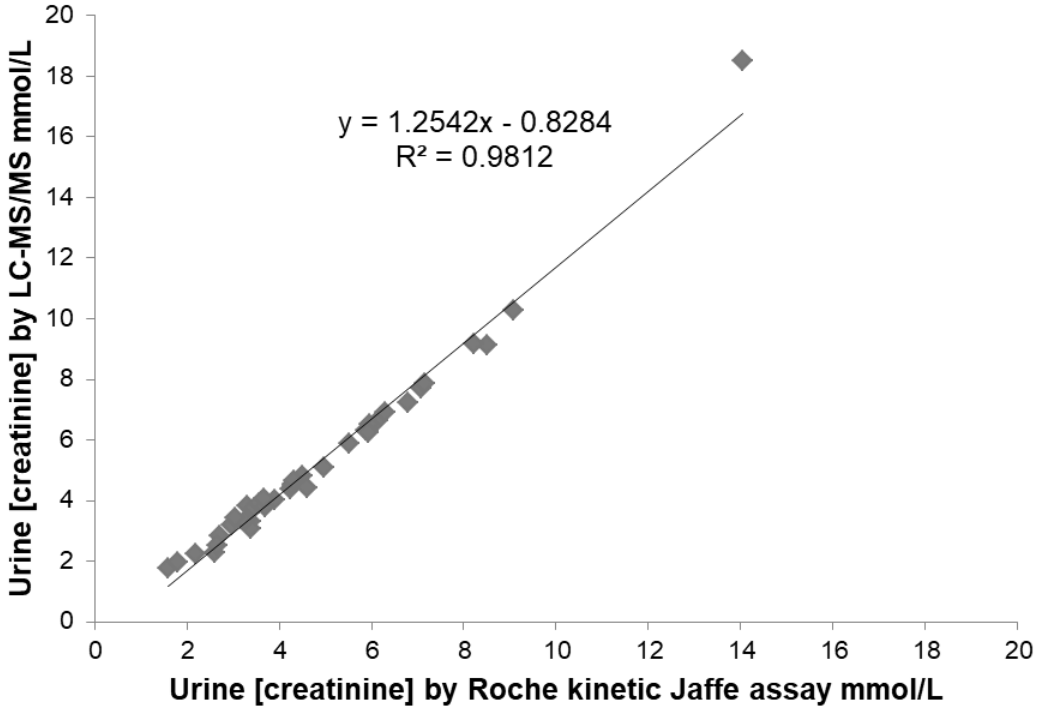

Figure 6.11: Correlation of Roche kinetic Jaffe assay with LC-MS/MS in urine samples from AKU patients.

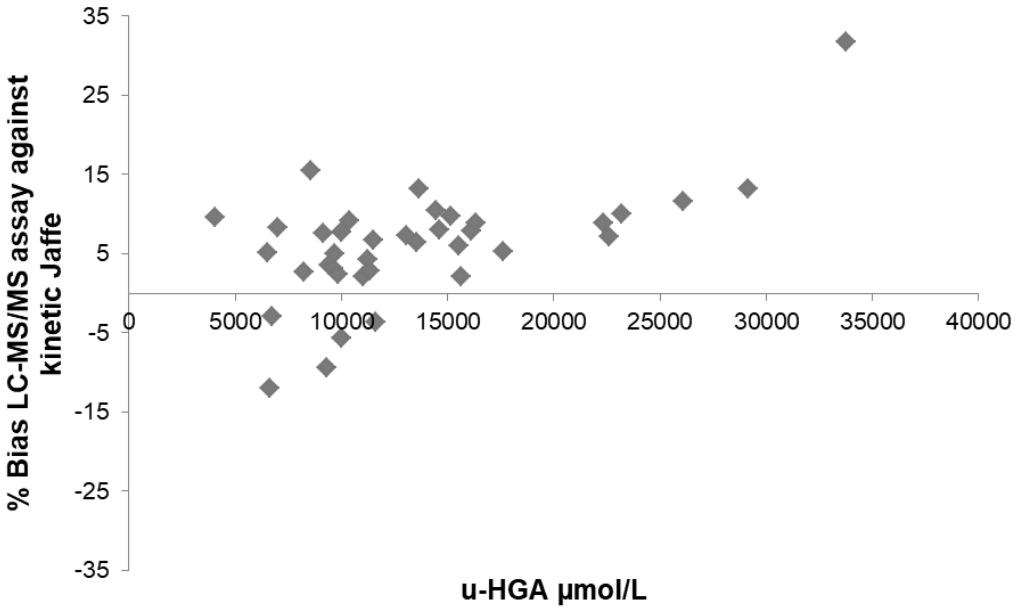

Figure 6.12: u-HGA against percentage bias of LC-MS/MS assay

6.5 Conclusions and Discussion

Although there are fully established automated assays for the large scale measurement of creatinine in busy analytical laboratories, there will always be samples that cause interferences. Our LC-MS/MS method described is not designed to supersede these assays, as mass spectrometers were never designed to be high throughput, but to provide the potential to offer a stable measurement for all possible sample types with the added advantage of being able to use sample volumes as low as 1 µl; which is particularly useful in trial studies when volume can be an issue particularly with neonates or animal model samples. As more interferences become apparent especially with assays using peroxidases then the backup of a robust LC-MS/MS method can be justified to provide an essential and accurate alternative measurement.

Our method although similar in respects to a previous published method (Owen et al., 2006) has utilised an analytical column. Using a Waters Atlantis C18 column (100 mm × 3.0 mm, 3 µm), gave the extra retention compared with the much smaller security guard column (4 mm x 3 mm), thus taking the chromatography and elution of the main peak out of the ion suppression zone which usually resides pre-1.5 min. The use of a binary gradient simplified the method and although not matrix-matched, our calibration curves for both serum and urine assays show excellent precision, accuracy and EQA returns that reveal the expected negative bias compared to automated assays.

Comparison with this published method demonstrated different correlations; whilst we have reported a negative bias by LC-MS/MS in serum samples compared with the Jaffe assay, the opposite was reported (Owen et al., 2006) with a small negative bias of the Jaffe at [creatinine] < 150 µmol/L which increased at higher [creatinine]. Differences between our method and the published one will be contributed to by calibration and likewise comparison with automated methods. For instance, automated methods are a one point calibration plus a zero, whereas the developed method in this

paper is six points and zero, providing stability across a more extensive concentration range. Much has been made of the pseudo-creatinine effect for which automated serum assays are adjusted. It is estimated that the creatinine over-estimation is 27 µmol/L which will have a greater impact at lower creatinine concentrations. Commercial calibrators for automated assays are ‘compensated’ to reduce the value by this amount and this will affect the intercept of the assay rather than the slope (Owen et al., 2006). Interestingly we have shown an intercept issue in the serum creatinine comparison.

Comparison of the urine methods, revealed the impact of HGA. HGA is present at an order of magnitude higher in urine than serum, mmol compared with µmol/L. The enzymatic creatinine assay was inhibited almost completely by all HGA concentrations in the samples examined. There was a slight effect on the Jaffe assay with a switch from a negative bias by LC-MS/MS to a positive bias, however a good correlation was achieved and the Jaffe assay is suitable to monitor renal function in AKU patients. Previous study (Loken et al., 2010) also deemed the enzymatic assay unsuitable for urine in AKU patients, with HGA concentrations of 2 mmol/L giving significant suppression. The hypothesis was that HGA competes with HTIB and 4-aminophenazone in the reaction forming the chromophore. This also has implications for using automated assays in animal model studies, as [u-HGA] have been shown to be higher in AKU mouse models (Hughes et al., 2020) and therefore a greater interference may be observed.

Serum comparison wasn’t able to be performed due to sample volume for reanalysis on the automated assays. Although the s-HGA concentrations are a factor of 1000 lower compared with u-HGA, creatinine is also lower, therefore the ratio of HGA:CRE would still interfere with the enzymatic assays (Curtis et al., 2014). We already routinely measure serum creatinine in AKU patients on the Jaffe assay due to the known HGA interference in the enzymatic assay.

Other methods have been published using a variety of chromatography techniques. Zhao (2016) suggested reverse phase did not demonstrate good chromatography due to the small molecular weight of creatinine choosing HILIC with water and acetonitrile mobile phases, wheras Fraselle et al., (2015) chose isocratic UPLC in basic conditions but a longer run-time compared with this author. Solid phase extraction methodology has also been used and overall gave a positive bias to LC-MS/MS methods (Huskova et al., 2004) unlike our negative bias which may be attributed to calibration differences, or exogenous factors due to the lack of a HPLC step within their method.

Overall, we have developed a method for urine and serum creatinine, by LC-MS/MS which can be utilised when simple interferences start to affects any of the established laboratory automated methods. Microsample volumes can be used which is particularly useful in clinical trials where volume, especially serum may be at a premium, and also in animal studies where volume is usually a limiting factor. The ability to dilute and analyse volumes as low as 1 µL has enabled us to tie creatinine measurements by this described LC-MS/MS method to previously described AKU methodologies (Hughes et al., 2014, 2015). The method described here was never intended to displace the established high throughput assays which are the mainstay within clinical laboratories, but as a viable clinical trial alternative. However as high throughput LC-MS/MS becomes more mainstream, this assay will certainly be a consideration.

Chapter 7

The effect of nitisinone on homogentisic acid and tyrosine: A two-year survey of patients attending the National Alkaptonuria Centre, Liverpool.

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Declaration and acknowledgments

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7.1 Abstract:

Background

Alkaptonuria (AKU) is a rare, debilitating autosomal recessive disorder affecting tyrosine (TYR) metabolism. Deficiency of homogentisate 1,2-dioxygenase leads to increased homogentisic acid (HGA) which is deposited as ochronotic pigment. Clinical sequelae include severe early onset osteoarthritis, increased renal and prostate stone formation and cardiac complications. Treatment has been largely based on analgaesia and arthroplasty. The National AKU Centre (NAC) in Liverpool has been using 2 mg nitisinone (NTBC) off-license for all patients in the United Kingdom with AKU and monitoring the TYR metabolite profiles.

Methods

Patients with confirmed AKU are commenced on 2 mg dose (alternative days) of NTBC for three months with daily dose thereafter. Metabolite measurement by LC-MS/MS is performed at baseline, day 4, three months, 6 months and one-year post-commencing NTBC. Thereafter, monitoring and clinical assessments are performed annually.

Results

Urine HGA concentration decreased from an average baseline 20,557 µmol /24hr (95^{th} percentile confidence interval 18,446-22,669 µmol/24hr) by on average 95.4% by 6 months, 94.8% at one year and 94.1% at two year monitoring. A concurrent reduction in serum HGA concentration of 83.2% compared to baseline was also measured. Serum TYR increased from normal adult reference interval to a mean ± SD of 594 ± 184 µmol /L at year-two monitoring with an increased urinary excretion from 103±81 µmol /24hr at baseline to 1,071 ± 726 µmol/24hr two years from therapy.

Conclusions

The data presented is the first longitudinal survey of NTBC use in an NHS service setting and demonstrates the sustained effect of NTBC on the TYR metabolite profile.

7.2 Introduction:

Alkaptonuria (AKU, OMIM: 203500) is a rare autosomal recessive disorder resulting from mutations in the gene that encodes for the enzyme homogentisate 1, 2-dioxygenase (HGD, E.C.1.12.11.5) (La Due al., 1958; Gallagher et al., 2016). The enzyme, a constituent part of the tyrosine (TYR) degradation pathway converts homogentisic acid (HGA) to maleylacetoacetic acid (figure 7.1). Deficiency of the enzyme results in the accumulation of HGA in tissues and excretion of gram quantities of HGA in the urine (Phornphutkul et al., 2002; Introne et al., 2011; Hughes et al., 2014; Ranganath et al., 2016). Through ancillary pathways, HGA oxidises to benzoquinone acetic acid (BQA), which is responsible for the black appearance of urine in AKU, upon standing. The conversion of BQA to melanin-like pigment and its binding to connective tissues are linked to the destructive nature of the disease such as seen in cartilage, bone and other connective tissues such as sclera (O'Brien et al., 1963; Helliwell et al., 2008; Taylor et al., 2012; Ranganath et al., 2013; Keenan et al., 2015; Roberts et al., 2015). This ochronotic process takes place over several years and affects virtually all connective tissues.

Treatment of the condition is largely via analgesics, joint replacement when required, dietary protein restriction and large doses of vitamin C, each with varying degrees of efficacy (Ranganath et al., 2013). There have been trials of 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3 cyclohexanedione (NTBC) more commonly referred to as nitisinone, in the treatment of AKU. NTBC inhibits 4-hydroxyphenylpyruvate dioxygenase (HPPD, E.C.1.13.11.27), the enzyme which produces HGA (Figure 7.1) (Ranganath et al., 2013; Lock et al., 2014).

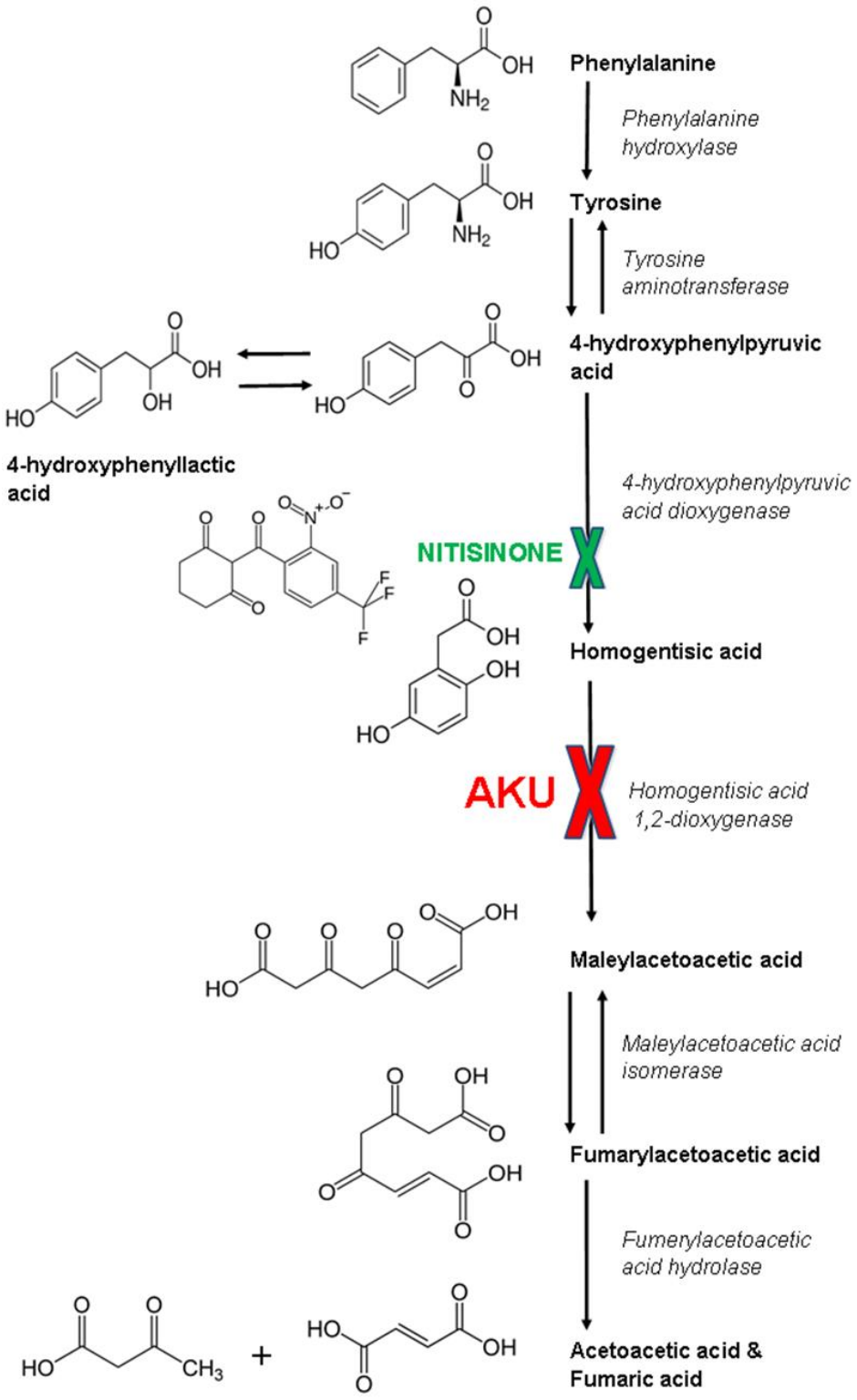

Figure 7.1: TYR degradation pathway indicating enzyme defect in AKU and where NTBC acts upon the pathway.

NTBC has been licenced for the treatment of hereditary tyrosinaemia type 1 (HT-1, OMIM 276700) since 2002 where deficiency of fumarylacetoacetate hydroxylase (FAH, E.C. 3.7.1.2) results in liver failure, hepatocellular carcinoma and renal tubular dysfunction (Ashorn et al., 2006). The efficacy of the therapy has resulted in NTBC replacing liver transplant as the treatment of choice in HT-1 (Lindstedt et al., 1992; McKiernan, 2013).

Previously published studies where NTBC has been used in the treatment of AKU have demonstrated the effect on blocking HGA production (table 7.1), all showing >94% reduction in urinary HGA. In all studies the concurrent rise in plasma TYR was evident with concentrations rising from within the reference interval to concentrations ranging from 500–1300 µmol/L (Phornphutkul et al., 2002; Suwannarat et al., 2005; Introne et al., 2011; Ranganath et al., 2013).

More recently, a large scale international trial (Suitability Of Nitisinone In Alkaptonuria 1, SONIA-1, DevelopAKUre, project number: 304985) demonstrated the short-term effects of various doses of NTBC (1, 2, 4 and 8 mg) over a four-week period (Ranganath et al., 2016). A clear, dose-dependent suppression of HGA was measured with a mean reduction of urine HGA concentration at four weeks, of 98.8% compared with baseline, on an 8 mg daily dose.

Study	Design	n	Dose	Duration	Results
Phornphutkul et al., 2002	Open, uncontrolled, proof of concept	2	0.35-1.4 mg twice daily	9 and 10 days	95% reduction in [HGA]
Suwannarat et al., 2005	Open, uncontrolled, proof of concept	9	0.35-1.05 mg twice daily	3 months	94% reduction in [HGA]
Introne et al., 2011	Randomised, parallel group, single blind	20	No treatment, 2 mg once daily	36 months	>95% reduction in [HGA]
Ranganath et al., 2016	Randomised, open label, parallel-group design	40	No treatment, 1, 2, 4, 8 mg once daily	4 weeks	98.8% reduction in [HGA] on 8 mg dose

Table 7.1: Summary of previous clinical trials using NTBC in AKU

(n=number of patients in the trial)

In 2012 the Robert Gregory National AKU Centre (NAC) was established by the NHS Highly Specialised Services Commissioning Group at The Royal Liverpool University Hospital, UK. With recognition that NTBC has potential for treatment of this debilitating condition, the centre aimed to provide NTBC, off-label, for all patients in the UK with AKU. The NAC is the first centre to provide long-term supportive and potentially disease-modifying medical care. This includes specialist dietary monitoring and biochemical assessment to ensure the safe and efficacious use of NTBC.

The present publication is an audit of the service at the NAC from a biochemical viewpoint. The NTBC is being used off-licence in a health service setting and the data described documents the effect of NTBC on TYR metabolites over two years of usage.

7.3 Patients and Methods:

7.3.1 Patients

The protocol for treatment is that patients with confirmed AKU are commenced on a 2 mg dose of NTBC, on alternative days for the first three months, which is then increased to 2 mg daily thereafter. Assessments are repeated on an annual basis to monitor response to therapy.

This longitudinal survey reports the biochemical data obtained from monitoring markers of the TYR metabolic pathway and includes data from patients that have attended the NAC for two years (three annual visits). To date 58 patients have enrolled at the NAC. Inclusion criteria are diagnosis of AKU; resident of either England or Scotland, and be aged over 16 years; exclusion criteria are pregnancy and lactation. NTBC is used in an open-label protocol here to investigate its safety and efficacy in the treatment of this rare disease. All patients are provided with written information about the scope of the centre and the assessments they will receive. All patients at the NAC are assessed at baseline with a four-day visit and then at annual follow-up. Most patients are medically fit enough to travel to Liverpool for assessments and treatments. Metabolite measurements are performed at baseline, day four (two-days post-NTBC), three months, 6 months and one

year; with annual monitoring thereafter. Confirmed diagnosis of AKU is based upon increased urinary HGA excretion and is mandatory for referral to the NAC. Urine HGA excretion in healthy volunteers has been demonstrated in the order of <2.92 µmol/24hr (Davison et al., 2015).

7.3.2 Data collection:

The NAC and all subsequent data collection and analyses are approved by The Royal Liverpool and Broadgreen University Hospital Trusts Audit Committee (Audit no. ACO3836). This is not a clinical trial and therefore ethical approval isn't required. Data obtained is following standard clinical assessments upon referral to the NAC. Patients are informed verbally and through patient information leaflets about the activities of the NAC. Patients are also explicitly informed that data may be used for publication and within the NAC patient information leaflet the following paragraph is included:

"We could publish results from the study but if we do, we will make sure that you cannot be identified in anyway. All data used for publicity or for other research purposes will ensure total anonymity. Please let us know when you are visiting the NAC that you understand and have no objections to this". No patient has objected to the use of their data.

7.3.3 Sample collection

Baseline 24 hr urine collection (acidified with 25 mL 2.5 mol/L H_2SO_4) and serum samples were collected. Serum samples were acidified by deproteinisation with perchloric acid (5.8 mol/L). This stabilises the HGA for short- and long-term storage and analysis (unpublished data).

7.3.4 Urine HGA and TYR analysis

Urine samples were analysed using a previously published liquid chromatography tandem mass spectrometry (LC-MS/MS) method (Hughes et al., 2014). The only modification was change from d_2 to a d_4-TYR internal standard (IS). In brief, all analyses were performed on an Agilent 6490 Triple Quadrupole mass spectrometer with Jet-Stream® electrospray ionisation coupled with an Agilent 1290 Infinity II UHPLC pump and autosampler.

10 µL of sample was diluted 1:1000 with 0.4 µmol/L $^{13}C_6$-HGA and 2 µmol/L d_4-TYR in 0.1% formic acid (v/v) in deionised water. Separation was achieved on an Atlantis dC18 column (100 x 3.0 mm, 3 µm, Waters) maintained at 35°C. Quantitation was achieved using a matrix-matched seven point calibration curve and two product ion transitions for each analyte of interest (HGA 167>122 and 167>108, negative ionisation; TYR 182>136 and 182>91 positive ionisation). The urine assay was linear for HGA concentrations up to 16 mmol/L (pre-NTBC therapy) and the lower limit of quantification (LLOQ) was 3 µmol/L (post-NTBC therapy). Dilution integrity was assessed with recovery 96.6 ± 6.6% (n=6) on a 1 in 10 dilution, when required (Hughes et al., 2014).

7.3.5 Serum HGA, TYR and NTBC analysis

Serum samples were analysed on a single method for all three analytes (Hughes et al., 2015) using a matrix-matched serum calibration curve. As for the urine assay, all analyses were performed by LC-MS/MS with 10 µL of deproteinsed sample diluted 1:1000 with 0.2 µmol/L $^{13}C_6$-HGA, 2 µmol/L d_4-TYR and 2 nmol/L $^{13}C_6$-NTBC in 0.1% formic acid/deionised water.
IS concentrations were lower than those used in the urine assay to reflect the lower concentrations in serum, compared with urine, requiring a more sensitive assay. TYR was linear from 10-2000 µmol/L covering both pre-NTBC and post-NTBC concentrations within one assay.

7.3.6 Creatinine measurement

Our group have published data demonstrating the interference of HGA on enzymatic creatinine assays and assays which utilise a peroxidase reaction (Curtis et al., 2014). Urine and serum creatinine analyses were therefore performed using the Jaffe creatinine assay where creatinine forms a yellow-orange complex with picrate at an alkaline pH measured on a Roche Cobas c501 analyser.

7.3.7 Statistical analysis

All statistical analyses were carried out using GraphPad Instat. A two-tailed unpaired t-test was used to make statistical comparison between groups to establish if there were any significant differences. Testing for Gaussian distribution was performed using Kolmogorov-Smirnov test. If data wasn't normally distributed a non-parametric Mann-Whitney test was performed to determine if there was any significant differences. Both the t-test and the Mann-Whitney test assume equal standard deviations in the groups; due to the large concentration changes upon commencing NTBC, standard deviations are not the same when comparing time points against baseline data; therefore an un-paired t-test with Welch correction has been applied.

7.4 Results

7.4.1 Patient demographics

To date (December 2015), 58 patients with AKU have been enrolled at the NAC for treatment with NTBC. Twenty-three females (mean age 53 years, range 22-75) and 35 males (mean age 48 years, range 22-70). This is an on-going service and at the time of submission for publication, 28 patients had reached two years of monitoring and were included in this survey. The remaining 30 patients have had less than two years of monitoring and were not included. One of the 28 patients included was already on NTBC prior to enrolling at the NAC. The average results at baseline of all NAC patients are with the exclusion of this patient; subsequent time points include this subjects' data (table 7.2).

	Baseline	Day 4	3 Months	6 Months	1 Year	2 Years
u-HGA_{24} (µmol/24h)	20,557 ± 5,445	4,120 ±3 691	2,295 ± 1,423	993 ± 678	1,029 ± 817	1,184 ± 709
u-HGA/creatinine (µmol/mmol)	2,264 ± 867	378 ± 272	213 ± 133	88 ± 48	94 ± 65	107 ± 73
u-TYR_{24} (µmol/24h)	103 ± 81	607 ± 610	1,010 ± 732	1039 ± 802	989 ± 573	1,071 ± 726
u-TYR/creatinine (µmol/mmol)	11.0 ± 7.7	56.3 ± 40.8	90.5 ± 50.6	95.7 ± 52.4	89.6 ± 38.0	96.5 ± 51.7
s-HGA (µmol/L)	30.0 ± 12.9	9.3 ± 3.5	8.3 ± 2.0	7.2 ± 2.3	4.6 ± 1.7	4.0 ± 1.6
s-TYR (µmol/L)	42.0 ± 13.3	373 ± 87.1	611 ± 165	603 ± 124	689 ± 119	594 ± 184
s-NTBC (µmol/L)	Baseline	0.46 ± .18	1.37 ± 0.57	2.11 ± 0.64	1.58 ± 0.52	1.05 ± 0.43

Table 7.2: Summary of the metabolic data from patients who have attended the NAC for two years.

7.4.2 Compliance issues

At both 6 months and one year, one patient has become non-compliant, evidenced by undetectable serum NTBC concentration and u-HGA_{24} (24hr urine HGA excretion) increasing to pre-treatment concentrations. The data is not included in the overall statistics.

7.4.3 Urine HGA concentrations

Urine HGA excretion, in relation to 24hr excretion (determined by volume) is shown in figure 7.2 and table 7.2. At baseline the average HGA excretion per day (u-HGA_{24}) was 20,557 µmol/24hr (95^{th} percentile confidence interval 18,446-22,669 µmol/24hr). At 2 days after commencing NTBC, repeat urine measurements showed that u-HGA_{24} decreased by 78.2% ($p<0.0001$), with average excretion of 4,120 µmol/24hr. Three month measurements revealed an 88.8% reduction from baseline with average u-HGA_{24} of 2,295 µmol/24hr. After three months the increase to a daily dose of 2 mg resulted in an additional decrease to 95.4% at 6 months ($n=25$, $p=0.0003$) which has remained steady at one year 94.8% ($n=27$). Twenty-six (compliant) patients have data at year two and still maintain a decrease in u-HGA_{24} of 94.1% two years post commencement of NTBC with an average u-HGA_{24} of 1,184 µmol/24hr.

Inherent issues with 24hr urine collections can occur with incomplete collection, over- and under-collection being the major problems. Relating the u-HGA to creatinine (u-HGA_{Cr}) demonstrated similar trends in reducing u-HGA with baseline average 2,264 µmol/mmol creatinine reduced to 204 µmol/mmol at year two (a 94.0% decrease).

7.4.4 Serum TYR concentrations

At baseline, the mean serum TYR concentration was 42 µmol/L (range 20-82 µmol/L). The serum reference interval in adults is approximately 21-87 µmol/L (Davison et al., 2015), so all patients with one exception are within the normal reference interval, which is characteristic of AKU. The exception is the patient already on NTBC and who is excluded from baseline statistics.

Figure 7.3 and table 7.2 demonstrate the trend of serum TYR pre- and post-NTBC treatment. There was a rapid increase two days after commencing NTBC, to a mean concentration of 373 µmol/L (range 249-546 µmol/L) with an average eight-fold increase (range 278-1552%). Following the rapid and significant increase ($p<0.0001$) there is an additional significant increase at three months ($p<0.0001$), between 6 months and 12 months ($p=0.181$) and 12 months and year two ($p=0.0366$). There is a large variation in TYR post-NTBC among patients reflecting the dietary protein contribution (unpublished data) and potentially the differing responses to NTBC.

7.4.5 Urine TYR

At baseline, the average urine TYR excretion per day (u-TYR_{24}) was 103.1 µmol/24hr (range 15.7–417 µmol/24hr). In relation to creatinine, the average at baseline was 11.0 µmol/mmol (range 1.7-38.8 µmol/mmol). With NTBC therapy, urine TYR excretion increased with increasing serum TYR (table 8.2). Mean u-TYR_{24} was 607 µmol/24hr at day four and at two years 1071 µmol/24hr, average six-fold and 10-fold increases respectively. Similar to observations on serum TYR concentrations, a significant rise was seen comparing baseline to 2 days post-commencing NTBC ($p=0.0001$) and day 4 to three months ($p<0.0375$). There was no significant difference between the average urine TYR concentrations at subsequent monitoring periods.

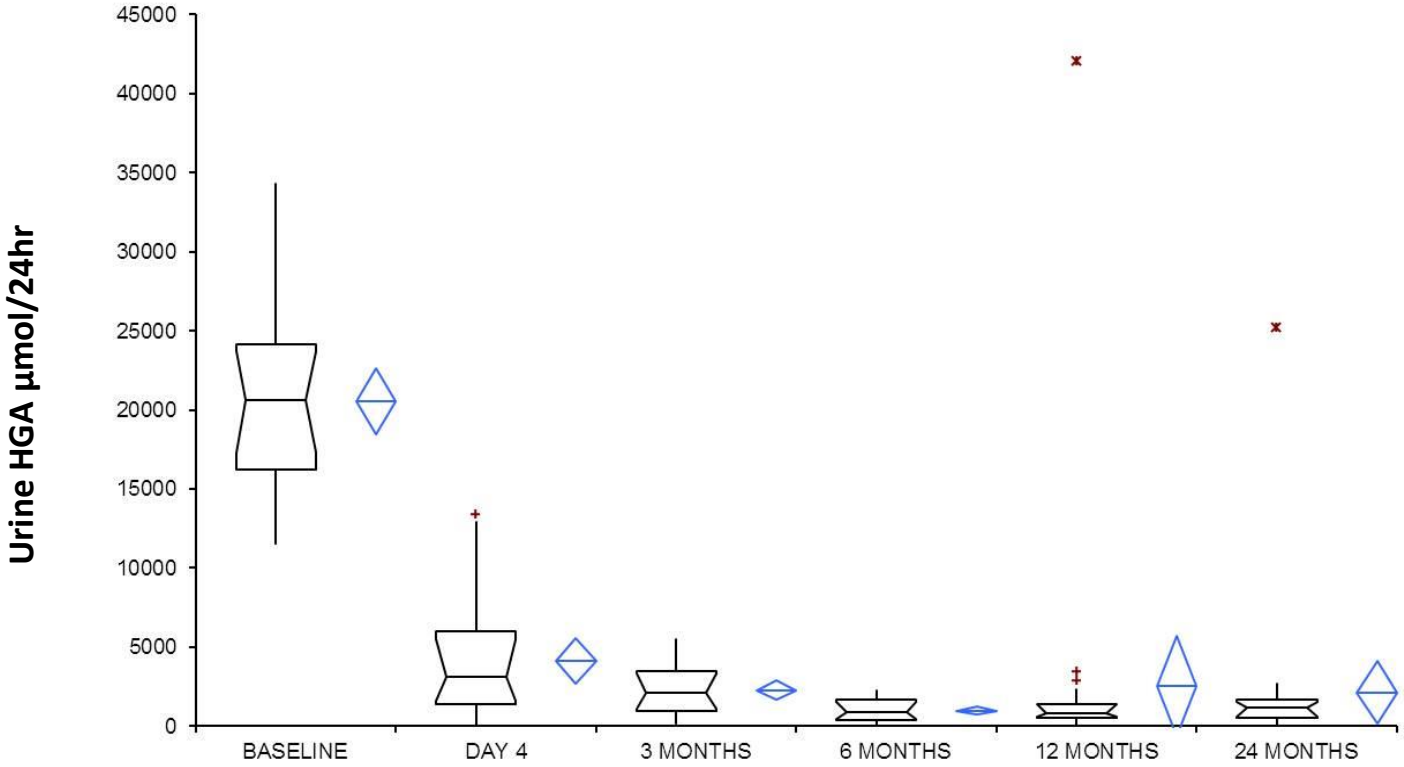

Figure 7.2: Urine HGA excretion (µmol/24hr) demonstrating the reduction in HGA excretion with oral administration of NTBC.

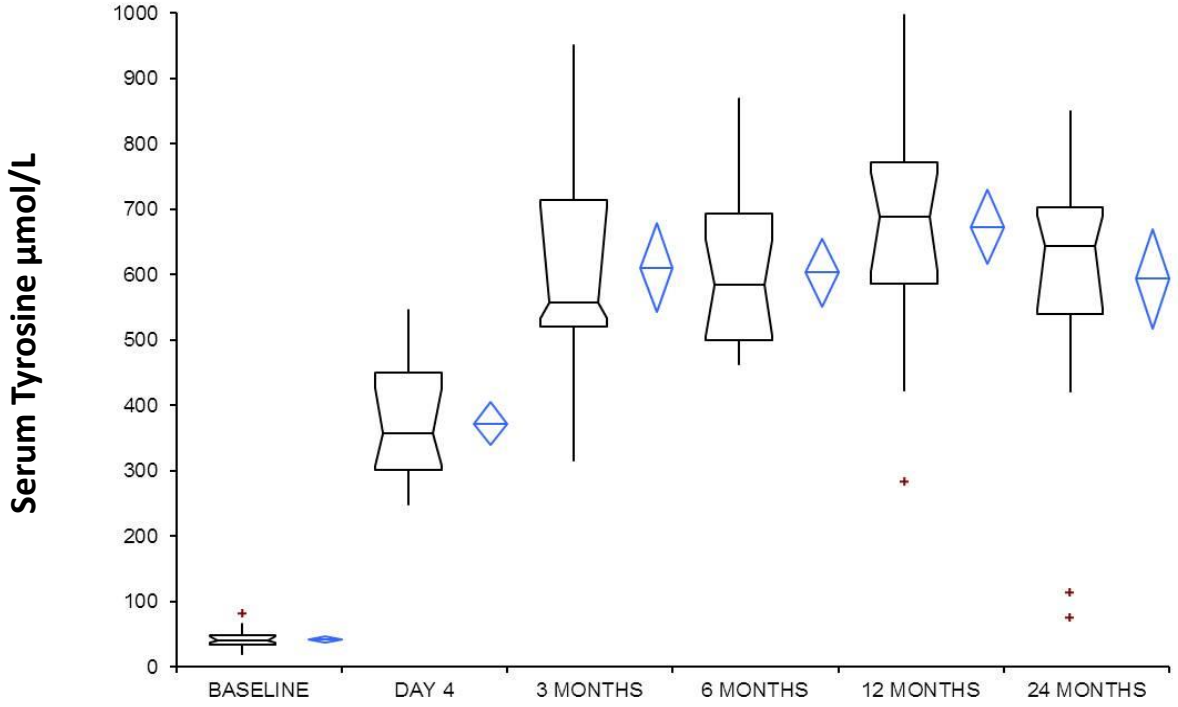

Figure 7.3: Serum TYR (µmol/L) demonstrating rapid increase in serum TYR upon commencing NTBC.

For figures 7.2 and 7.3 - Boxplot represents 95% confidence intervals, line is median, tail the minimum and maximum results (excludes outliers). Diamond is mean and 95% confidence intervals (includes outliers). **x** = outliers >3 inter quartile range.

7.4.6 Serum HGA

Serum HGA demonstrates a significant decrease upon commencing NTBC (table 7.2). At baseline there was a wide range of baseline serum HGA concentrations; similar to the range seen for urine HGA. Of note all patients had an eGFR >60 ml/min/1.73m^2 at baseline. The average serum HGA at baseline was 30.0 µmol/L (range 11.9-75.2 µmol/L). At two days after commencing NTBC, the serum HGA has decreased by 64.0% ($p<0.0001$) and by three months 67.0% of baseline. The NTBC was increased to 2 mg daily at three months which results in a further decrease in serum HGA; at 6 months by 74.3% (n=24), 12 months 82.7% (n=26) and at 24 months 83.2% (n=24). The difference seen between 6 months and 12 months is significant ($p<0.0001$) with no further significant difference seen comparing 12 month to 24 month time periods.

No correlation was seen between serum and urine HGA concentrations at baseline (R^2=0.1896 with µmol/24hr excretion; R^2=0.223 with µmol/mmol excretion).

7.4.7 Serum NTBC

Serum NTBC was measured and at three months following treatment with 2 mg every other day, the mean serum NTBC concentration was 1.37 ± 0.57 µmol/L; the six month time window reflects the increase in dose to 2 mg every day with an average of 2.11 ± 0.64 µmol/L, a significant increase ($p=0.0003$). NTBC concentrations then decrease significantly ($p=0.0056$) at year one, compared with 6 months, with an average of 1.58 ± 0.52 µmol/L and by 24 months an average of 1.05 µmol/L ($p=0.0003$) compared with 12 months (table 7.2). Evidence of non-compliance was evident in one patient at 6 months and one year, with NTBC <0.2 µmol/L – these were excluded from the significance testing above. Of note, the patient was compliant for year two assessment.

7.4.8 Adverse events

One adverse event occurred in a 21 year old male who developed TYR keratopathy and a skin rash at seven weeks following 2 mg NTBC on alternative days (Stewart et al., 2014). NTBC was discontinued and the ocular symptoms, keratopathy and rash all resolved. NTBC was recommenced at 2 mg weekly which was tolerated with no reoccurrence of the ocular or skin problems. His u-HGA_{24} at baseline was 15,733 µmol/24hr, which decreased to 80% of baseline at one year, with weekly NTBC. Serum TYR at the time of the symptoms was 941 µmol/L and 582 µmol/L at year one.

7.5 Discussion:

This is the first longitudinal survey of the off-licence use of NTBC for the treatment of AKU in a specialist hospital setting. Herein we report the impact of NTBC therapy on the TYR metabolic pathway over a two year period. Previous authors have reported on the use of NTBC in treatment of AKU demonstrating efficacy of therapy (Phornphutkul et al., 2002; Suwannarat et al., 2005; Introne et al., 2011; Ranganath et al., 2016), but only in a clinical trial setting. Introne et al., (2011) demonstrated the utility of a 2 mg dose of NTBC over a three year period. The methodology used for metabolite measurement resulted in 60% of serum HGA measurements being undetectable and therefore classified as in the normal reference range.

Sensitive and specific, LC-MS/MS methodology developed at The Royal Liverpool University Hospital (Hughes et al., 2014, 2015) has enabled the first measurement of serum and urine HGA in a non-AKU population. Studies performed in-house have demonstrated that serum HGA in non-AKU individuals is <3.1 µmol/L (Davison et al., 2015) and as a consequence of our methodology we have measured serum HGA concentrations in 96% of all samples. Therefore, a 2 mg daily dose of NTBC is not adequate to suppress serum HGA within the normal reference range, as previously thought. This is supported by the recent clinical trial published (Ranganath et al., 2016) where over a short period of time (four weeks), a dose-dependent decrease was seen up to the highest tested dose of 8 mg daily.

Urine HGA was also measurable in all cases and again was not suppressed to the non-AKU reference range of <2.92 µmol/24hr (Davison et al., 2015) on a daily 2 mg dose of NTBC. The lowest u-HGA concentration measured was 43 µmol/24hr at year two. There was significant reduction in u-HGA_{24} and serum HGA concentrations at 6 months when the NTBC dose was increased from 2 mg on alternative days to daily, at three-month time period. The reduction of urine HGA excretion by 94% at year two supports previous studies where a >95% reduction was observed (Introne et al., 2011). To obtain a greater reduction, it is suggested the NTBC dose may need to be increased; however, the long-term safety and efficacy of this is currently unknown. The circulating HGA concentrations (s-HGA) are of interest regarding the pathological progression of AKU; urine HGA represents the amount filtered and excreted whereas s-HGA is effectively what is available for conversion to the ochronotic pigment, via the BQA pathway. Within the NAC, the serum HGA concentrations are reduced by an average 83% from baseline on a 2 mg daily dose, suggesting there is still potential for pigment deposition, albeit on a reduced scale. The rapid decrease in both s-HGA and u-HGA seen at day 4 (post two doses of NTBC) has implications in cases where a rapid decrease in HGA is required, such as seen in haemolysis due to renal failure (Davison et al., 2016).

TYR concentrations increased dramatically upon commencing NTBC which has previously been observed in both AKU and in the treatment of HT-1 (McKiernan, 2013). However, the dose of NTBC used in HT-1 is 1-2 mg/kg compared with 2 mg daily at the NAC. Due to the side effects and implications for development in children, serum TYR is ideally maintained at <400 µmol/L (McKiernan, 2013), largely through diet restriction. Non-compliance with protein restriction in HT-1 has led to several reported ocular symptoms, including corneal crystals and opacities (Holme and Lindstedt, 1998; Ahmad et al., 2002; Gissen et al., 2003); similar corneal opacities have been reported in AKU (Introne et al., 2011; Stewart et al., 2014). In all cases, the corneal opacities resolved upon cessation of NTBC and normalisation of TYR concentrations. The mechanism for the keratopathy has not been fully elucidated however there were patients in the NAC who had higher serum

TYR concentrations and did not develop any ocular symptoms or side effects, suggesting a pre-disposition, independent of TYR concentrations. Diet restriction in adults is difficult and although patients at the NAC are advised about diet and protein intake, and food diaries are maintained, no restriction is applied.

NTBC concentrations were determined when at steady state. NTBC has a relatively long half-life of approximately 50hr which means steady state occurs at a minimum, ten days post commencing treatment. Average serum concentrations were 2.11 ± 0.64 µmol/L at 6 months with a significant decrease at one year to 1.58 ± 0.52 µmol/L. Therapeutic concentration in HT-1 are 20-120 µmol/L NTBC (Davit-Spraul et al., 2012), however there is no suggested therapeutic target for AKU. Previous studies have demonstrated similar serum concentrations with 1.39 ± 0.57 µmol/L on a 1 mg dose twice daily (Suwannarat et al., 2005) and 2.22 ± 0.54 µmol/L, again on a 2 mg daily dose (Introne et al., 2011). Although 2 mg daily dose is well tolerated, compliance has been an issue for a few individuals. This is clearly determined by routine biochemical measurements of TYR and HGA in both serum and urine. Urine HGA returns to high baseline levels and serum TYR to within the normal reference range. The significant dose-dependent decrease in serum NTBC concentrations is not reflected by any concurrent rise in serum HGA concentrations or normalisation of serum TYR, in fact, the serum HGA significantly decreases between 6 months and 12 month monitoring periods. Analytical issues were excluded with stability assays and reanalysis of earlier samples. Little is known about NTBC metabolism; it is known to bind tightly to the HPPD-inhibitor complex with a slow dissociation rate (Ellis et al., 1995; Kavana and Moran, 2003). Studies have demonstrated dose proportional pharmacokinetics using daily 1 mg to 8 mg NTBC doses (Olsson et al., 2015), however this was only over a four-week period. Metabolism of NTBC is via CYP3A4 and certain medications are inducers or inhibitors of this enzyme; there were no notable changes to the medications of the patients in this cohort. Ongoing studies into the metabolomics profiling of these patients on NTBC therapy will hopefully aid in understanding this change.

The survey of metabolic changes described herein provides biochemical evidence for the safe and efficacious use of NTBC therapy in lowering both urine and serum HGA in the treatment of AKU, with off-licence use of NTBC. Additional studies are on-going to determine if this will lead to a reduction in ochronosis, as seen previously in a mouse model (Keenan et al., 2015).

Chapter 8

The effect of nitisinone on the alkaptonuria pathway metabolites: Eight years of data on patients attending the National Alkaptonuria Centre, Liverpool.

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Declaration and acknowledgments

All sample analysis and interpretation undertaken by Andrew T Hughes.

In preparation

8.1 Abstract

Background

Alkaptonuria (AKU) is a rare, debilitating autosomal recessive disorder affecting tyrosine (TYR) metabolism. Deficiency of homogentisate 1,2-dioxygenase leads to increased homogentisic acid (HGA) which is deposited as ochronotic pigment. The aetiology of this disorder is well understood and the National AKU Centre in Liverpool have been treating all UK AKU patients with 2 mg nitisinone (NTBC) off-license and monitoring the TYR metabolite profiles since 2012. Reported within this manuscript is 8 year data analysis of NAC patient monitoring.

Methods

Patients with confirmed AKU are commenced on 2 mg dose (alternative days) of NTBC for three months with daily dose thereafter. LC-MS/MS was performed on serum and urine samples to measure all metabolites at baseline and annually thereafter. To date there were 83 patients that have been treated at the NAC.

Results

Urine HGA concentration has remained consistently suppressed by 93.8% after eight years. The average reduction of serum HGA remains ≥84%, remaining detectable and above our new reference range (<0.1 μmol /L). Although as expected both urine TYR (9 fold) and serum (13 fold) TYR increased, but levels have remained consistent throughout. In addition, there is also a consistent rise and levelling of HPPA and HPLA, expected with the effects of NTBC.

Conclusions

The data presented is the first and most comprehensive longitudinal survey of NTBC use in an NHS service and now provides long-term information upon the sustained effect of NTBC on the TYR metabolite profile. This data provided can now be positive evidence for the introduction of an increased dose of NTBC from 2 mg to 10 mg.

8.2 Introduction

The initial publication reviewed the major metabolites, namely tyrosine (TYR), homogentisic acid (HGA) and nitisnone (NTBC) over a two year period, incorporating alkaptonuria (AKU) patients attending the National AKU Centre (NAC). Although at the time of the initial publication, there were some patients on later visits than their two year review, however the small number did not warrant inclusion. The additional data presented within this paper provides data up to visit 9, which is 96 months (eight years) since the commencement of NTBC and encompasses the 83rd patient to attend the NAC for AKU.

Currently within the NHS, there are differences between the provision of services in England and Wales. A cohort of patients from Wales who although they attend the NAC for routine clinical monitoring and assessments, are unable to be prescribed NTBC; this group act as a control group within the NAC and demonstrate the steady state of metabolites in AKU, with normal serum TYR and raised urine and serum HGA.

Within this paper, data is provided up to December 2020, although during 2020 the NAC was unable to see patients for the majority of the year due to the COVID pandemic. Some patients have been clinically monitored virtually and have had blood spot TYR monitoring performed for NTBC safety (data not included).

8.3 Materials and Methods

8.3.1 Dose of NTBC

The protocol for treatment remains standard, with patients commenced on a 2 mg NTBC dose every other day for the first three months; after which this is increased to 2 mg daily. Assessments are annual unless the patient reports any side effects and an extra visit is included.

8.3.2 Data collection

The data collection from the NAC is covered by The Royal Liverpool and Broadgreen University Hospital Trusts Audit Committee (Audit no. ACO3836). This is not a clinical trial and therefore ethical approval wasn't required with patients consenting to their data being used for publication.

8.3.3 Sample collection

Data obtained is following standard clinical assessments upon referral to the NAC. 24hr urine samples (acidified with 1% 5N H_2SO_4) and serum samples were collected at all visits. Serum samples were acidified by deproteinisation with perchloric acid (5.8 mol/L).

The analytical service has continued to offer a fast turnaround for monitoring bloods and urine from the NAC ensuring that patient safety is monitored when commenced on NTBC.

The methodology described within Chapters 2, 3 and 4 has enabled a full metabolite panel to be measured in both serum and urine namely TYR, HGA, phenylalanine (PHE), hydroxyphenyllactate (HPLA), hydroxyphenylpyruvate (HPPA) and NTBC (in serum only). All methodology refinement has been described within these chapters and Chapter 7.

8.3.4 Statistical analysis

All statistical analyses were performed using GraphPad Instat (v3). A two-tailed unpaired t-test was used to make statistical comparison between groups to establish if there were any significant differences. Testing for Gaussian distribution was performed using Kolmogorov-Smirnov test. If data wasn't normally distributed a non-parametric Mann-Whitney test was performed to determine if there was any significant differences. Both the t-test and the Mann-Whitney test assume equal standard deviations in the groups; due to the large concentration changes upon commencing NTBC, standard deviations are not the same when comparing time points against

baseline data; therefore an un-paired t-test with Welch correction has been applied.

8.4 Results

8.4.1 Patient demographics

To date, (December 2020), 83 patients have been enrolled in the NAC. Thirty-two females (mean age 56 years, range 22-80) and 51 males (mean age 52 years, range 17-75). It should be noted this age range includes all patients initially enrolled and does not distinguish those who are actively attending from those who have discontinued their visits to the NAC (including ill health, deceased or withdrawn from the NAC).

The data presented within this paper encompasses the following numbers of results (including those not on NTBC) and is the largest and longest monitoring data set for NTBC in AKU (table 8.1). Due to the volume of data, 3 and 6 month data has been excluded from this addendum and the focus has been on annual visit monitoring.

Visit	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
	Baseline	12M	24M	36M	48M	60M	72M	84M	96M
n=	83	65	59	53	41	38	31	14	6

Table 8.1: Recorded number of AKU patients and visits to the NAC for monitoring over eight years.

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	Baseline (V1)	1 Year (V2)	2 Year (V3)	3 Year (V4)	4 Year (V5)	5 Year (V6)	6 Year (V7)	7 Year (V8)	8 Year (V9)
u-HGA_{24} (µmol/24h)	22,865 ± 9,067	1,503 ± 1,639	1,416 ± 1,434	1,351 ± 1,808	1,337 ± 1,023	1,208 ± 929	1,073 ± 656	1,832 ± 2,039	1,015 ± 745
u-TYR_{24} (µmol/24h)	107 ± 84	930 ± 528	1017 ± 619	1106 ± 652	1013 ± 564	905 ± 482	1049 ± 603	968 ± 582	991 ± 454
u-PHE_{24} (µmol/24h)	58 ± 36	49 ± 25	53 ± 54	55 ± 36	52 ± 29	46 ± 19	51 ± 34	60 ± 31	41 ± 24
u-$HPLA_{24}$ (µmol/24h)	105 ± 210	9,568 ± 4,405	9,966 ± 4,643	11,953 ± 5,299	10,717 ± 3,878	10,023 ± 3,918	11,147 ± 3,404	9,621 ± 4,699	10,850 ± 6,575
u-$HPPA_{24}$ (µmol/24h)	164 ± 239	12,394 ± 6,563	12,478 ± 7,365	15,786 ± 7,419	13,146 ± 4,614	14,216 ± 4,809	13,897 ± 5,729	12,927 ± 6,572	11,722 ± 6,875
s-HGA (µmol/L)	29.4 ± 14.1	4.6 ± 2.1	4.5 ± 2.8	3.8 ± 1.1	4.4 ± 2.4	3.8 ± 1.7	4.0 ± 1.1	4.7 ± 1.9	3.5 ± 1.1
s-TYR (µmol/L)	50.5 ± 14.2	685 ± 200	717 ± 227	802 ± 147	760 ± 210	738 ± 182	706 ± 222	701 ± 175	657 ± 239
s-PHE (µmol/L)	64.3 ± 14.1	56.4 ± 9.4	56.7 ± 9.6	58.3 ± 10.0	59.1 ± 8.3	56.2 ± 10.4	60.4 ± 10.6	65.2 ± 6.9	60.7 ± 10.8
s-HPLA (µmol/L)	ND	45.8 ± 19.6	50.8 ± 15.2	51.4 ± 21.4	50.5 ± 18.3	47.1 ± 17.2	49.1 ± 20.3	52.5 ± 21.0	48.2 ± 17.1
s-HPPA (µmol/L)	ND	34.3 ± 22.3	31.0 ± 10.8	37.8 ± 14.6	34.8 ± 14.0	36.4 ± 15.8	38.3 ± 14.2	43.5 ± 16.4	31.8 ± 9.3
s-NTBC (µmol/L)	ND	1.2 ± 0.6	1.0 ± 0.4	1.0 ± 0.4	1.0 ± 0.7	1.0 ± 0.4	1.1 ± 0.4	1.0 ± 0.4	1.6 ± 0.2

Table 8.2: Summary of the metabolic data from patients over the eight years of monitoring at the NAC. This data only includes patients on NTBC.

8.4.2 Urine HGA

Urine HGA remains the test for screening for AKU and the NAC have received upwards of seven urine samples over the last 12 months with '? Dark urine - ?Alkpatonuria' evidencing that the service is the national referral centre for diagnosis and screening as well as monitoring for the NAC patients.

All patients not receiving NTBC have been excluded (except at baseline). Data in table 8.2 demonstrates a significant reduction ($p<0.0001$) in u-HGA_{24} once the dose becomes 2 mg per day, with urine HGA remaining on average >1000 µmol/24hr across all visits. The average reduction from baseline is 93.4% at V2 (12 months) and 93.8% at V3 (24 months). This remains consistent with the previously published data where u-HGA_{24} was reduced by 94% at 24 months, the difference in the number of patients included was 26 versus 50 within this additional data set. Reduction in u-HGA_{24} remains consistent (figures 8.1 and 8.2) with only a slight reduction at V8 (n=14) where the reduction measured is on average, 92%. Closer review of one patient shows someone who has a reduced NTBC dose due to side-effects, and if this is excluded the u-HGA_{24} reduction returns to 93.8%. All visits demonstrate a significant reduction in u-HGA_{24} compared with the baseline ($p<0.0001$). Comparison of the remaining visits with each other (figure 8.2) reveals there is no significant difference once patients are on 2 mg daily dose of NTBC between any visit.

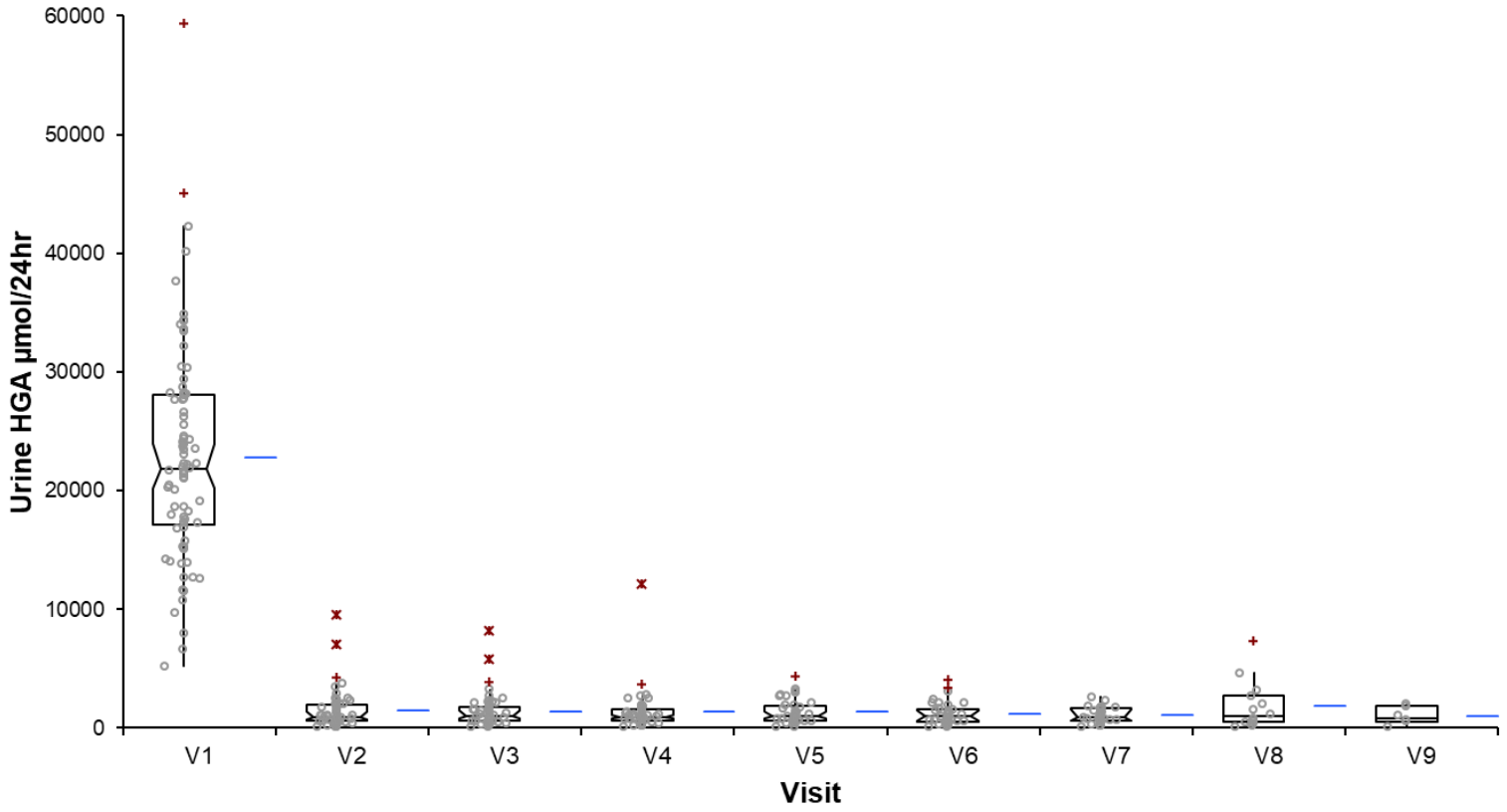

Figure 8.1: Urine HGA excretion (u-HGA_{24}) for all visits (blue line represents the mean; red crosses the outliers and box plots 95% confidence interval).

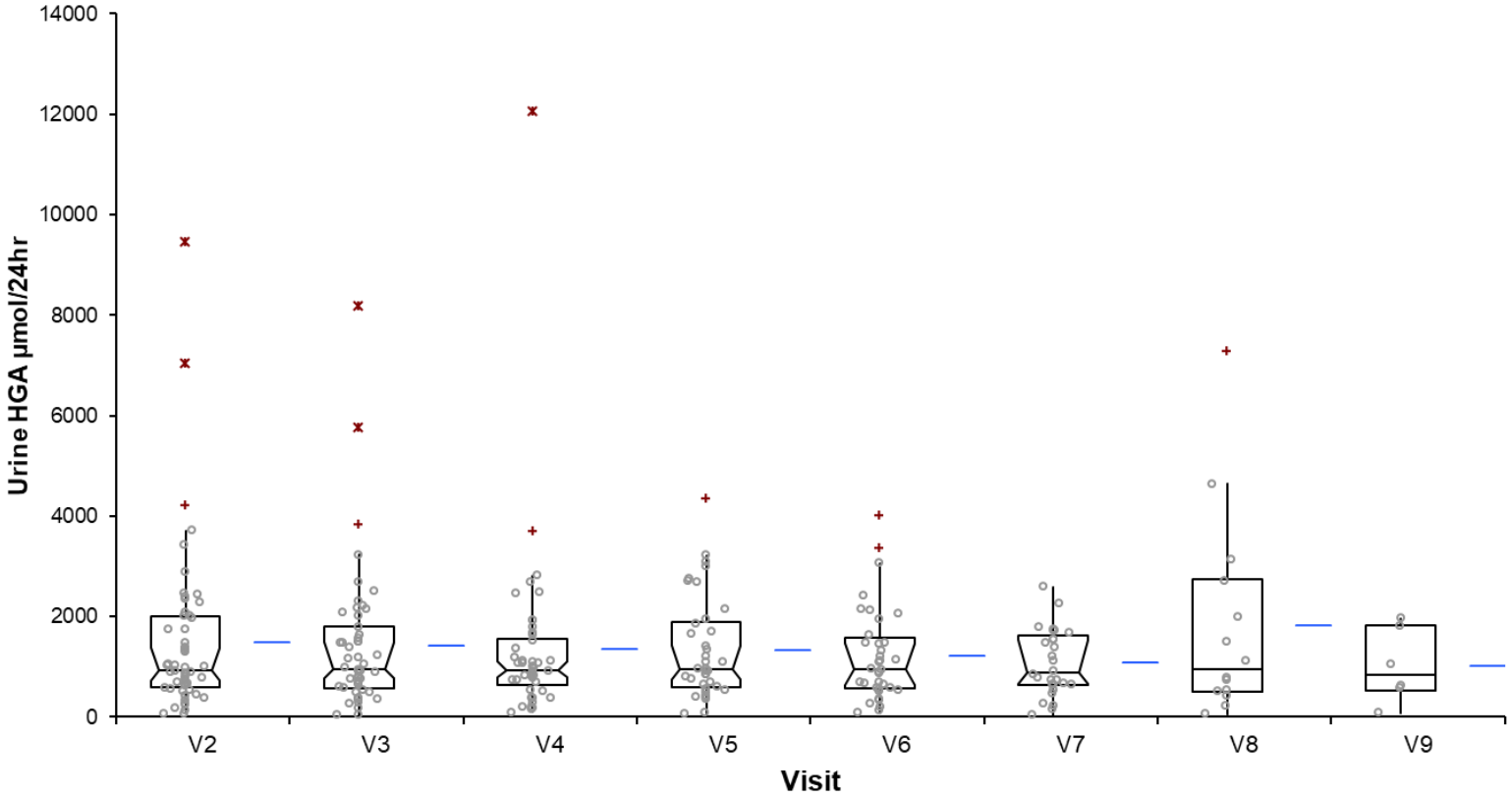

Figure 8.2: Urine HGA excretion (u-HGA_{24}) for visits 2 to 9 (blue line represents the mean; red crosses the outliers and box plots 95% confidence interval).

8.4.3 Urine TYR

At baseline the average urine TYR excretion per day (u-TYR_{24}) was 107 µmol/24hr (table 8.2), which compares with the previously reported of 103.1 µmol/24hr and provides confidence in the assay as well as consistent performance of the assay. At V3 (24 months post NTBC) the mean u-TYR_{24} was 1017 µmol/24hr with a significant nine fold increase compared to baseline concentrations ($p<0.0001$). At all visits, compared with baseline, the u-TYR_{24} is significantly elevated ($p<0.001$, at V9 $p=0.0122$). There are no other significant differences measured between the average u-TYR_{24} concentrations during all subsequent monitoring periods.

Figure 8.3 displays the trend of u-TYR_{24} from baseline (V1) until V9 and although there is a wide scatter of results similar to serum TYR, the average is consistent across the time period assessed (blue line on graph).

8.4.4 Urine PHE

Urine PHE was not reported in the original manuscript, therefore this paper demonstrates the full profile of urine PHE across all nine visits at the NAC.

It is evident that there is no increase post commencing NTBC and urine PHE concentrations remain consistent across the time course (table 8.2). Figure 8.4 displays u-PHE_{24} and compared with the baseline data, there is only a significant decrease at V6 ($p=0.0292$). This reflects the smaller SD for this visit as the mean u-PHE_{24} was 46 µmol/24hr compared with V9 which is 41 µmol/24hr, but is not significantly different from baseline excretion values. Of note there is no significant difference between any other visits.

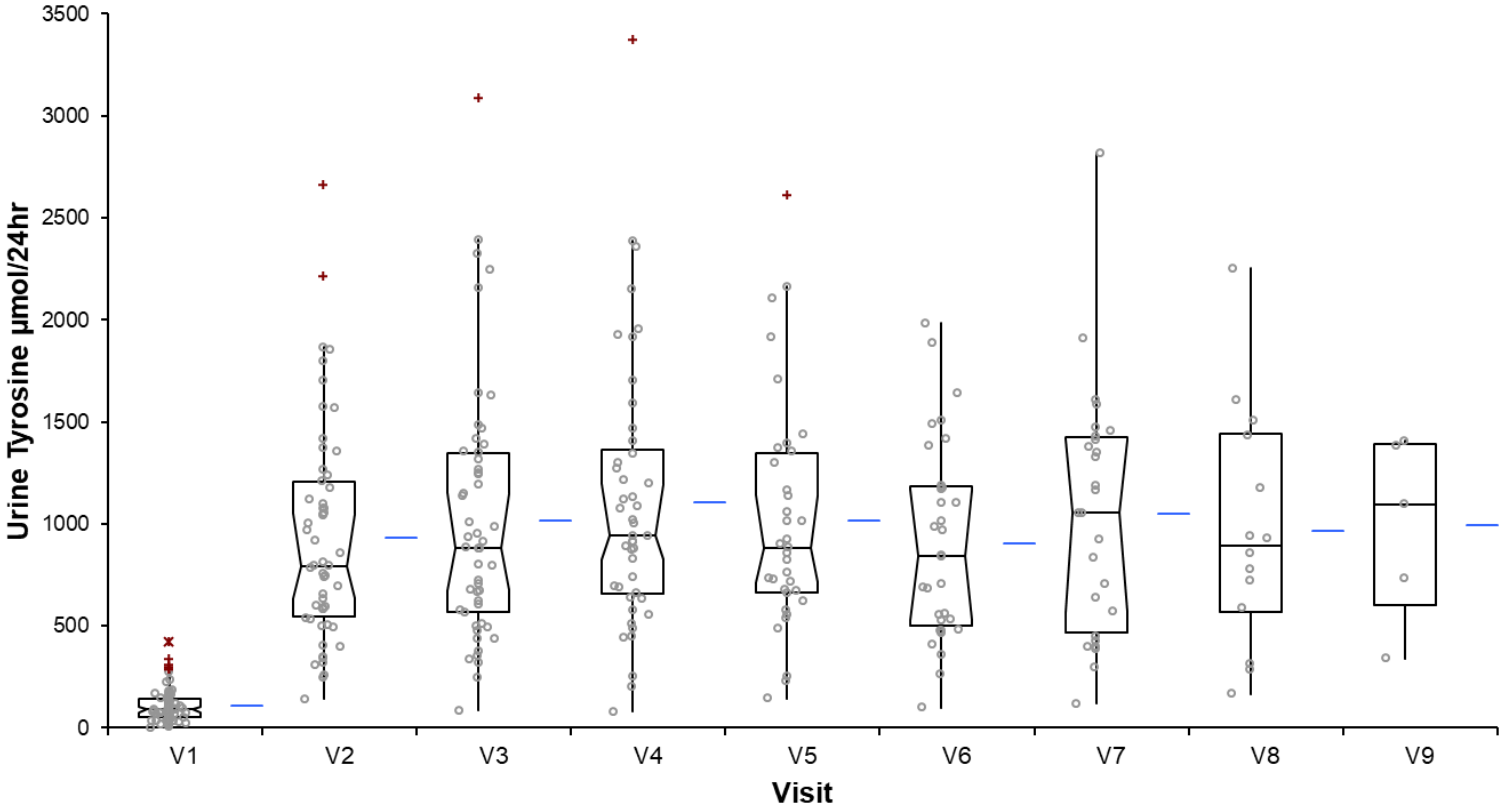

Figure 8.3: Urine TYR excretion (u-TYR_{24}) for all visits (blue line represents the mean; red crosses the outliers and box plots 95% confidence interval).

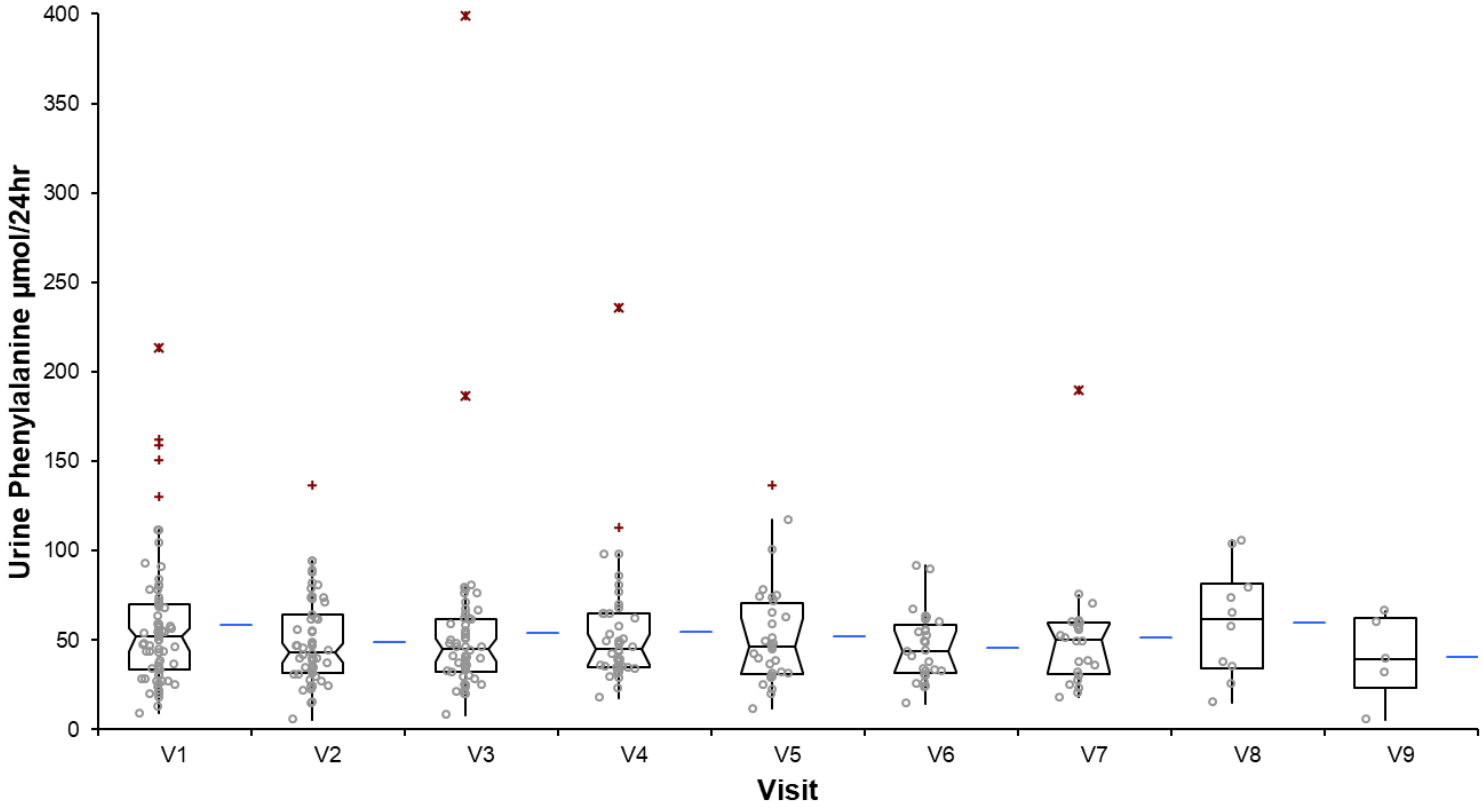

Figure 8.4: Urine PHE (u-PHE$_{24}$) for all visits (blue line represents the mean; red crosses the outliers and box plots 95% confidence interval).

8.4.5 Urine HPLA and HPPA

This paper reports the u-$HPLA_{24}$ and u-$HPPA_{24}$ in the NAC cohort for the first time, and the trend is opposite of that for u-HGA_{24}. At baseline u-HPLA is only quantifiable in 24 patients and similarly u-HPPA. The limit of quantification is <20 µmol/L for u-HPLA and <50 µmol/L for u-HPPA. Evident from table 8.2 and figures 8.5 and 8.6 is the rapid rise in concentrations of HPLA and HPPA, post commencing NTBC. At V2 there is a significant ($p<0.0001$) increase from 105 µmol/L HPLA to 9,568 µmol/24hr (over a 90 fold increase) and for HPPA a similar increase is seen from 164 µmol/L to 12,394 µmol/24hr (over a 70 fold increase). However this is then consistent over all visits with the only significant difference for u-$HPLA_{24}$ being between V2 and V4 ($p=0.0293$). No other pairing of visits demonstrates any significance. Similarly with u-$HPPA_{24}$ there is only a significant difference between V2 and V4 ($p=0.0215$) and V3 and V4 ($p=0.0354$), all other pairings have no significant difference.

As production of HPLA and HPPA occurs when patients are treated with NTBC, little is known about this metabolic diversion. However with the data available, the ratio of urine HPPA:HPLA was calculated (it was chosen in this order as HPPA is directly in the pathway and HPLA forms reversibly from HPPA). Figure 8.7 demonstrates the ratio with evidence that this is maintained both at baseline and post-treatment with NTBC. The mean ratio is 1.5 at V2 and V4, 1.3 at V3, V5 and V9, 1.4 at V7 and 1.7 at V8. At baseline the ratio is 2.0, however, although there are less measurable values at baseline, there are less pairs of data.

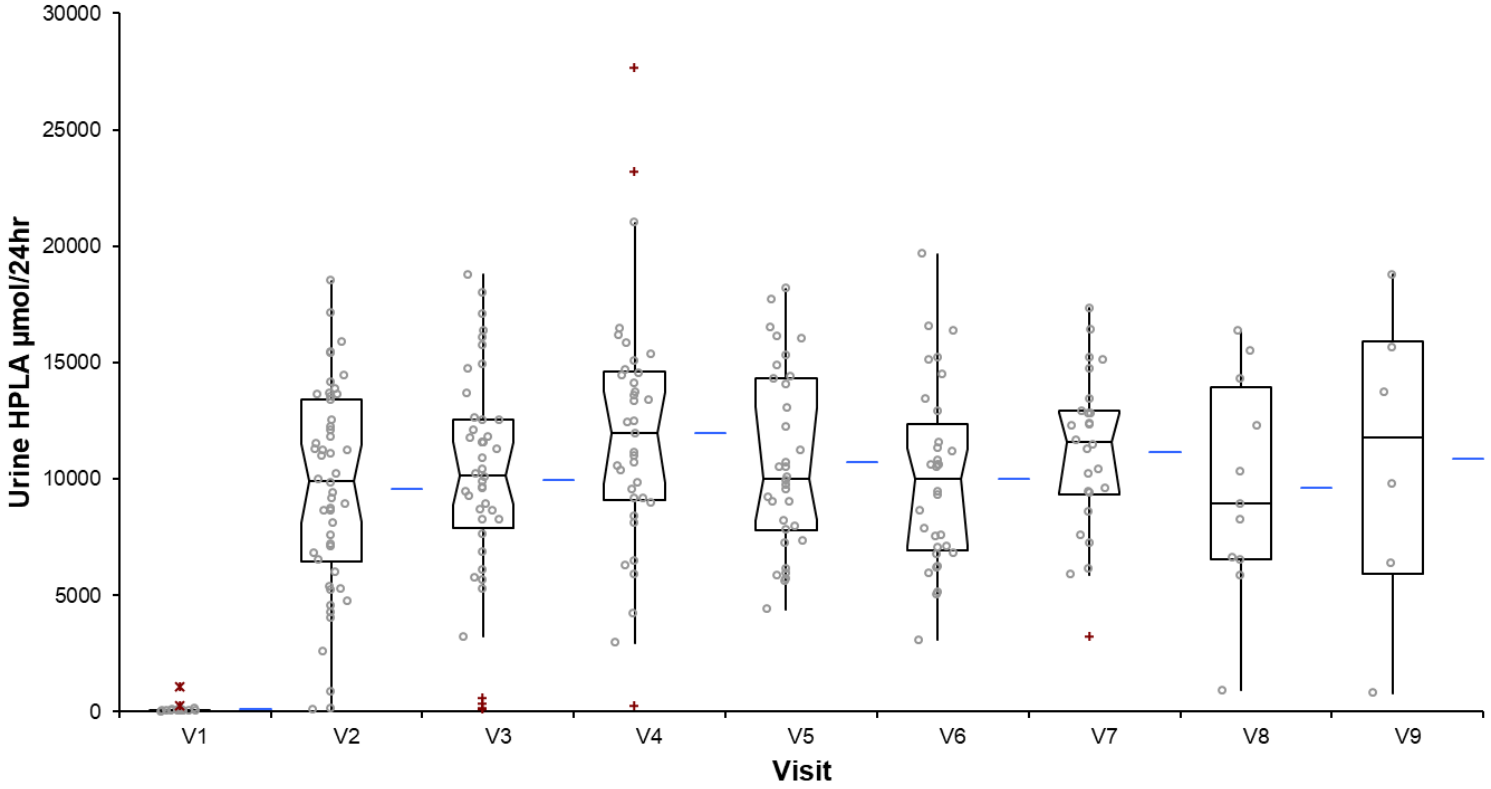

Figure 8.5: Urine HPLA (u-HPLA$_{24}$) for all visits (blue line represents the mean; red crosses the outliers and box plots 95% confidence interval).

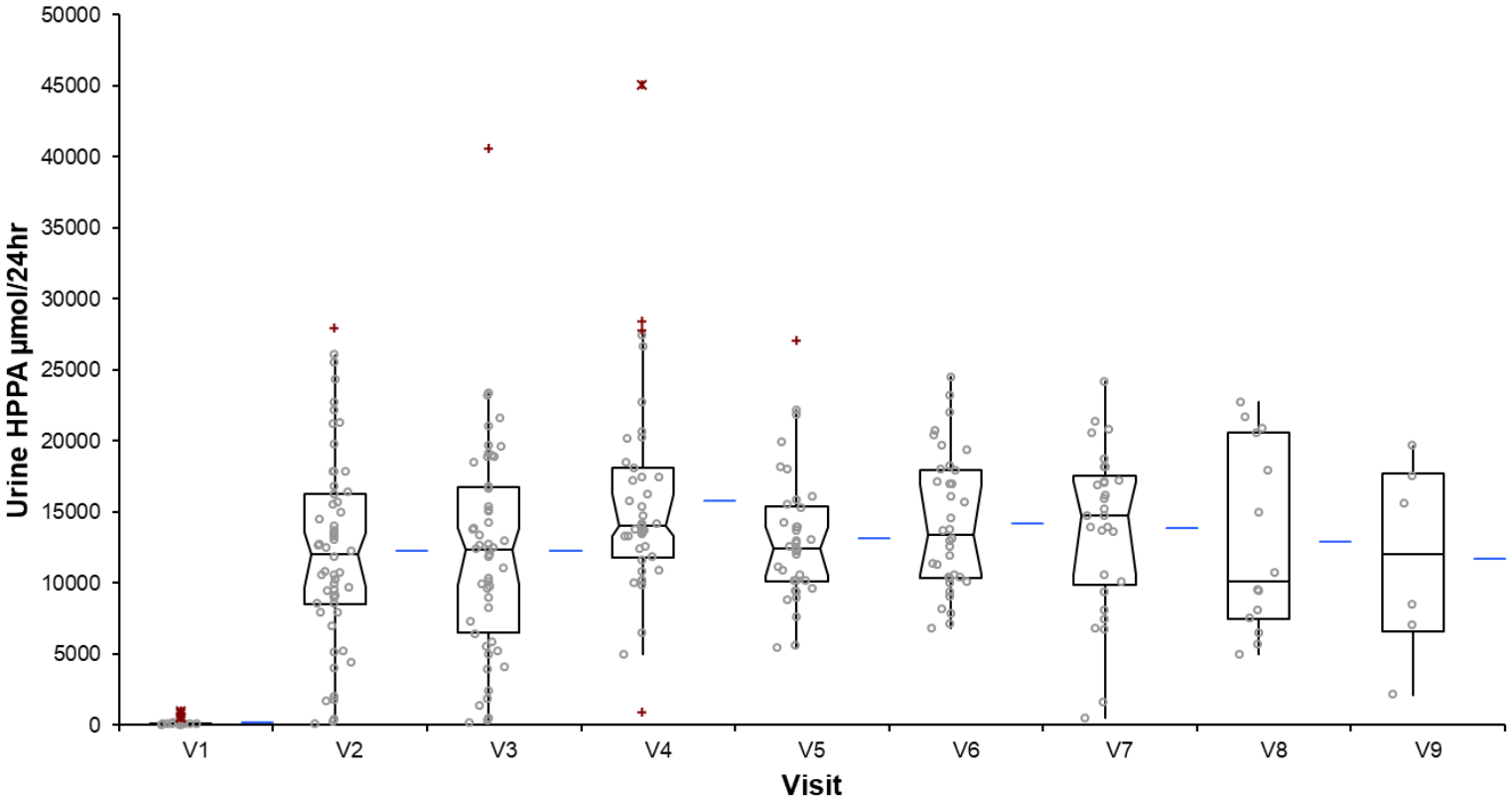

Figure 8.6: Urine HPPA (u-HPPA$_{24}$) for all visits (blue line represents the mean; red crosses the outliers and box plots 95% confidence interval).

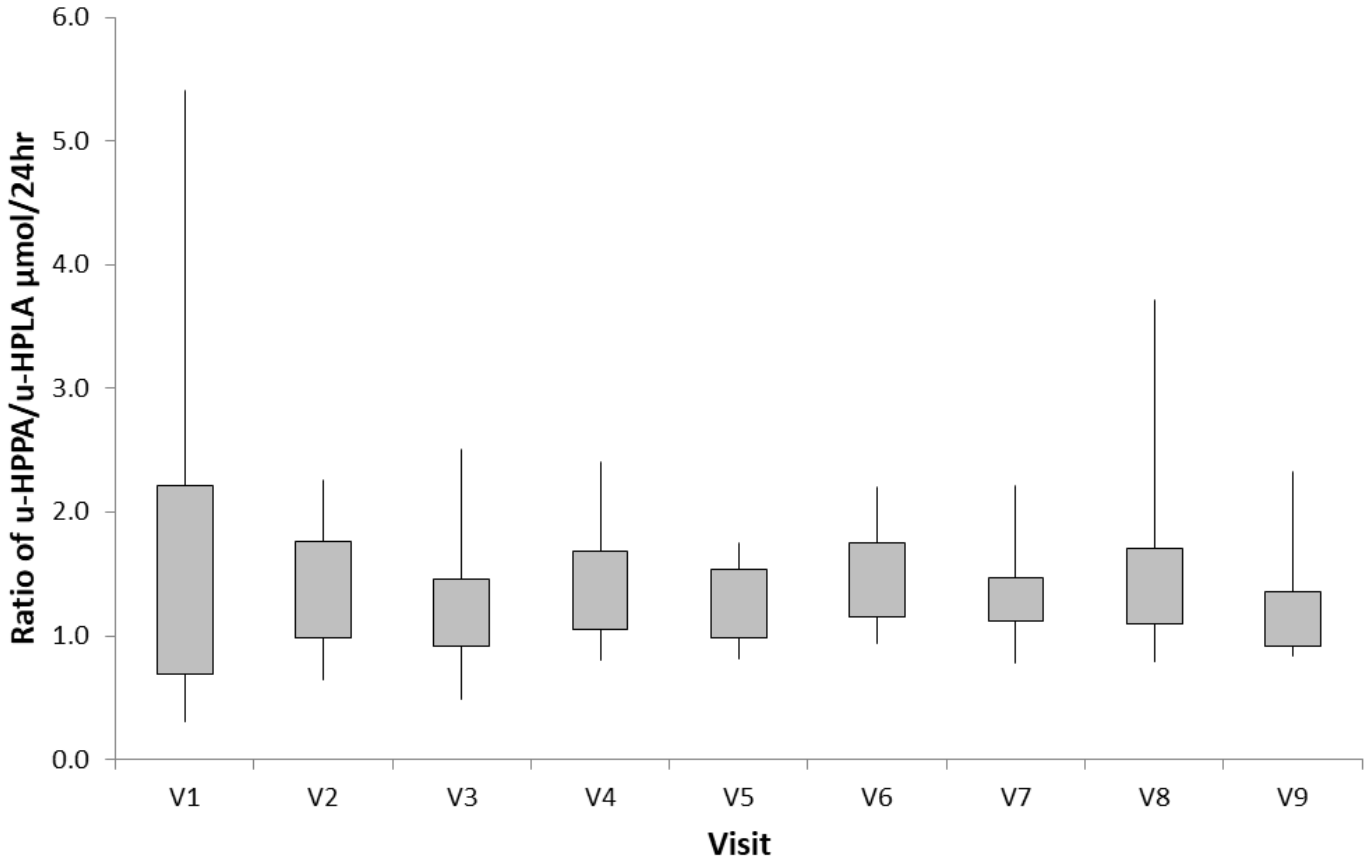

Figure 8.7: Plot demonstrating the ratio of u-HPPA:U-HPLA, using 24hr excretion values. The boxes represent 25th and 75th percentile and the tails, 5th and 95th percentile.

8.4.6 Serum HGA

Circulating concentrations of HGA are considered the precursor for pigment deposition. NTBC is effective at reducing these concentrations however, it still remains detectable (either in the standard assay or the ultra-low HGA assay) thus corroborating the urine HGA data discussed earlier, as the kidneys still excrete measurable quantities of HGA. Data from SONIA-1 and SONIA-2 support the knowledge that 2 mg daily is not adequate to suppress HGA within the 'normal' reference range. Figures 8.8 and 8.9 show the effects of NTBC treatment on serum HGA levels with and without the baseline level. As per urine HGA, serum HGA is a characteristic of AKU and is measurable at baseline with a mean of 29.4 µmol/L. At all other visits the serum HGA is <5 µmol/L (table 8.2), representing a significant decrease ($p<0.0001$).

At V2, the updated data set shows the percentage reduction from baseline is 84.4% (n=43) which compares favourably with the reported reduction of 82.7% (n-26) at V2 in the original manuscript. Similarly the reduction at V3 reported as 83.2% (n=24) is calculated to remain at 84.7% (n=38); again comparing favourable with the original reported data (Chapter 7). Figure 8.9 displays the data without the baseline values and although there is variability of the mean across all visits, there is no significant difference between any visit. The average reduction of serum HGA remains ≥84% (88% at V9 and 84% at V8).

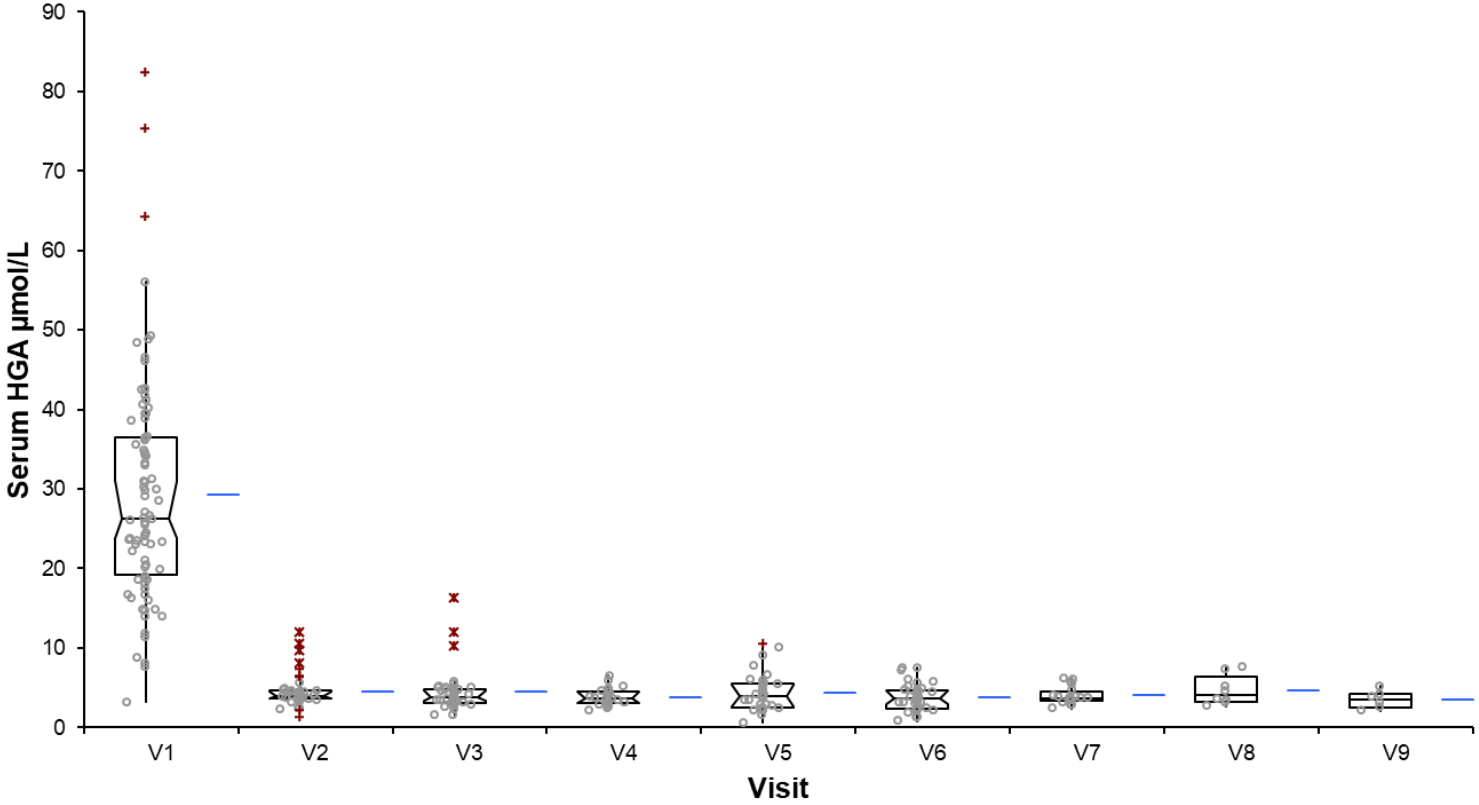

Figure 8.8: Serum HGA all visits for all visits (blue line represents the mean; red crosses the outliers and box plots 95% confidence interval).

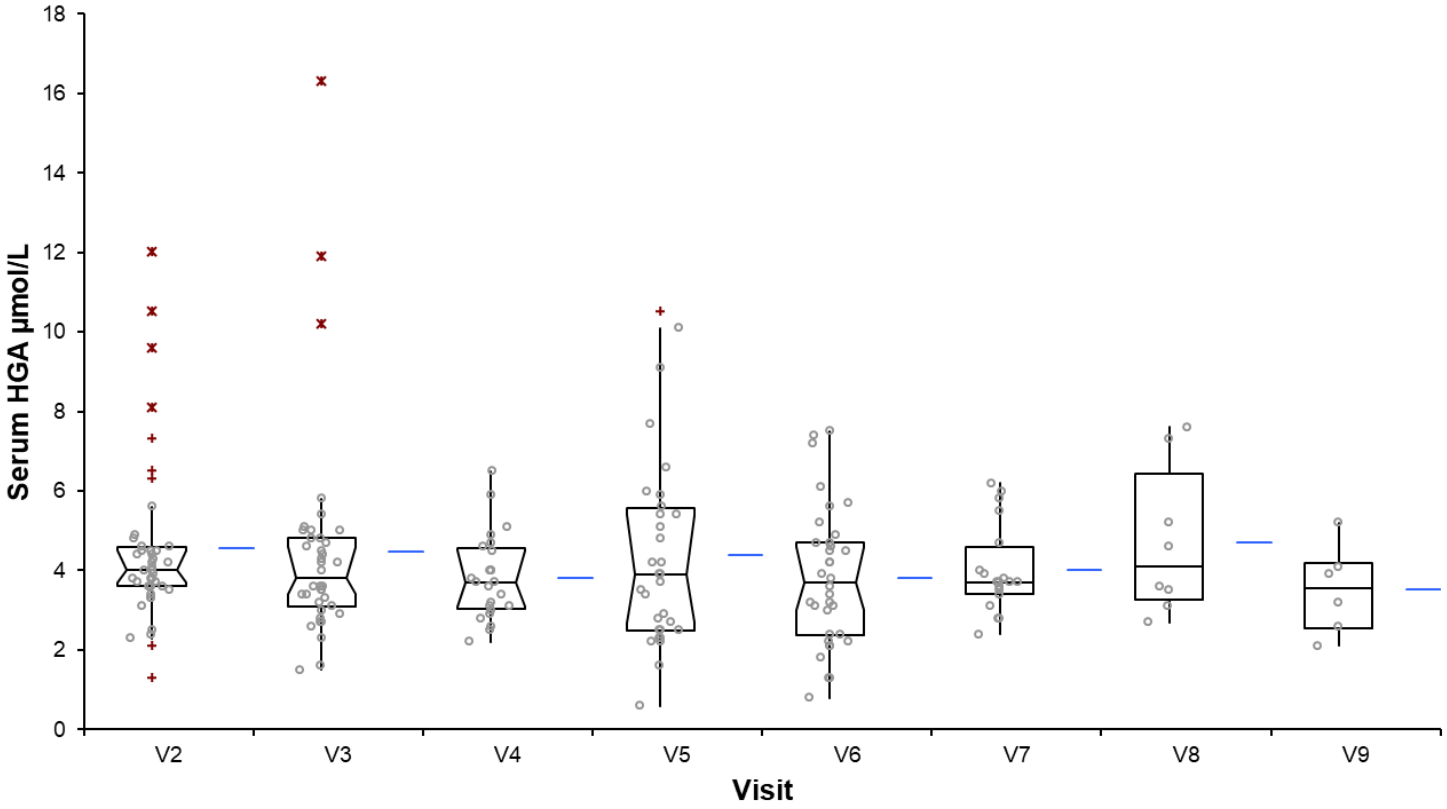

Figure 8.9: **Serum HGA visits 2-9 for all visits (blue line represents the mean; red crosses the outliers and box plots 95% confidence interval).**

8.4.7 Serum TYR

At baseline the mean serum TYR concentration was 50 µmol/L (range 20-96 µmol/L). The serum reference interval determined by our method (Davison et al., 2015) of 21-87 µmol/L fits this data set, with one outlier at either side of the range. Table 8.2 demonstrates the trend of serum TYR through the visits with a 13 fold increase at V2 compared with baseline. Figures 8.10 and 8.11 (without baseline data) show that this significant increase compared with the baseline is consistent across all visits ($p<0.0001$). There is variation and this will reflect dietary protein intake (and was also seen in the u-TYR_{24} data). There is no significant difference between visits apart from V3 compared with V4 ($p=0.0298$) and V4 compared with V7 ($p=0.0424$). V4 data has the highest mean TYR and the tightest SD range, and although the same statistical test has been used throughout (unpaired t-test, Welch corrected) this is unlikely to have a clinical implication.

8.4.8 Serum PHE

Figure 8.12 and table 8.2 demonstrate that s-PHE like u-PHE_{24} show little variability across all visits. Baseline s-PHE was 64 µmol/L (range 44-105 µmol/L). The average variability between visits ranges from an average 12.6% decrease at V6 (compared with baseline) and a 1.4% increase at V7. However statistically there is a significant decrease comparing baseline to V2, V3, V4, V5 and V6 ($p=<0.0001$, $p=0.0002$, $p=0.0047$, $p=0.0106$ and $p=0.005$ respectively). In addition there is a significant difference compared with V8 (for V2, V3 and V6). The baseline and V8 have the highest average s-PHE concentrations of 64 and 65 µmol/L. Again, although a significant difference has been identified, the clinical impact is minimal as s-PHE remains within the reference range of 0 to 80 µmol/L (local referral lab, Alder Hey Hospital, Liverpool UK).

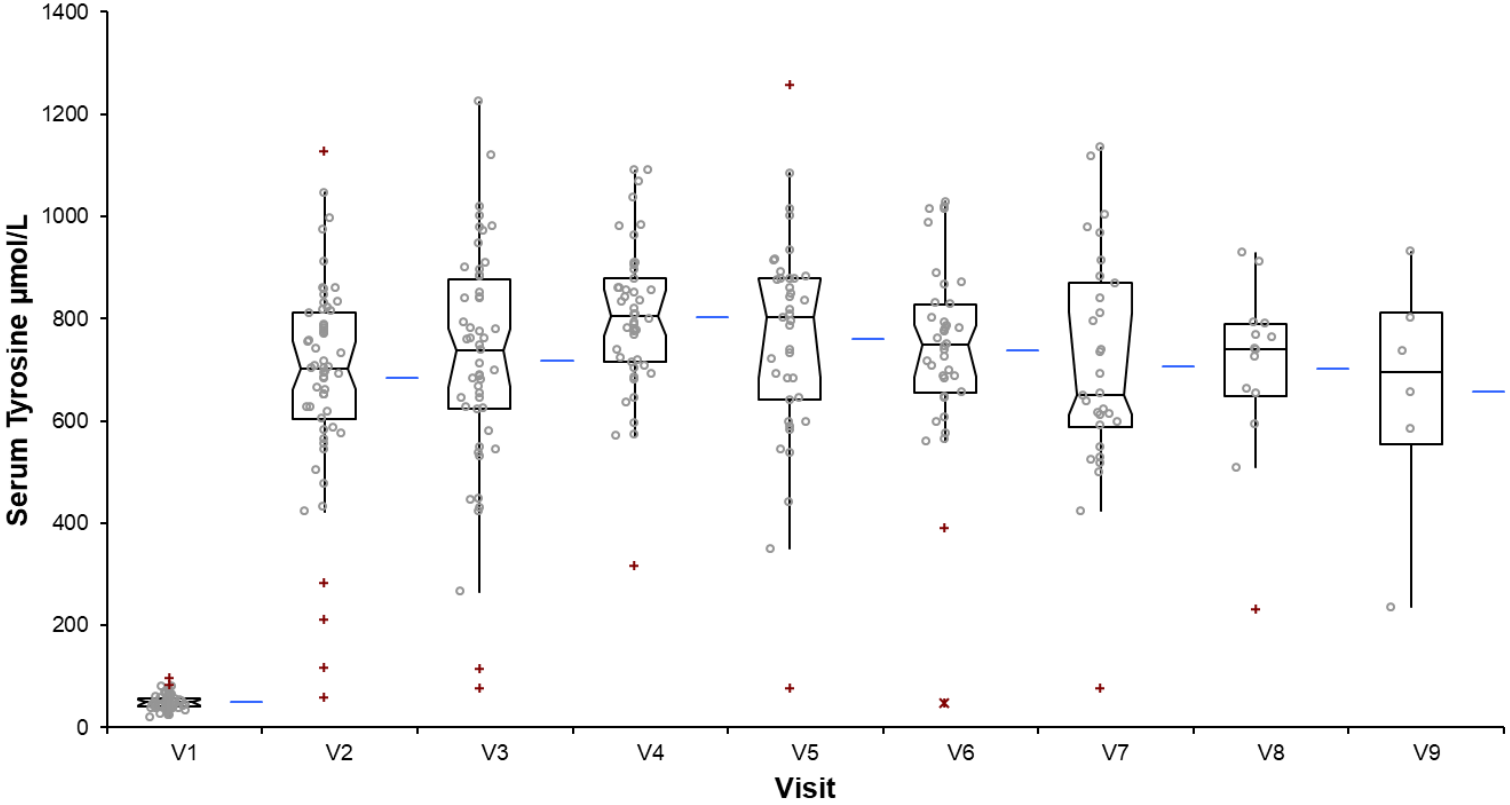

Figure 8.10: **Serum TYR all visits (blue line represents the mean; red crosses the outliers and box plots 95% confidence interval).**

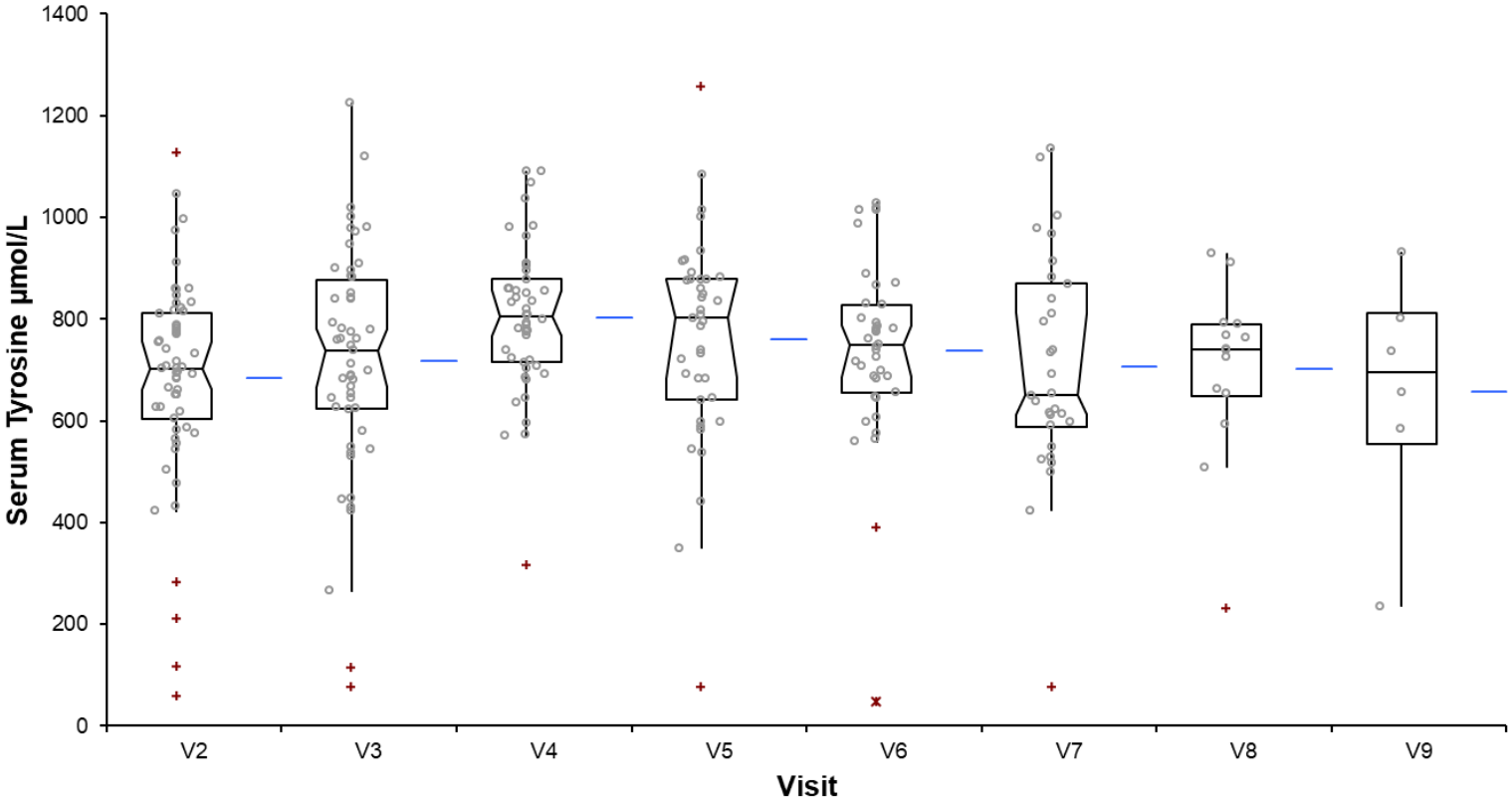

Figure 8.11: Serum TYR visits 2-9 (blue line represents the mean; red crosses the outliers and box plots 95% confidence interval).

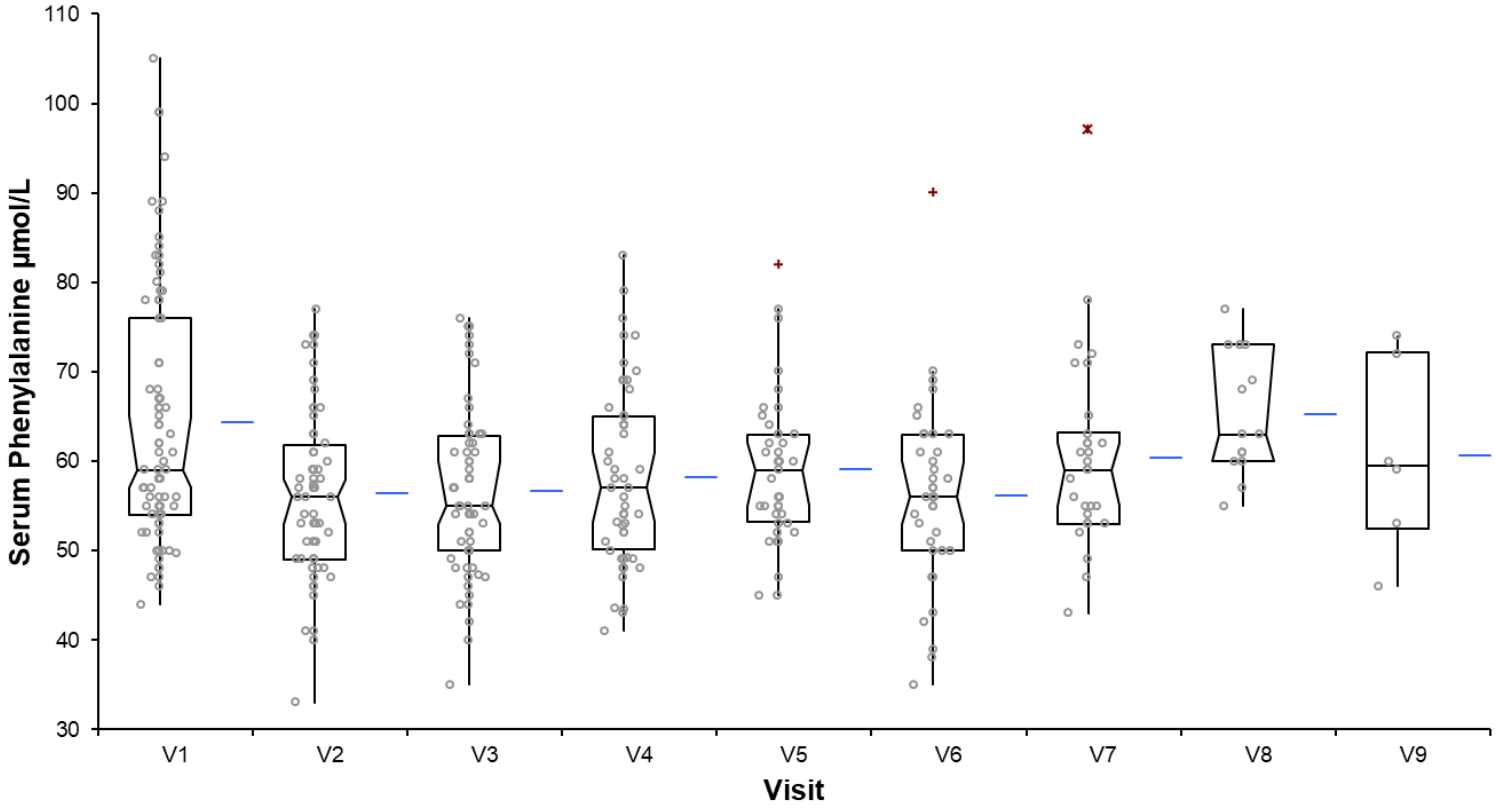

Figure 8.12: Serum PHE (blue line represents the mean; red crosses the outliers and box plots 95% confidence interval).

8.4.9 Serum HPLA and HPPA

At baseline, only two patients had detectable serum HPLA concentrations (>5 µmol/L) and therefore any statistics and data comparisons cannot be performed with baseline comparison. Similarly with HPPA, a very small number had detectable serum HPPA (>10 µmol/L), however as there were five patients, this has enabled a baseline mean value.

Table 8.2 demonstrates the trend of serum HPLA which is displayed in figure 8.13. The mean serum HPLA at V2 is 45.8 µmol/L and this is consistent across all visits. There is no significant difference between the serum HPLA concentrations at all visits.

Similarly for serum HPPA (figure 8.14) the mean is 34.3 µmol/L at V2 and although there is a rise at V8 to a mean of 43.5 µmol/L, the average trend is consistent. Statistically there is a significant difference comparing V3 data to V4 ($p=0.0121$), V7 ($p=0.0209$) and V8 ($p=0.0161$), however the overall trend provides a steady picture of the circulating HPPA concentrations post-NTBC treatment.

The ratio of serum HPPA:HPLA (figure 8.15) shows a similar trend to that seen for the urine (section 8.3.5) in that the ratio is consistent across the visits. There is a slight decrease in the ratio at V7 and V8 however as with the urine excretion ratios, there is little variation across the time windows and a consistent amount is excreted and retained in circulation.

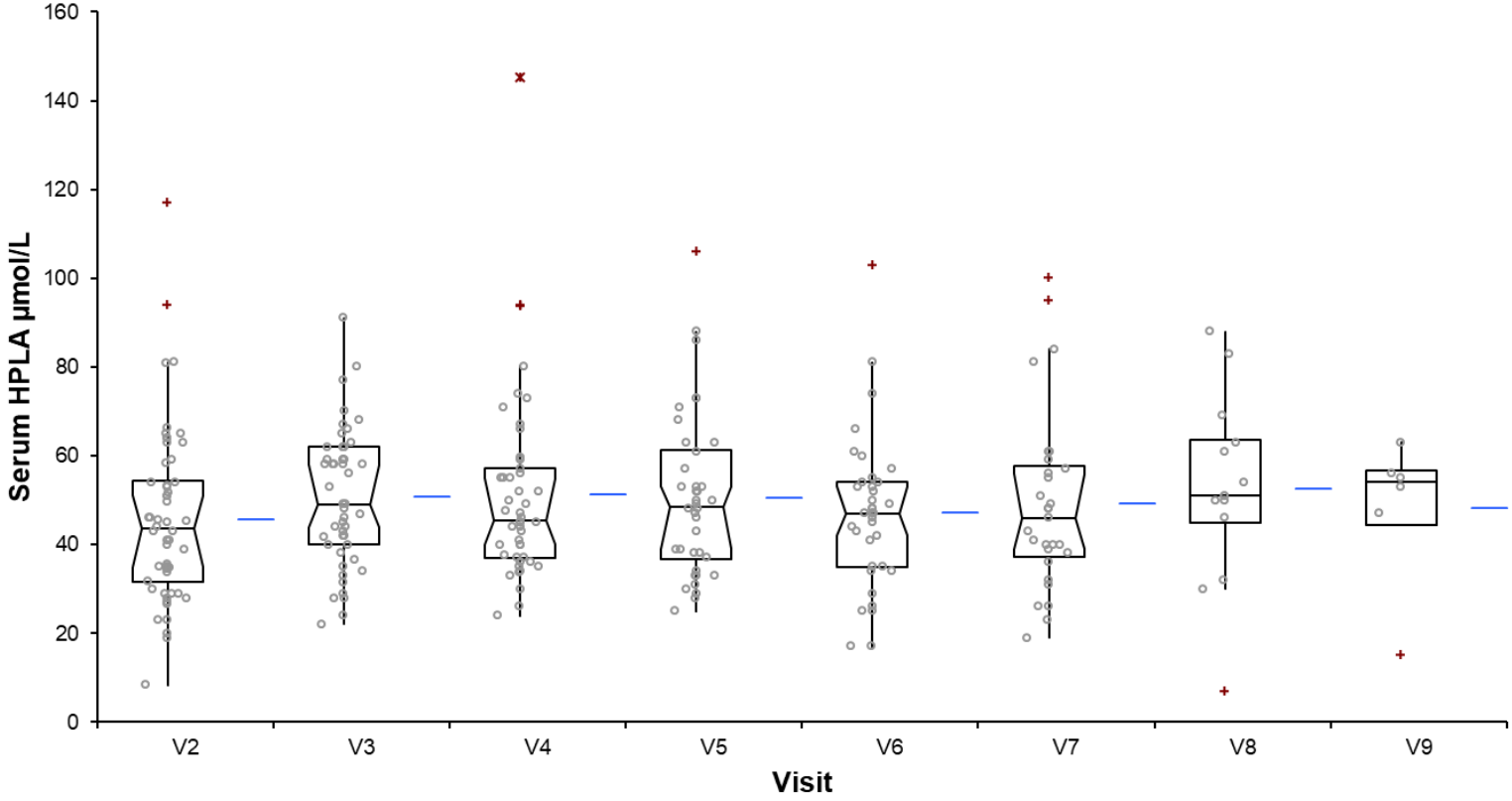

Figure 8.13: Serum HPLA (blue line represents the mean; red crosses the outliers and box plots 95% confidence interval).

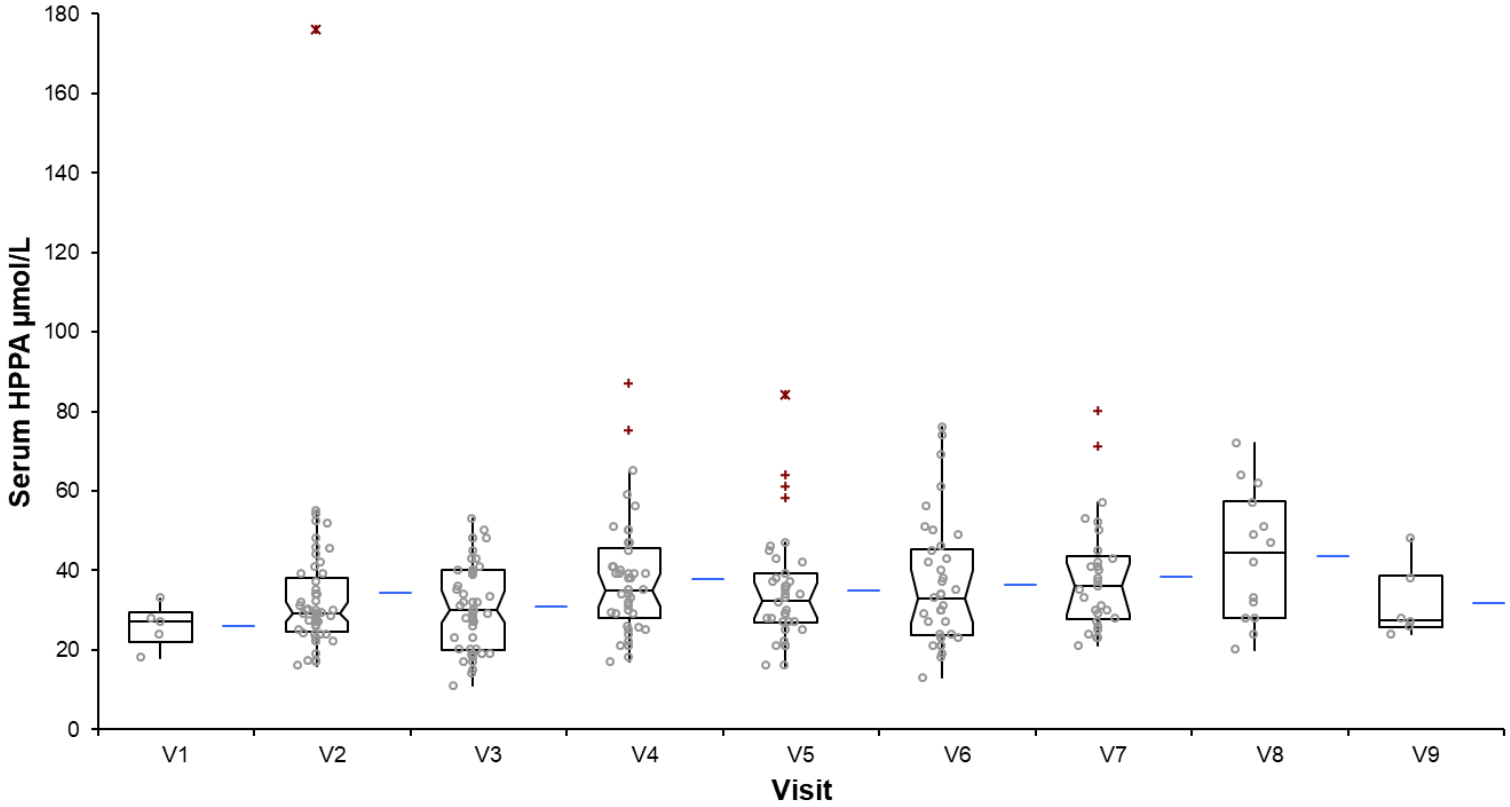

Figure 8.14: Serum HPPA (blue line represents the mean; red crosses the outliers and box plots 95% confidence interval).

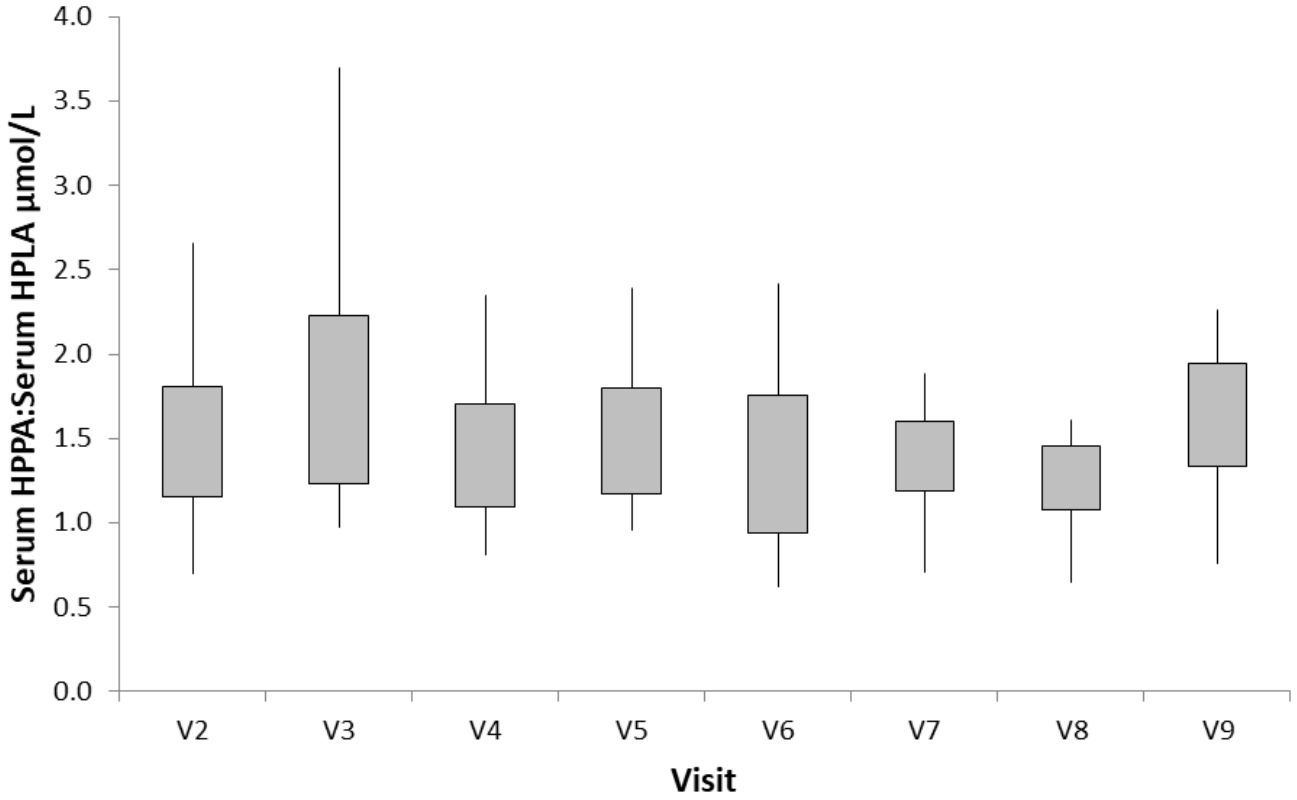

Figure 8.15: Ratio of serum HPPA:serum HPLA in V2 to V9. The boxes represent 25th and 75th percentile and the tails, 5th and 95th percentile.

8.4.10 Serum NTBC

NTBC is provided at a dose of 2 mg every other day from V1 until three months and then is increased, providing there are no complications, to 2 mg every day (reported in original manuscript, section 7.4.7). This dose is still a small dose in comparison with that used in SONIA-1 and SONIA-2 and also in HT-1 where 1-2 mg is used per kg of body weight. Serum concentrations are on average steady across all visits as shown in table 8.2 and figure 8.16.

Increasing numbers corroborate previous findings, with the average NTBC concentration at 24 months being 0.97 µmol/L. Comparison across the visits suggest a significant rise in mean NTBC at V9, (significant for all visits p values ranging from 0.0001 to 0.0025), however there are only six patients currently in the V9 cohort, who are the longest standing ones attending the NAC. In addition, four of these six patients have consistently had NTBC concentrations >1.2 µmol/L across all of their visits which demonstrates the individual variation in NTBC absorbance and metabolism. This will be revisited once there is more V9 patient data.

Currently there is no therapeutic target or range for NTBC monitoring on a 2 mg daily dose. The metabolic target for these patients is reducing serum and urine HGA and preventing serum TYR from concentrations which may predispose to keratopathy or other side effects. Using the NTBC data set, once patients are on 2 mg daily it has been possible to suggest a target range which incorporates the 5^{th} to 95^{th} percentile of the data. With all data up to V9, this is calculated as 0.46 – 1.80 µmol/L. Despite the rise seen at V9, this range was previously calculated three years ago, using V2-V6 data and it was almost identical at 0.47 – 1.80 µmol/L.

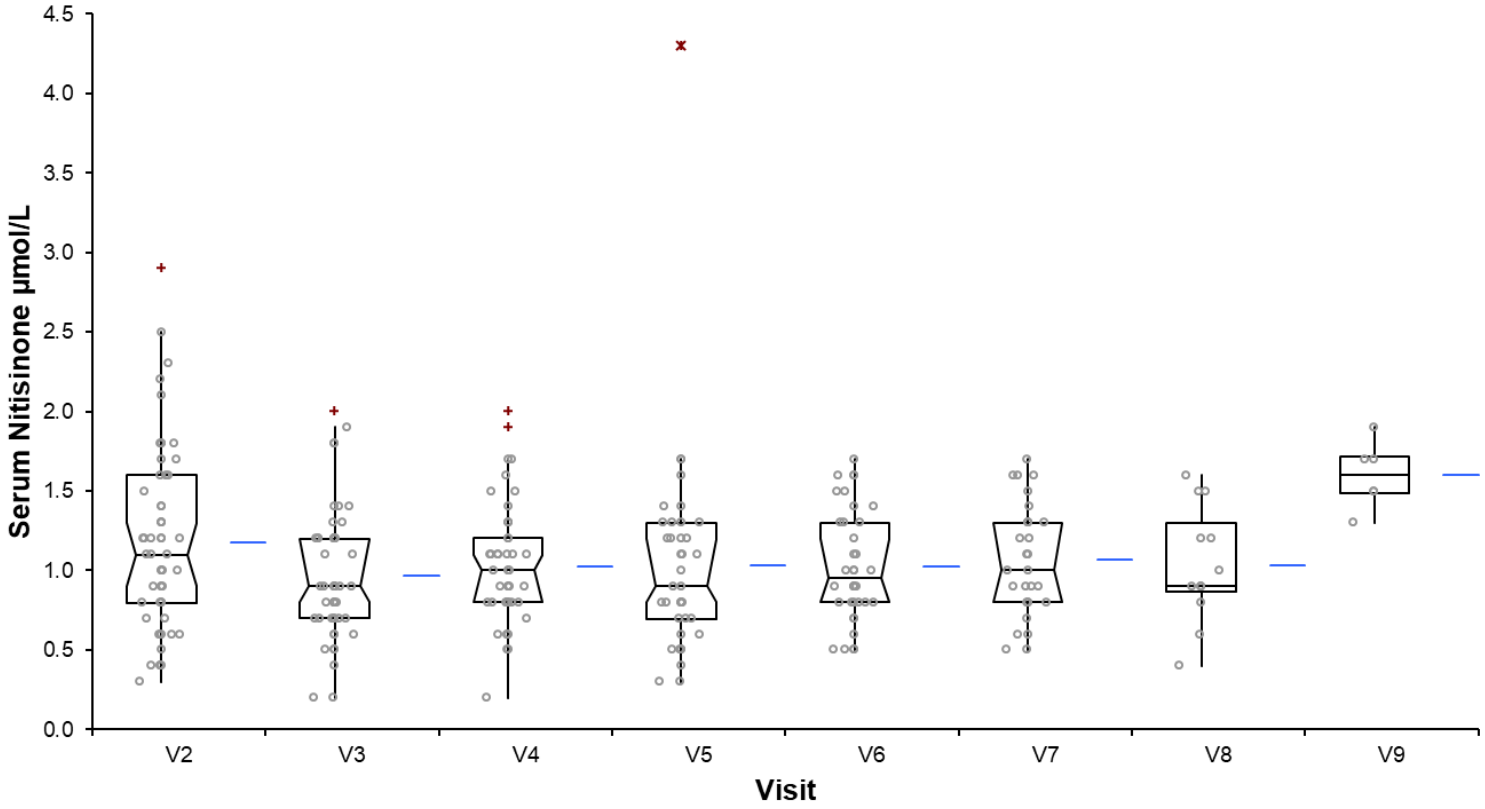

Figure 8.16: Serum NTBC (blue line represents the mean; red crosses the outliers and box plots 95% confidence interval).

8.5 Discussion

In conclusion, the data reported within this paper provides the most comprehensive longitudinal survey of AKU patients on NTBC. So far 83 patients have registered with the NAC at the time of writing, and this number is still increasing, suggesting that as a specialised centre we are engaged with a substantial number of patients from within the United Kingdom. In total eight years of NAC data has now been processed utilising the established assays described in the earlier chapters of this thesis. To provide confidence in the assay stability, in-house calibration standards and robustness of the procedures in place is reassuring and satisfying. The earlier published data presented in Chapter 7 has been corroborated in this additional paper, with similar average values of metabolites and percentage changes in serum and urine HGA suppression. In addition, this addendum reports PHE, HPLA and HPPA in the NAC cohort, in both serum and urine.

It is important to remember that not all V1 samples are eight years old, each visit pool is constantly being added to when new patients join and at any one month clinic, there can be for example V8, V4 and new patients attending. The consistent findings support our confidence in the assay. HGA levels in urine and serum remain stable after initial suppression, and TYR concentrations are consistent across visits.

Reviewing the HPLA and HPPA data in both urine and serum, there are consistent trends. Clearance and total metabolite ‘pools’ have been discussed by Milan et al., (2019), whereby in using an assumption of total body water in conjunction with renal clearance, it is evident that there is a greater increase in HPLA and HPPA compared with the concentration of HGA suppressed. This leads to the suggestion of alternative pathways of generation of HPLA and HPPA, once on NTBC and also the hypothesis of release from potential pigment reversal.

The LLOQ for the urine and serum assays for these two analytes are different, with the serum assay being more sensitive. During assay

development, a multi-analyte assay will always have winners and losers in terms of sensitivity, therefore to modify a sample preparation which has worked well for HGA, TYR and NTBC may then have implications for the sensitivity of the established analytes. The rationale for not focussing on refining the LLOQ for these analytes, particularly in the urine, is that in health they have little suggested role, being not routinely quantitated in any UK lab and thus generally not detected in health. The interest for the AKU population was what happened further up the pathway once NTBC blocked or partially inhibited the enzyme 4-hydroxy-phenylpyruvate dioxygenase (HPPD). The methodology validated for this thesis has been able to quantitate and demonstrate this in all patients who attend the NAC in both serum and urine.

Use of statistics within this paper has demonstrated several times, a surprising significance where it would appear there is not one when visually reviewing the data, for example serum PHE and comparison with the baseline average data. There was a calculated significance compared with all visits from V2 to V6 despite the means and SD's appearing to overlap. One would suggest that although being statistically significant, the subtle changes are not clinically significant and with additional sample numbers the subtle statistical signficiances may alter.

As patients continue to be monitored for all the metabolites discussed and the drug NTBC which has received a licence to treat AKU patients following successful SONIA-1 and SONIA-2 clinical trials, the next question is about dose. For SONIA-2 it was 10 mg daily which is significantly greater than that used in the NAC. The higher dose as discussed has a more significant impact on suppressing s-HGA and u-HGA and may in the long-term, with careful monitoring of the s-TYR, be the choice of dose for all AKU patients. The balance and final end point will be improving the quality of life for patients with AKU, and even preventing the debilitating effects by commencing NTBC earlier in life.

Chapter 9

Suitability Of Nitisinone In Alkaptonuria 1 (SONIA-1): An international, multicenter, randomized, open-label, no-treatment controlled, parallel-group, dose-response study to investigate the effect of once daily nitisinone on 24-hour urinary homogentisic acid excretion in patients with alkaptonuria after 4 weeks of treatment

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Full author address available on-line and in printed version.

Declaration and acknowledgments

This was a large scale consortium publication ensuring that all partners were included and acknowledged from trial design through tostatistical analysis and HGD mutations.

All methods were developed and metabolite analysis was undertaken by Andrew T Hughes. This paper is included within the thesis as application of the validated methodology for primary and secondary endpoint of the trial, interpretation of the data and to enable continuity of the AKU story.

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9.1 Abstract:

Background

Alkaptonuria (AKU) is a serious genetic disease characterized by premature spondyloarthropathy. Homogentisate-lowering therapy is being investigated for AKU. Nitisinone (NTBC) decreases homogentisic acid (HGA) in AKU but the dose-response relationship has not been previously studied.

Methods

SONIA-1 was an international, multicenter, randomized, open-label, no-treatment controlled, parallel-group, dose-response study. The primary objective was to investigate the effect of different doses of once daily NTBC on 24hr urinary HGA excretion (u-HGA_{24}) in patients with AKU after 4 weeks of treatment. Forty patients were randomized into 5 groups of 8 patients each, with groups receiving no treatment or 1, 2, 4 and 8 mg of NTBC.

Findings

A clear dose-response relationship was observed between NTBC and the urinary excretion of HGA. At 4 weeks, the adjusted geometric mean u-HGA_{24} was 31.53, 3.26, 1.44, 0.57 and 0.15 mmol for the no treatment or 1, 2, 4 and 8 mg doses respectively. For the most efficacious dose, 8 mg daily, this corresponds to a mean reduction of u-HGA_{24} of 98.8% compared to baseline. An increase in tyrosine (TYR) levels was seen at all doses but the dose-response relationship was less clear than the effect on HGA. Despite tyrosinaemia, there were no safety concerns and no serious adverse events were reported over the 4 weeks of NTBC therapy.

Conclusion

In this study in AKU patients, NTBC therapy decreased urinary HGA excretion to low levels in a dose-dependent manner and was well tolerated within the studied dose range.

9.2 Introduction:

Alkaptonuria (AKU) is a serious, autosomal recessive, multisystem disorder (O'Brien et al., 1963; Helliwell et al., 2008; Ranganath and Cox, 2011) affecting approximately one in every 250,000 people (Phornphutkul et al., 2002), although some countries such as Slovakia and the Dominican Republic have a higher prevalence rate of around one in 19,000 (Milch, 1960; Zatkova, 2011). Morbidity in AKU is caused by increased levels of homogentisic acid (2,5-dihydroxyphenylacetic acid, HGA) due to a deficient enzyme, homogentisate 1,2-dioxygenase (HGD) (La Du et al., 1958). Despite efficient urinary excretion of HGA (Phornphutkul et al., 2002), some of it is oxidized to a melanin-like polymeric pigment via benzoquinone acetic acid (BQA). This pigment polymer is deposited in connective tissues, particularly cartilage, a process termed ochronosis (Zannoni et al., 1969), leading especially to severe premature arthritis with an early onset, affecting the spine and synovial joints, large and small (Phornphutkul et al., 2002; Ranganath and Cox, 2011).

Current treatments are limited to palliative analgesia and arthroplasty (Ranganath et al., 2013). Nitisinone (NTBC), a competitive inhibitor of the enzyme 4-hydroxyphenyl-pyruvate dioxygenase (HPPD), decreases the formation of HGA (Lindstedt et al., 1992; McKiernan, 2006). NTBC has been used for the treatment of hereditary tyrosinaemia type 1 (HT-1) for more than 20 years (Lindstedt et al., 1992; McKiernan, 2006). HT-1 is due to accumulation of highly toxic metabolites (maleylacetoacetate and fumarylacetoacetate among others) further downstream from HGA, which leads to progressive liver and renal failure, and is fatal if not treated (Lindstedt et al., 1992; McKiernan, 2006).

It is hypothesized that if HGA levels are reduced before the onset of overt ochronosis, this might prevent the development of the debilitating features of AKU. While NTBC has been shown to reduce plasma HGA levels and urinary excretion (Suwannarat et al., 2005; Introne et al., 2011), and in a mouse model of AKU (Preston et al., 2014), it has not undergone any formal clinical development for AKU, although three published investigator-initiated

studies have been completed (Phornphutkul et al., 2002; Suwannarat et al., 2005; Introne et al., 2011). The 3-year study by Introne et al., (2011) showed a significant reduction in urine HGA excretion by about 95% using a daily dose of 2 mg of NTBC but no significant effect on clinical parameters (Introne et al., 2011). One possible factor for the inconclusive effect on clinical parameters may be the use of a sub-optimal dose.

AKU is a very slow progressive disease with overt manifestations presenting in the third decade onwards. The present study was designed to investigate the metabolic effects of nitisinone, namely the relationship between different doses of nitisinone and u-$HGA_{24,}$ serum HGA and TYR.

9.3 Methods:

9.3.1 Patients

Patients with a well-documented AKU verified by increased urine HGA excretion and who were at least 18 years old were eligible for inclusion in the study. Details of inclusion and exclusion criteria are described in the supplementary appendix (table S1, available online). In all patients, diagnosis of AKU was confirmed by HGD gene mutation identification performed during the study (data not shown).

9.3.2 Study Design and Intervention

SONIA-1 was a randomized, open-label, parallel-group study with a no-treatment control group. Patients were randomized to receive either 1, 2, 4 or 8 mg NTBC once daily or no treatment (control). Forty patients were randomized, equally distributed amongst the groups (8 patients per group). The treatment period consisted of 4 weeks, during which study drug was administered. At 6 weeks a follow up telephone call concluded the study. The study design is summarized in figure 9.1 and study procedures described in table S2 (available online).

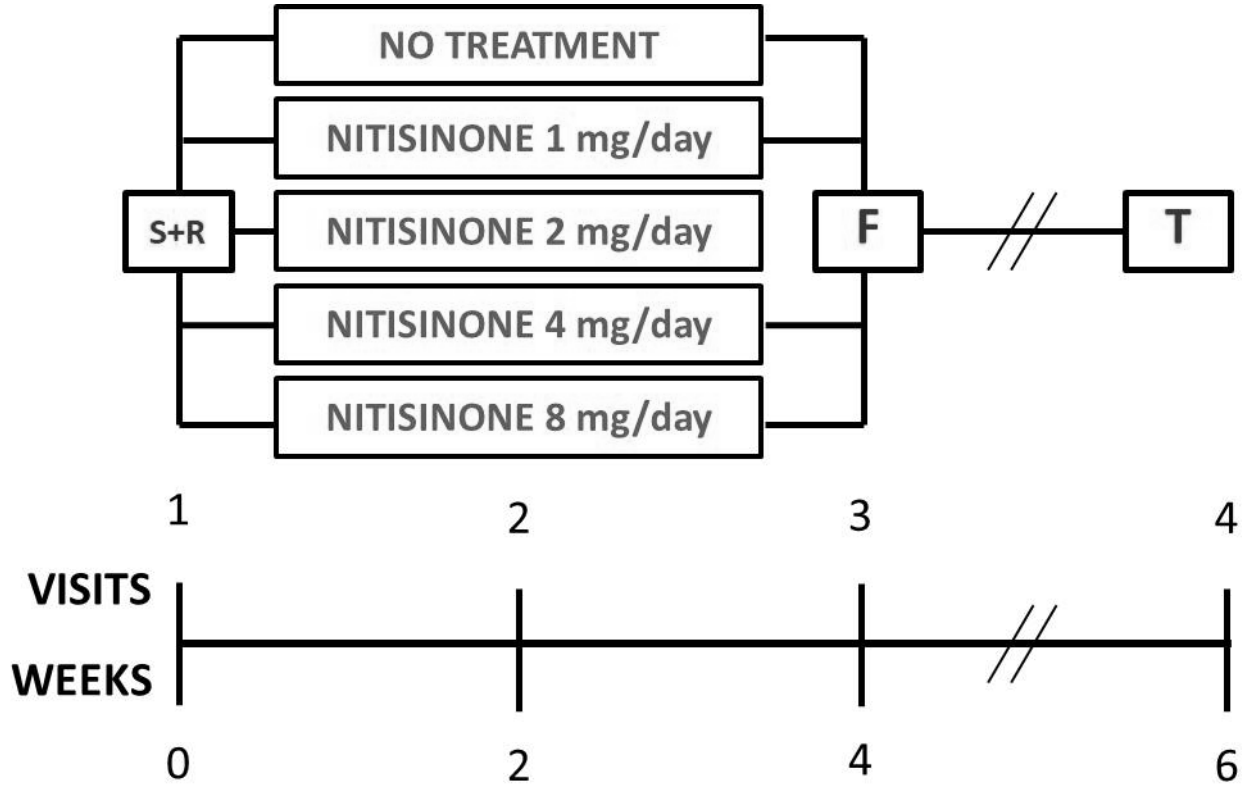

Figure 9.1: SONIA 1 Study Design.

The study consisted of two main periods: treatment, and follow-up. After screening, patients were randomized at baseline (1:1:1:1:1) to no-treatment (control), and oral daily doses of NTBC of 1, 2, 4 and 8 mg. The treatment period consisted of 4 weeks, during which study drug was administered. At 6 weeks a follow up telephone call concluded the study. (Abbreviations: S+R = Screening, Baseline and Randomization Visit; F = Final Treatment Visit; T = Telephone Follow-Up Visit)

The study was open label, since it is not feasible to blind a study with HGA-lowering treatment in AKU. One of the cardinal signs of AKU is urine darkening on standing as HGA is oxidized; patients could therefore easily know whether they were on NTBC or placebo. Furthermore, any personnel involved at the investigative sites who were involved in the processing of urine samples would also be able to see this difference. However, the only subjective reporting in the study was that of adverse events. Patients were requested to maintain stable dietary habits during the 4-week study period in order not to change their dietary protein intake. Altering the dietary protein intake could affect serum HGA concentrations and urine HGA excretion; the SONIA-1 study was carried out with no modulation or monitoring of diet in order to observe the effect of NTBC alone on both HGA and TYR.

9.3.3 Rationale for dose selection

The choice of doses used in the present study was based on current knowledge regarding the HGA-lowering effect of NTBC in AKU. In one previous study, mean u-HGA_{24} was reduced from 4 g/day to 230 mg/day using a dose of 2.1 mg of NTBC daily (Suwannarat et al., 2005). In a 3-year study, using a dose of 2 mg daily, mean u-HGA_{24} decreased from 5.1 g/day to values ranging from 113 to 203 mg/day during the course of the study, which on average corresponded to a 95% decrease (Introne et al., 2011). We wanted to investigate the effect of NTBC on HGA at higher doses than the ones used in previous studies, and to find a dose that reduces HGA by close to 100%. At the same time, we were interested in determining the effect of NTBC on serum TYR levels at a dose lower than 2 mg daily. Therefore, doses of 1, 2, 4 and 8 mg were used in this study.

The investigational medicinal product (IMP) used in the SONIA-1 study was a suspension of NTBC (Orfadin®) containing 4 mg/mL administered in the morning was used as it allowed easy administration of the selected doses. The daily dosing frequency in our study is based on the long half-life of NTBC (McKiernan, 2006).

9.3.4 Randomization procedures

Patients were randomly assigned to one of five groups, in a 1:1:1:1:1 ratio stratified by study centre using randomly permuted blocks. Results for HGA and TYR were not accessible to the medical monitors, sponsor personnel, or study site personnel until study completion.

9.3.5 Prior and concomitant therapy

Patients were allowed to continue on any chronic medication and any changes during the study were recorded, from the time of the screening and randomization visit until the follow-up telephone call. Patients were not allowed to have used NTBC within the 60 days prior to randomization.

9.3.6 Treatment compliance

Product accountability records were kept by the pharmacy and investigator. All unused IMP was returned to the clinical study sites and measured. The amount consumed was compared to the expected consumption for the randomized dose.

9.3.7 Chemical measurements

9.3.7.1 Urine sample collection and handling

At baseline, and weeks 2 and 4, urine was collected over 24hr into 2.5 L bottles containing 30 mL of 5N H_2SO_4 and stored away from bright light in cool conditions. The weight of the collected urine was recorded and used as the volume in the calculations of u-HGA_{24} assuming a density of 1g/mL. An aliquot of the collected urine was frozen and kept at -20ºC until analysis.

9.3.7.2 Serum sample collection and handling

Measurements of serum TYR (s-TYR) and HGA (s-HGA) concentrations were performed at weeks 0, 2 and 4. At each visit one sample was collected pre-dose in fasting patients. Blood samples were collected in non-gel serum tubes. An aliquot of serum was immediately acidified using perchloric acid (10% v/v 5.8 M), and kept frozen at -20ºC until analysis.

9.3.7.3 Analyses of HGA and TYR

The concentrations of TYR and HGA in serum and urine were measured by liquid chromatography tandem mass spectrometry (LC-MS/MS) (Hughes et al., 2014; 2015). All analyses were performed on an Agilent 6490 Triple Quadrupole mass spectrometer with Jet-Stream® electrospray ionisation (ESI-MS/MS) coupled with an Agilent 1290 infinity UHPLC pump and HTC autosampler. This method incorporates reverse-phase chromatographic separation on an Atlantis C18 column (100 mm x 3.0 mm, 3 µm). Initial conditions of 80:20 water:methanol with 0.1% formic acid (v/v) increased linearly to 10:90 over five minutes. Matrix-matched calibration standards and quality controls were utilized with appropriate isotopically labelled internal standards (IS). Quantitation was achieved in multiple reaction monitoring (MRM) mode with two product ion transitions for both TYR (positive ionisation) and HGA (negative ionisation). Samples were prepared by dilution in a combined IS solution (final concentrations of 0.4 µmol/L $^{13}C_6$-HGA and 2 µmol/L d_2-TYR in 0.1% formic acid (v/v) in deionised water). All serum and urine quantitation analyses were performed by the Department of Clinical Biochemistry and Metabolic Medicine at the Royal Liverpool University Hospital (RLUH). The analytical performance for s-HGA, s-TYR and u-HGA was satisfactory with acceptable coefficients of variation for both within and between batches (Supplementary table S4, online).

9.3.8 Endpoints

The primary endpoint was the u-HGA_{24} in patients with AKU after 4 weeks of NTBC treatment. Secondary endpoints supporting the primary objective

included u-HGA_{24} after 2 weeks, as well as the uHGA/u-creatinine ratio at weeks 2 and 4. Secondary endpoints included the pre-dose s-HGA and s-TYR concentrations at weeks 2 and 4.

9.3.8.1 Safety Assessment

At each visit, adverse events (AEs) and laboratory values were recorded. Routine laboratory processes at each clinical study site were employed to measure biochemistry and haematology profiles. At each visit a corneal slit lamp examination was performed to check for possible corneal toxicity. TYR can occasionally cause idiosyncratic reversible corneal dendritiform keratopathy and skin rash with no consistent NTBC dose or TYR concentration relationships (Stewart et al., 2014).

9.3.9 Statistical analysis

The primary variable, u-HGA_{24} at Week 4, was analysed using a Mixed Model for Repeated Measures (MMRM). The model included the study site, treatment group, visit, and the interaction between treatment group and visit as fixed factors and the baseline u-HGA_{24} as a covariate. Model-based least square means and associated 95% confidence intervals for each treatment group were calculated. As the distribution of u-HGA_{24} was skewed, log-transformation was applied prior to analysis. The estimates were then back transformed to the original scale and thus the estimates correspond to adjusted geometric means. In addition, post-hoc pairwise comparisons were performed with no adjustment for multiplicity. Linear contrasts from the MMRM model were used for these comparisons. The analyses were conducted on the full analyses set (FAS), using the statistical software SAS v9.3.

9.3.10 Analysis of safety and tolerability data

9.3.10.1 Adverse events

All AEs during the study were coded using the Medical Dictionary for Regulatory Activities (MedDRA version 16.0). The incidence of adverse events was summarized in frequency tables. The changes in safety

laboratory parameters from baseline to all post-baseline visits were summarized by treatment group and visit using descriptive statistics.

9.3.11 Study funding and oversight

SONIA-1 is part of the DevelopAKUre program, which has received funding from the European Union Seventh Framework Program (FP7). The EC had no direct participation in any aspect of design and conduct of the study, drug supply or reporting. The objective of the clinical development program is to investigate the possibility of an effective and safe treatment of AKU.

The study was conducted at two sites, Liverpool (United Kingdom) and Piešťany (Slovakia) from May to October 2013. Data was recorded by investigators at each site, collected, and monitored by the Contract Research Organization PSR Group (Amsterdam, Netherlands). The protocol and amendments were approved by the relevant ethics review boards and national regulatory authorities. Written informed consent was obtained from all patients before any study procedures. An external Data and Safety Monitoring Board was assigned to evaluate the safety data. The study has EudraCT no is 2012-005340-24 and is registered at ClinicalTrials.gov with number NCTO1828463.

9.4 Results:

9.4.1 Patients and Study Treatment

15 and 25 patients with AKU from the clinical study sites, in Liverpool and Piešťany respectively, were randomized into 5 groups (no treatment, 1, 2, 4 and 8 mg groups). All randomized patients completed the study. Patient demographics and baseline characteristics were similar across the 5 groups (table 9.1). The majority (67.5%) were male, and the mean age for all patients was 47.2 years ranging from 19 to 63 years; 37 were Caucasian and 3 were Asian. Baseline s-HGA, s-TYR and u-HGA_{24} are shown in table 9.2. No patient had abnormal renal function with decreased eGFR. Serum creatinine and eGFR data are shown in table 9.1. There were no missing

data for the primary or secondary variables. There were no obvious protocol deviations that affected the interpretation of the results.

All measurements of u-HGA, s-HGA and s-TYR performed with intra- and inter-assay coefficients of variation of less than 6% and 7% respectively across a large linear range of concentrations as shown in table S4 (online only).

The daily HGA excretion in the no-treatment arm was not significantly different over the 3 visits suggesting that there were no major change in dietary habits in that group.

Table 9.1: SONIA 1 Patient demographics, and related baseline data [Mean (SD)] *

		Untreated (n=8)	1 mg (n=8)	2 mg (n=8)	4 mg (n=8)	8 mg (n=8)	Total (n=40)
Age (years)		45.9 (15.3)	44.4 (10.9)	43.9 (13.7)	47.3 (10.7)	54.4 (7.3)	47.2 (11.9)
Body weight (kg)		71.0 (23.5)	86.9 (15.9)	74.6 (10.9)	76.9 (14.3)	81.1 (13.7)	78.1 (16.3)
Height (cm)		165.3 (12.1)	170.6 (7.1)	167.1 (9.4)	168.4 (5.9)	165.9 (6.7)	167.5 (8.3)
S-Creatinine (mmol/L)		55.7 (13.5)	61.3 (10.2)	50.4 (10.7)	63.2 (13.3)	60.4 (11.2)	58.2 (12.2)
S-ALT/SGPT (u/L)		34.0 (25.5)	27.6 (11.4)	35.6 (16.6)	30.9 (16.5)	26.8 (6.9)	31.0 (16.1)
eGFR ($mL/min/m^2$) §		124.5 (18.2)	122.5 (16.4)	149.0 (29.7)	110.8 (17.9)	120.5 (27.1)	125.4 (24.9)
Gender n (%)	Female	4(50.0)	1(12.5)	3(37.5)	3(37.5)	2(25.0)	13(32.5)
	Male	4(50.0)	7(87.5)	5(62.5)	5(62.5)	6(75.0)	27(67.5)
Race n (%)	White	7(875)	7(875)	8(100)	8(100)	7(875)	37(925)
	Asian	1(125)	1(125)	0(0)	0(0)	0(0)	2(5.0)
	Others	0(0)	0(0)	0(0)	0(0)	1(12.5)	1(2.5)

*Data in parentheses are SD or %
§Modification of Diet in Renal Disease calculation was employed

Table 9.2: Mean (SD) and range for u-HGA_{24}, u-HGA/creatinine, s-HGA and s-TYR

	Untreated n=8	**1 mg n=8**	**2 mg n=8**	**4 mg n=8**	**8 mg n=8**
u-HGA_{24} (mmol)					
Baseline	29.8(5.1) 22.3–38.8	36.7(14.6) 20.7–69.5	31.4(7.4) 20.0–40.7	35.4(13.6) 14.4–55.4	27.3(5.2) 17.1–32.9
Week 4	31.0(4.6) 22.2–35.4	3.9(1.7) 1.8–6.7	1.6(0.8) 0.6–3.5	0.7(0.4) 0.2–1.5	0.1(0.05) 0.1–0.2
u-HGA/creatinine (mmol/mol)					
Baseline	2.8(0.9) 2.0–4.3	2.3(0.3) 1.9–2.8	3.1(0.3) 2.5–3.6	2.8(0.4) 2.3–3.3	2.7(0.6) 2.0–3.7
Week 4	3.0(0.9) 1.8–4.2	0.2(0.1) 0.1–0.4	0.1(0.04) 0.1–0.2	0.05(0.03) 0.01–0.1	0.01(0.005) 0.01–0.02
s-HGA (µmol/L)					
Baseline	27.5(8.9) 14.6–45.5	28(11.1) 5.8–41.4	30.3(7.7) 20.6–41.9	32.1(6.6) 22.2–41.4	28.3(7.8) 15.6–37.6
Week 4	30.5(12.4) 14.4–53.3	ND	ND	ND	ND
s-TYR (µmol/L)					
Baseline	54(15) 39-87	68(20) 49-113	62(10) 47-78	60(9) 46-71	55(5) 48-63
Week 4	56(15) 42-91	653(106) 450-806	715(171) 506-965	803(155) 657-1155	813(145) 523-927

[§] Lower row in cell is range of data; ND = not determined (below 3.1 µmol/L); u-HGA_{24}: urine HGA excretion over 24 hours; u-HGA/creatinine: urine HGA excretion adjusted per mol of urine creatinine; s-HGA: serum HGA in acidified fasting sample; s-TYR serum TYR in fasting sample

9.4.2 Primary Endpoint

9.4.2.1 Urinary HGA excretion

Baseline u-HGA_{24} varied substantially between individuals and ranged from 14.4 to 69.5 mmol (corresponding to 2.43 to 11.7 grams) (table 9.2).

At week 4, a clear dose-response relationship between NTBC and u-HGA_{24} was observed (table 9.2). This is also illustrated in figure 9.2a (all patients) and in figure 9.2b (data at week 2 and 4 for treated patients only). The adjusted geometric means and associated 95% confidence intervals were 31.53 (27.19 to 36.57), 3.26 (2.27 to 4.69), 1.44 (1.00 to 2.06), 0.57 (0.39 to 0.81) and 0.15 (0.11 to 0.22) mmol for the untreated, 1, 2, 4, and 8 mg doses, respectively. The greatest reduction in u-HGA_{24} was measured using the 8 mg dose.

From the MMRM, treatment group, visit, treatment group-visit interaction and baseline u-HGA_{24} were statistically significant (p values: <0.0001, 0.008, 0.020, 0.002 respectively) and site was not significant (p=0.523). All post-hoc pairwise comparisons between doses at week 4, were statistically significant (p=0.002 or lower in all cases). A similar pattern was observed for pairwise comparisons at 2 weeks.

The u-HGA/u-creatinine ratios at baseline and week 4 are presented in table 9.2. They confirm the results seen for the u-HGA_{24} values without creatinine correction, and indicate acceptably complete 24hr urine collection.

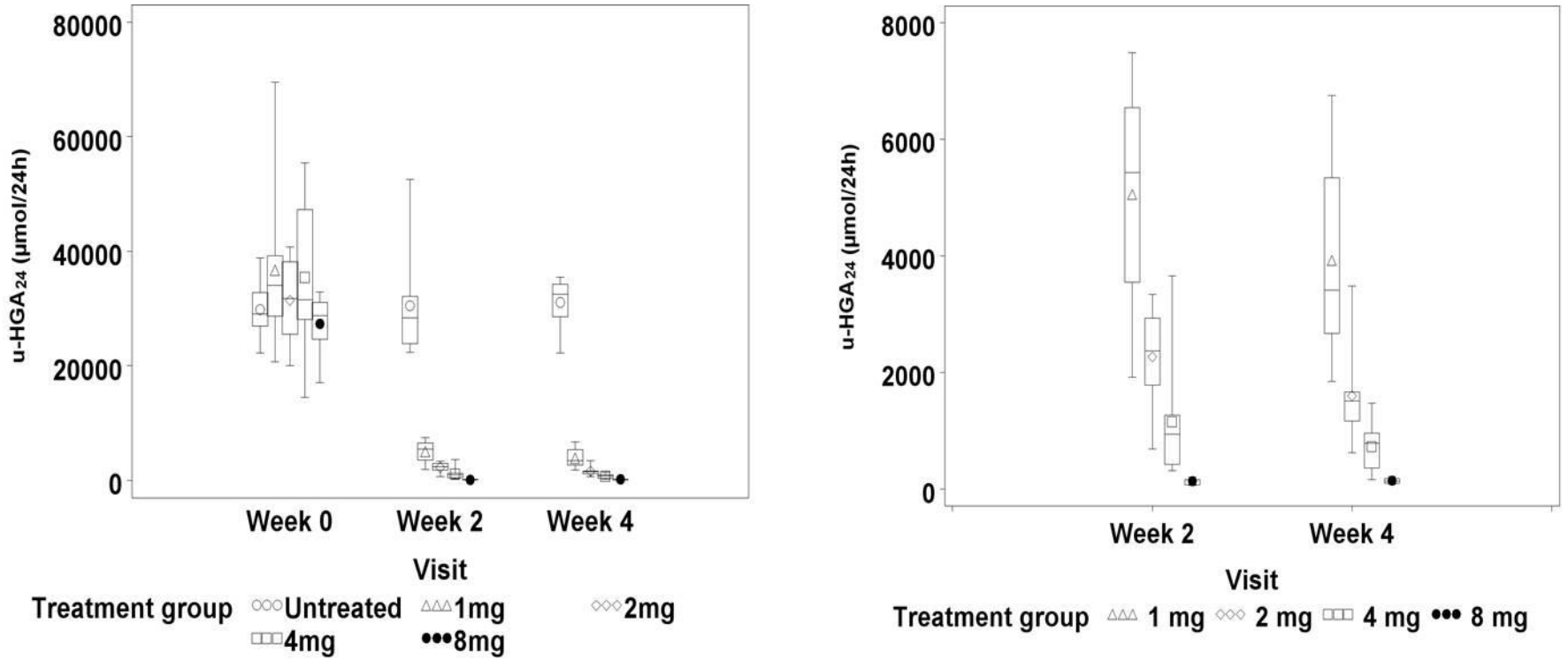

Figure 9.2: 24hr urinary excretion of HGA

9.2a: Box-plots of 24-hour urinary excretion (µmol/24 hours) of HGA in untreated and nitisinone-treated AKU patients over time.

9.2b: Box-plots of 24-hour urinary excretion (µmol/24 hours) of HGA in nitisinone-treated AKU patients at weeks 2 and 4.

9.4.3 Secondary endpoint

9.4.3.1 Serum HGA

Serum HGA was quantifiable in all patients before starting NTBC treatment. Supplementary data are shown for relationship between baseline s-HGA and u-HGA_{24} at baseline. The correlation between s-HGA and u-HGA_{24} at baseline was weak (r=0.286, p=0.074) (figure S1). After treatment, s-HGA values were below the lower limit of quantification (LLOQ, 3.1 µmol/L) in 56% of all samples collected in treated patients (table 9.2) (1 mg: 1 patient; 2 mg: 3 patients, 4 mg: 7 patients; 8 mg: 7 patients at week 4). No calculation of descriptive statistics for s-HGA was therefore performed.

9.4.3.2 Serum TYR

The s-TYR data, pre-treatment and after 4 weeks of treatment, are presented in table 9.2 and figure 9.3. Mean s-TYR increased with dose post-NTBC in all patients. However, as seen in figure 9.3, there was large inter-individual variability in the data, and with few exceptions, all nitisinone-treated patients had levels above 500 µmol/L, with the highest observation (1,117 µmol/L) seen for a patient in the 4 mg group. The relationship between change in u-HGA_{24} and change in s-TYR are described further in the supplementary data section (figure S2). There was no correlation between these two variables (r=-0.025, p=0.890).

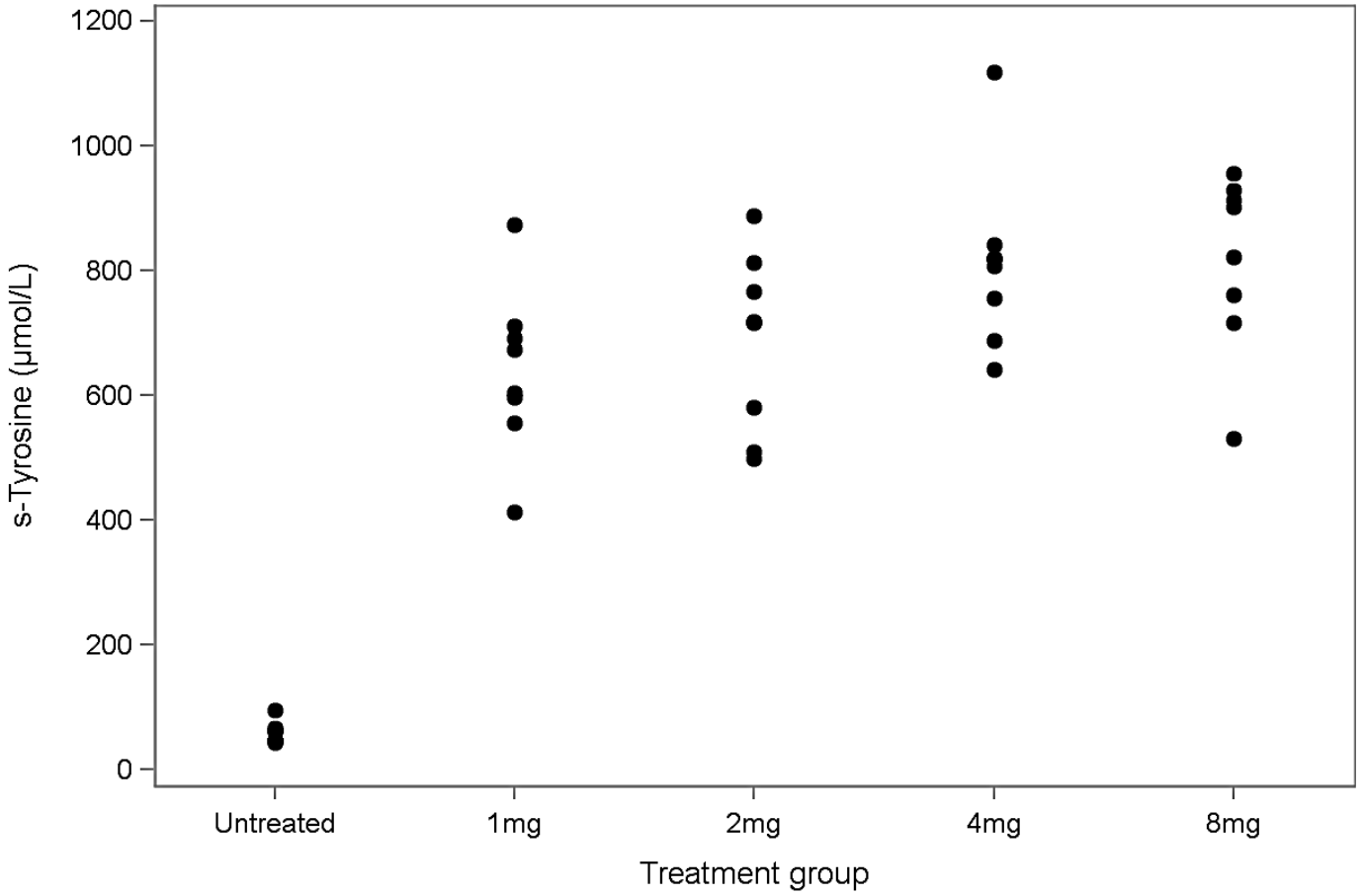

Figure 9.3: Fasting pre-dose serum concentrations of TYR at week 4 (all patients).

9.4.4 Other safety results

No safety concerns were identified in this 4 week study. AEs are summarized in table S3. There were no SAEs and no event occurred in more than one patient, except for back pain that was reported by two patients in different dose groups. All events were considered mild, except for back pain in one patient in the 4 mg dose group. No abnormalities or changes in clinical chemistry or haematology laboratory data were observed. No patient experienced any corneal effects of elevated TYR.

The use and effect of concomitant medication taken during the conduct of the study was reviewed. No known CYP3A4 inhibitors or inducers were used in the study.

9.5 Discussion:

A clear dose-response relationship was observed for the effect of NTBC on the urinary excretion of HGA, with excretion decreasing consistently across the studied dose interval of 1 to 8 mg. Since SONIA-1 used objective assessments of efficacy (changes in HGA levels), the open design was unlikely to have introduced bias. The 8 mg dose resulted in a mean reduction of u-HGA_{24} of 98.8% compared to baseline. While it is not possible to guarantee full compliance, the data on the s-HGA, s-TYR and u-HGA_{24} are sufficiently consistent for us to believe that compliance was not an issue during the study. The fact that each patient consumed at least 80% of the prescribed dose is supportive of this statement.

Doses in the treatment of HT-1 are considerably higher than those used in this study (McKiernan, 2006). But in that disease, the accumulating TYR metabolites are extremely toxic, and the disease is fatal if not treated. Thus in HT-1, doses must be high enough to secure a near 100% inhibition of HPPD in all patients at all times, which is not considered necessary for AKU.

There is currently no approved pharmacological therapy for AKU. Treatment therefore relies on palliative analgesia and joint replacement surgery. Current

experience with NTBC is limited to three studies carried out at the National Institutes of Health (NIH), USA (Phornphutkul et al., 2002; Suwannarat et al., 2005; Introne et al., 2011). The last NTBC study carried out by the NIH, a 3 year outcome study, was inconclusive for the rheumatological endpoint (hip rotation) (Introne et al., 2011). One possible reason for the inconclusive NIH study is that an optimal dose may not have been used. We therefore investigated the HGA-lowering effect of different doses of NTBC to find a dose that could lower u-HGA_{24} by close to 100%.

There is a lack of data correlating levels of serum and urine HGA with the evolution of AKU in patients. Therefore the level of HGA post-nitisinone that would prevent ochronosis if treatment is started sufficiently early, or the level that would arrest or delay ochronosis in humans if treatment is started later in AKU, is currently unknown. Lifetime treatment of AKU mice with NTBC resulted in an 88% plasma HGA reduction, down to less than 10 µmol/L and completely prevented ochronosis (Preston et al., 2014). Furthermore in vitro evidence also indicates that 10 µmol/L HGA is insufficient to cause ochronosis (Tinti et al., 2011). It is reasonable to assume that the low serum HGA achieved by NTBC in this study will prevent ochoronosis.

The effect of NTBC on clinical symptoms and long-term safety needs to be further investigated. A longer study (SONIA-2) is underway to answer this question.

9.6 Conclusions:

Treatment of AKU patients with NTBC at doses of 1 to 8 mg reduced u-HGA and s-HGA in a dose-dependent manner. No safety concerns were raised from this short-term study.

9.7 Appendix to Chapter 9:

Within the original manuscript, primary and secondary endpoints were published, which were the u-HGA_{24} and s-HGA and s-TYR. There have been secondary publications after the original manuscript was accepted for publication. The first one reported the s-NTBC concentrations (Olsson et al., 2015) and confirmed that it increased in proportion to dose and that NTBC decreased u-HGA_{24} up to s-NTBC concentrations of 3 µmol/L. To achieve a s-NTBC concentration of 3 µmol/L required a daily NTBC dose of 8 mg, and was one of the factors which led to a daily dose of 10 mg being chosen for the SONIA-2 study. Another reason was NTBC was not manufactured in 8mg doses.

The measurement of hydroxyphenylpyruvate (HPPA) and hydroxyphenyllactate (HPLA) in SONIA-1 was reported in a paper entitled "Quantification of the flux of TYR pathway metabolites during NTBC treatment of AKU" (Milan et al., 2019). The paper discussed the unexpected increase in the total 'pool' of metabolites, post-NTBC. Analysis of urine HGA, TYR, PHE, HPLA and HPPA confirmed that regardless of NTBC dose, there was no change in net excretion of totalled urine metabolites. With the suppression of u-HGA there was a counter-balanced rise of u-TYR, u-HPPA and u-HPLA. There was no dose-effect in keeping with the relatively free renal excretion of HGA, HPLA and HPPA and the maintained renal function during the four-week trial.

For both the manuscript used for Chapter 9 (re-published with permission, appendix 1) and Milan et al., 2019, I was solely responsible for analysing all of the samples through the validated methods described within the earlier chapters of this thesis. In addition, I contributed to writing and reviewing of both manuscripts.

Chapter 10

Metabolite stability in alkaptonuria – implications and considerations for long-term clinical trials.

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All sample analysis and interpretation undertaken by Andrew T Hughes.

In preparation

10.1 Abstract

Background

The aetiology of AKU is well understood and the National AKU Centre in Liverpool have been treating all UK AKU patients as well as undertaking large-scale clinical trials investigating the effects of the drug nitisinone (NTBC) on patients. What was less understood was the stability of samples, reagents, inter- and intra-assay variables and their long-term implications.

Methods

Using validated and established in-house assays for the measurement of tyrosine (TYR) metabolites and nitisinone (NTBC), serum and urine samples were reanalysed to assess long-term stability.

Results

Both urine and serum metabolites trended a positive bias on reanalysis that was statistically significant, with no mean bias exceeding 10%. No obvious sample deterioration had occurred.

Conclusions

The positive bias indicates calibration variance as opposed to sample degradation when stored at -80^{o}C. Three sets of calibrators have been assigned during the sample analysis and storage period, strict laboratory protocols were adhered to so ‘natural’ variance is to be expected and although statistically speaking there is a difference, both a clinical and analytical knowledge determines there is no issue with the assay or protocols.

10.2 Introduction

Longitudinal analysis of serum, urine and other biological fluids is frequently encountered in clinical trials and confidence in trial outcome data is reliant on reproducible, credible and robust analytical processes within clinical and analytical laboratories. Laboratory uncertainty can be contributed to by pre-analytical, analytical and post-analytical issues, all of which should be assessed during method validation and verification.

Pre-analytical variability can encompass sample collection, processing, transport and storage which are even more relevant if samples are taken in multi-site locations and transported to a central analytical facility.

Analytical variability has many contributors including operator, room temperature, reagent and consumable variability, lot number changes, equipment maintenance and malfunction, but key to analytical performance is adequate and robust quality procedures to minimise and detect analytical variability.

Post-analytical procedures are the processes implemented to ensure quality procedures are followed; assay acceptance criteria are defined, result transcription and data reporting mechanisms are suitable, and analyses are performed in a timely manner. Key to all clinical laboratories is the quality management system which includes standard operating procedures, training records, competency assessments, monitoring of internal and external quality assurance schemes and equipment maintenance schedules. These process which are underpinned by ISO 15189, the standard for laboratory accreditation (United Kingdom Accreditation Service UKAS) ensures monitoring and improvement where required, of procedures and processes in all stages of sample analysis to ensure result authenticity.

We have previously undertaken development and validation of LC-MS/MS methods for analysis of serum and urine samples (Hughes et al., 2014, 2015) from both the National Alkaptonuria (AKU) Centre (NAC) (Milan et

al.,2017) and DevelopAKUre clinical trials – Suitability of Nitisinone in Alkaptonuria 1 and 2 (SONIA-1 and SONIA-2) (Ranganath et al., 2016, 2020a). SONIA-1 was a short-term, two-centre, multi-dose trial (Ranganath et al., 2016) and SONIA-2 a longitudinal study comparing patients on 10 mg daily dose of nitisinone (NTBC) against a control group, not on NTBC, over 4 years of follow-up (Ranganath et al., 2020a).

The methodology developed was bespoke for the NAC in that the urine assay quantitated urine tyrosine (u-TYR), phenylalanine (u-PHE) and homogentisic acid (u-HGA) in acidified urine. The serum assay additionally quantitated serum NTBC (s-NTBC). Further assay modifications added hydroxyphenyllactate (HPLA) and hydroxyphenylpyruvate (HPPA) in both urine and serum. During method validation, short-term stability was assessed and in addition the effect of pre-analytical acidification of urine, and protein precipitation of serum samples with perchloric acid (Hughes et al., 2014, 2015). The characteristic feature of patients with AKU is darkening of the urine upon standing or with alkalinisation which occurs as the HGA converts to benzoquinones via oxidation (Zannoni et al., 1969). For accurate analysis of u-HGA, this processes was halted by acidification of urine samples. Little was known about serum stability as prior to our method, s-HGA wasn't quantitated directly, either due to sample instability, lack of sensitive methodologies for quantitation or minimal interest in pathology (Davison et al., 2020). Characteristic of AKU is the mmol quantities of HGA excreted in the urine, however we demonstrated that the mean circulating s-HGA is an order of magnitude lower at approximately 30 µmol in AKU patients and <3.1 µmol/L in health (Davison et al., 2015; Milan et al., 2017).

In clinical trials, samples are often stored and analysed at time of completion, or analysed at pertinent time points, with trial outcomes summating data to reach conclusions. Key to data interrogation is knowledge on analyte and sample stability otherwise quantitative outcomes may be null and void if analytes degrade or assay performance isn't stable across the trial period. This study describes stability of serum and urine TYR, PHE, HGA, HPLA, HPPA and s-NTBC which not only provides data for long-term monitoring of

patients on NTBC at the NAC, but also the validity of trial data in SONIA-1 and SONIA-2 studies (Ranganath et al., 2016, 2020a) and any metabolic studies and disease monitoring where measurement of TYR and PHE is utilised, for example hereditary tyrosinaemia type I (HT-1).

10.3 Methods

10.3.1 Chemicals and materials

TYR, PHE, their respective isotope-labelled internal standards (IS) (d_4-TYR, d_5-PHE), HPLA, HPPA and HGA were obtained from Sigma-Aldrich UK. HGA isotope-labelled IS, $^{13}C_6$-HGA was obtained from Larodan Fine Chemicals (Sweden). NTBC was generously provided by Swedish Orphan Biovitrum AB. NTBC isotope-labelled IS, $^{13}C_6$-NTBC was obtained from Toronto Research Chemicals (Canada). LC-MS/MS grade methanol and acetonitrile were obtained from Sigma Aldrich, UK. Formic acid was obtained from Biosolve. Water was purified in-house by Elix Essential 5UV with Synergy UV Millipore water purification system. All dilutions and sample preparation were performed in glass. Oxygen-free nitrogen was supplied by a Genius 2010 Peak nitrogen generator.

10.3.2 Instrumentation and operating conditions

All analyses were performed on an Agilent 6490 Triple Quadrupole mass spectrometer with Jet-Stream® electrospray ionisation (ESI-MS/MS) coupled with an Agilent 1290 infinity UHPLC pump and 1290 multi-sampler. All data processing both qualitative and quantitative analysis was performed using Mass Hunter software package (Version B.06.00).

Chromatographic separation was achieved on an Atlantis C18 column (100 mm x 3.0 mm, 3 µm, Waters) maintained at 35°C. Initial conditions were 80:20 water:methanol with 0.1% formic acid (v/v) increasing linearly to 10:90 by 2.5 min. The mobile phase was maintained for 1.1 min, increasing to 100% organic for 1 min and then returned to starting conditions 80:20. The flow rate was maintained at 0.4 ml/min throughout the run. The column was reconditioned for 2 min prior to the next injection. 20 µl of sample was

injected with a total run-time of 7.0 min. Optimum operating ESI conditions were gas temperature set at 150°C, gas flow 17 L/min; nebulizer pressure 40 psi, sheath gas temperature 320°C and sheath gas flow 12 L/min. Capillary voltages were optimised to 3500V in positive mode and 2500V in negative mode with equal nozzle voltages (1500V) in both modes. The iFunnel parameters were optimised in both negative and positive mode as 60V for low pressure RF and 110V for high pressure RF.

10.3.3 Preparation of standards and internal standards

Standards were prepared as previously described (Hughes et al., 2014, 2015) with super-stock individual solutions added to matrix (either steroid depleted serum for the serum assay or acidified urine for the urine assay) to create final standards. NTBC was only included in serum calibration standards. Quality controls (QC) were prepared separately but in a similar mode.

Internal standards (IS) were prepared as previously described (Hughes et al., 2014, 2015) with the change from d_2-TYR to d_4-TYR and inclusion of d_5-PHE. The IS mix was used as the assay diluent with final concentrations for urine samples of 500 nmol/L d_4-TYR, 12.5 nmol/L d_5-PHE and 1 µmol/L $^{13}C_6$-HGA per 500 mL deionised water with 0.1% formic acid. The serum assay was adjusted for the lower concentrations and consisted of 250 nmol/L d_4-TYR, 12.5 nmol/L d_5-PHE, 100 nmol/L $^{13}C_6$-HGA and $^{13}C_6$-NTBC per 500 mL deionised water with 0.1% formic acid.

10.3.4 Sample preparation

Samples used for the stability evaluation have already been reported and therefore the patient consent and ethics are detailed in the SONIA-1 and SONIA-2 manuscripts (Ranganath et al., 2016, 2020a). All samples, calibrators and QC were diluted 1 in 1000 in IS diluent. All samples have been stored at -80^{o}C with no additional freeze-thaw post the initial analysis.

10.3.5 Statistics:

Correlation of results has been performed using excel and GraphPad Instat version 3. Repeat analysis has been compared using the paired, non-parametric, two tailed Wilcoxan matched pairs test. This test was selected as data is not normally distributed due to the spread of analytes pre- and post-NTBC treatment; however data is paired and changes can be either increase or decrease.

10.4 Results

Short-term stability and the effect of urine acidification has already been published (Hughes et al., 2014) and no significant difference was observed following three freeze-thaw cycles, room temperature and 4°C storage over a short time period.

10.4.1 Urine stability

Acidified urine samples analysed after 5 years storage at -80°C demonstrated minimal degradation. Urine TYR (range <20 to 2906 µmol/L) demonstrated an average bias of -1.1% (figure 10.1B) with an R^2=0.9793 (y=0.9239x+13.6, figure 10.1A). A Bland-Altman plot showed even distribution of bias across the range of urine TYR concentrations quantitated (figure 10.1B). Statistically the difference was signficant (p=0.0024).

Urine PHE (range <10 to 132 µmol/L) exhibited an average bias of -6.0% upon reanalysis (figure 10.1D) with an R^2 of 0.9672 (y=0.9994x-2.789, figure 10.1C). Bland-Altman of % bias demonstrates a concentration trend with negative bias at concentrations <40 µmol/L switching to a slight positive bias >40 µmol/L (Figure 10.1D). The difference was statistically signficant (p<0.0001).

Urine HGA concentrations ranged from low (<1000 µmol/L) in those on treatment to >20,000 µmol/L pre-NTBC treatment (untreated AKU patient) therefore correlation is demonstrated in figure 10.2A and 10.2C with an R^2=0.9889 at <1000 µmol/L (y=1.0698x+9.5898) and figures 10.2B and

10.2D at >1000 µmol/L (y=1.0721x-147.2, R^2=0.9916). Overall bias at concentrations <1000 µmol/L was + 6.03% (figure 10.2B) and at concentrations >1000 µmol/L was + 1.38% (figure 10.2D). Comparison of the change between each pair of data was deemed signficant (p=0.0011).

Urine HPLA comparison (figure 10.3A) demonstrated a correlation of R^2=0.9800 upon reanalysis (y-1.1639x – 32.737) with an average percentage bias of +7.1% (figure 10.3B). Statistical comparison of the initial and repeat analysis was significant (p<0.0001). Urine HPPA correlation (figure 10.3C) demosntrated a correlation of R^2=0.9625 (y=0.998x + 153.59). The percentage bias of reanalysis (figure 10.3D) demonstrated an overall average bias of + 0.6%. However as the figure displays there is a uniform positive and negative bias for u-HPPA which was not significant upon reanalysis.

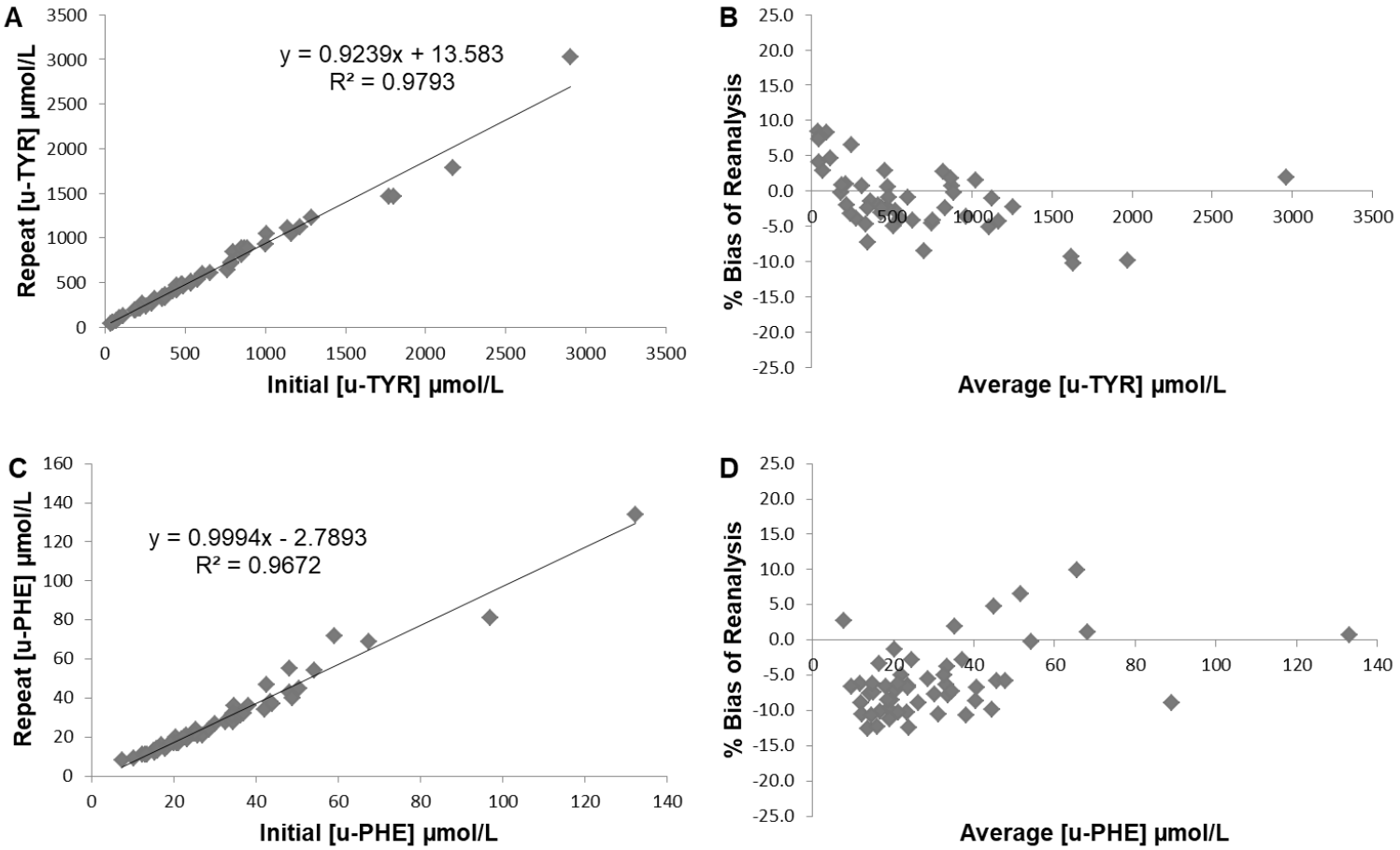

Figure 10.1: A and B urine TYR reanalysis of patient samples after 5 years storage at -80°C; Figure C and D urine PHE reanalysis of patient samples after 5 years storage at -80°C

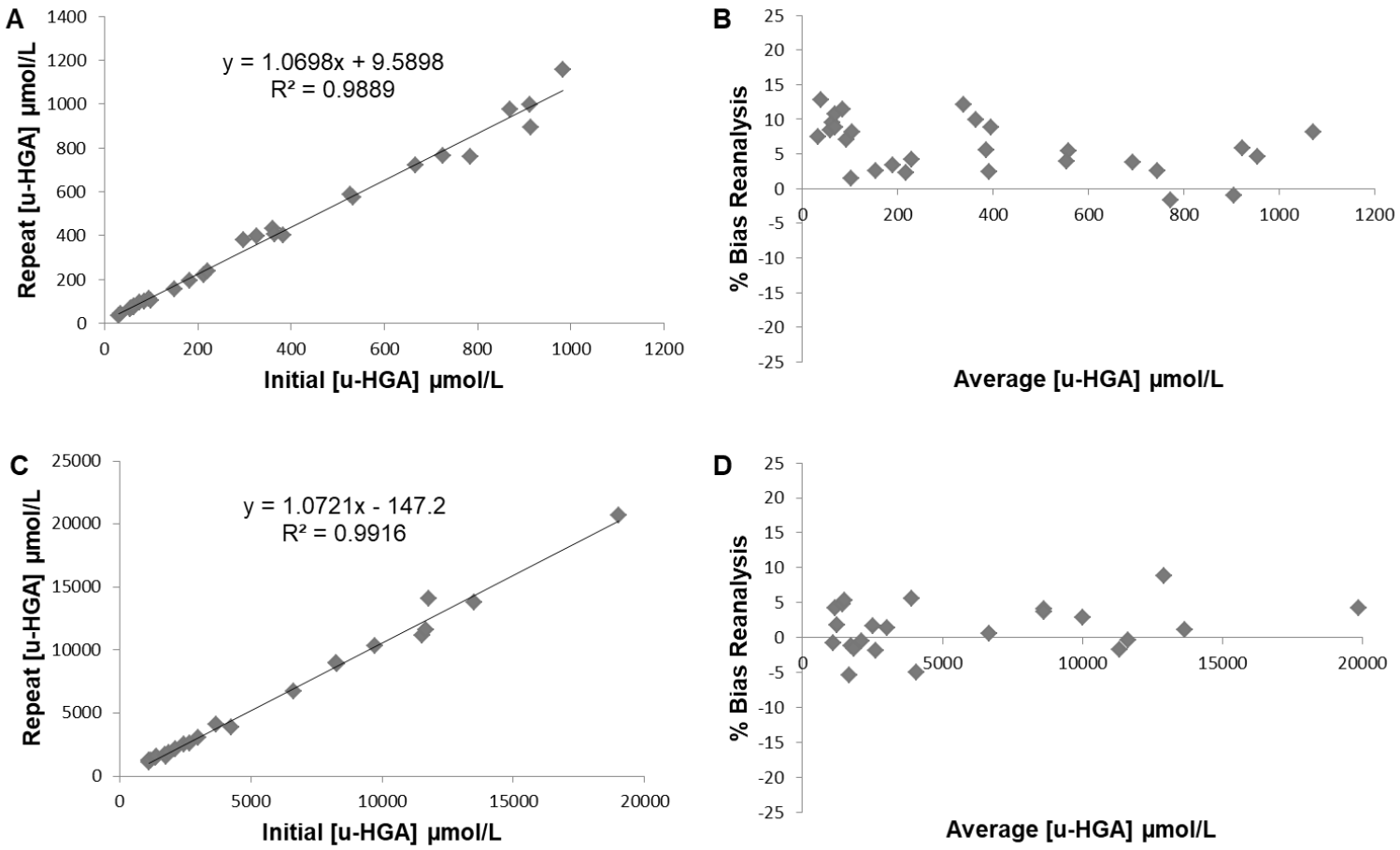

Figure 10.2: A and B urine HGA concentrations of NTBC treated patient samples over 5 years storage at -80°C; Figure C and D urine HGA concentrations of pre-NTBC treated patient samples over 5 years storage at -80°C.

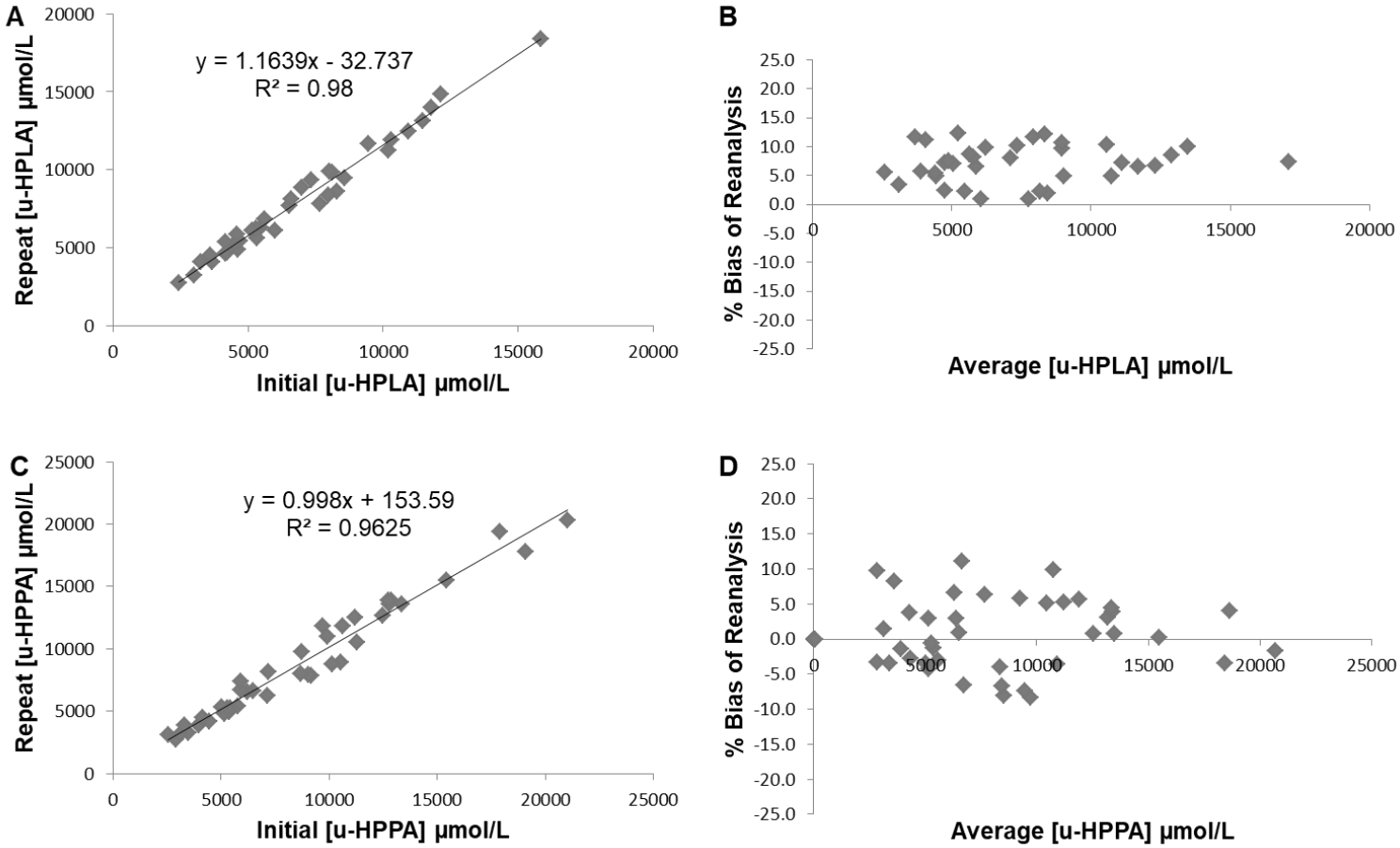

Figure 10.3: A and B urine HPLA reanalysis of patient samples after 5 years storage at -80°C; Figure C and D urine HPPA reanalysis of patient samples after 5 years storage at -80°C

10.4.2 Serum stability:

Short-term stability has previously been reported with no statistically significant difference following three freeze-thaw cycles, 24hr at room temperature and 24hr storage at 4^{o}C ($p>0.05$) (Hughes et al., 2015). All serum samples assessed during this long-term stability report were deproteinised (acidified) serum, as per standard sample protocol for AKU sample preparation.

Serum TYR demonstrated a positive bias upon reanalysis of 7.4% on average, with both pre-treatment values and post-NTBC treatment concentrations behaving similarly (figure 10.4A and 10.4B). Although a plot of absolute difference isn't shown, this change appears to be calibration related; and across the time window from the initial analysis to reanalysis, three different sets of calibration standards had been utilised. Verification of calibration standards were performed according to standard protocols, with calibration values assigned following n=20 analysis; then a subsequent sample comparison performed for verification. For serum TYR and serum PHE, there was a change in the calibration range once a more detailed clinical understanding of the metabolite concentration changes were obvious. In SONIA-2, the TYR concentrations were higher than seen in SONIA-1 as the NTBC dose used was higher (although no dose-dependent effect was seen, Ranganath et al., 2016).

Serum PHE demonstrated minimal change (figures 10.4C and 4D), and again a slight positive bias was determined upon reanalysis (average 7.2%). Serum HGA reanalysis is displayed in figures 10.5A and 10.5B. Initial analysis was undertaken blind, with no published range of s-HGA values expected in AKU patients, as raised u-HGA was the primary diagnostic tool in AKU. The calibration range was tailored after the initial analysis to enable focus on the 5-60 µmol/L range (initial calibration range was >100 µmol/L). Although there is an overall positive bias upon reanalysis, this ranges from +12% to -12% with an average of 3.2%.

Serum NTBC demonstrated an overall negative bias ($p<0.0001$) upon reanalysis (figures 10.5C and 10.5D). Overall there was a 5.3% negative bias, with a range from -13.7% to 14.5%. It should be noted that at the low concentrations of 0.3-1.0 a 10% negative bias is equivalent to very small changes numerically and clinically.

Serum HPLA comparison (figure 10.6A) revealed a good correlation ($R^2=0.9375$) with an average percentage positive bias of 6.1% (figure 10.6B). There is a trend to a positive bias at s-HPLA concentrations of <120 µmol/L ($p<0.0001$). Similarly s-HPPA (figure 10.6C) demonstrated a good correlation ($R^2=0.9323$) with an average bias (figure 10.6D) of -0.1% (not signficant, $p=0.3161$) with a range from -9.2 to +9.7%.

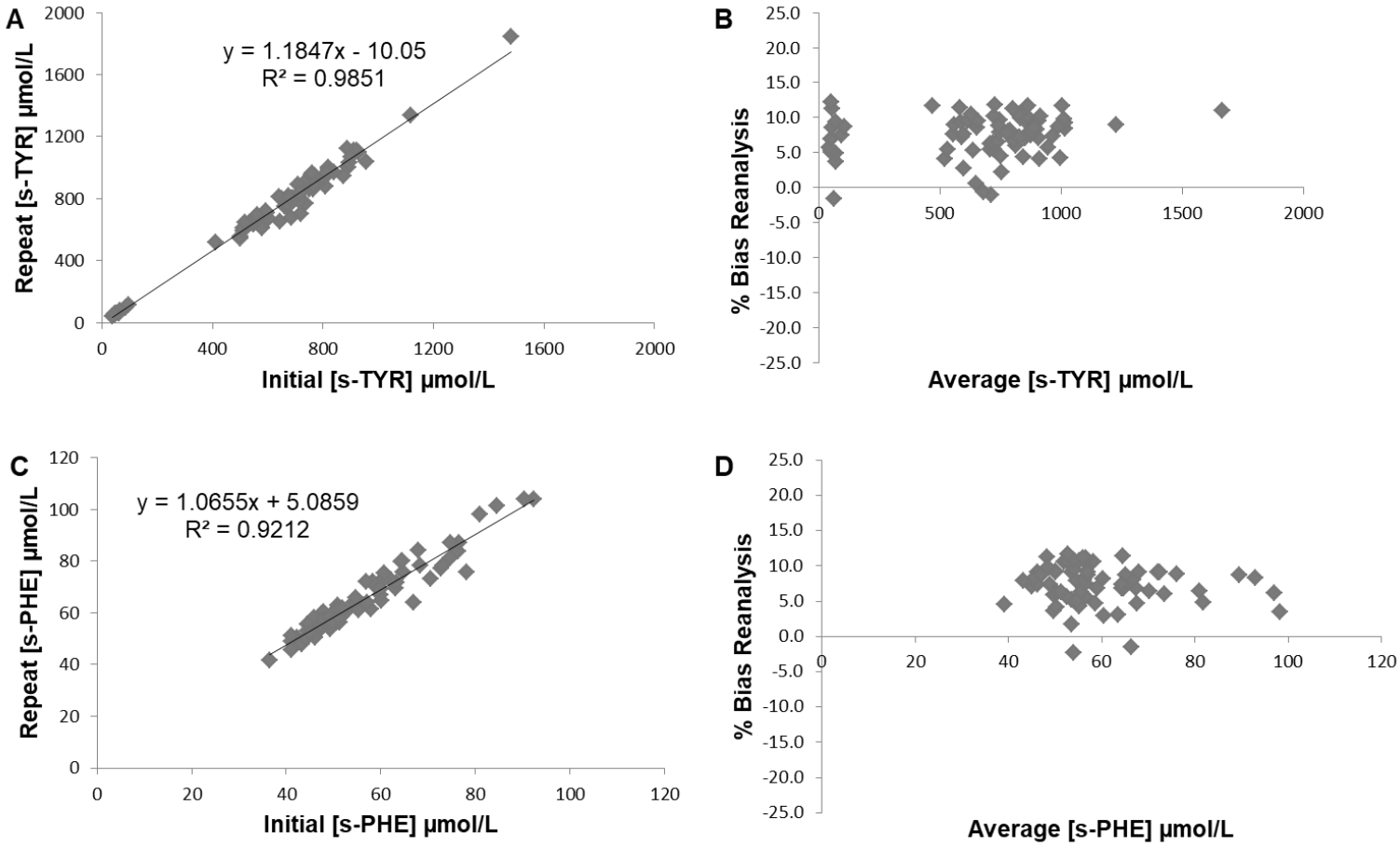

Figure 10.4: A and B s-TYR concentrations covering both pre-NTBC and treated patient samples over 5 years storage at -80°C. C and D s-PHE patient samples over 5 years storage at -80°C

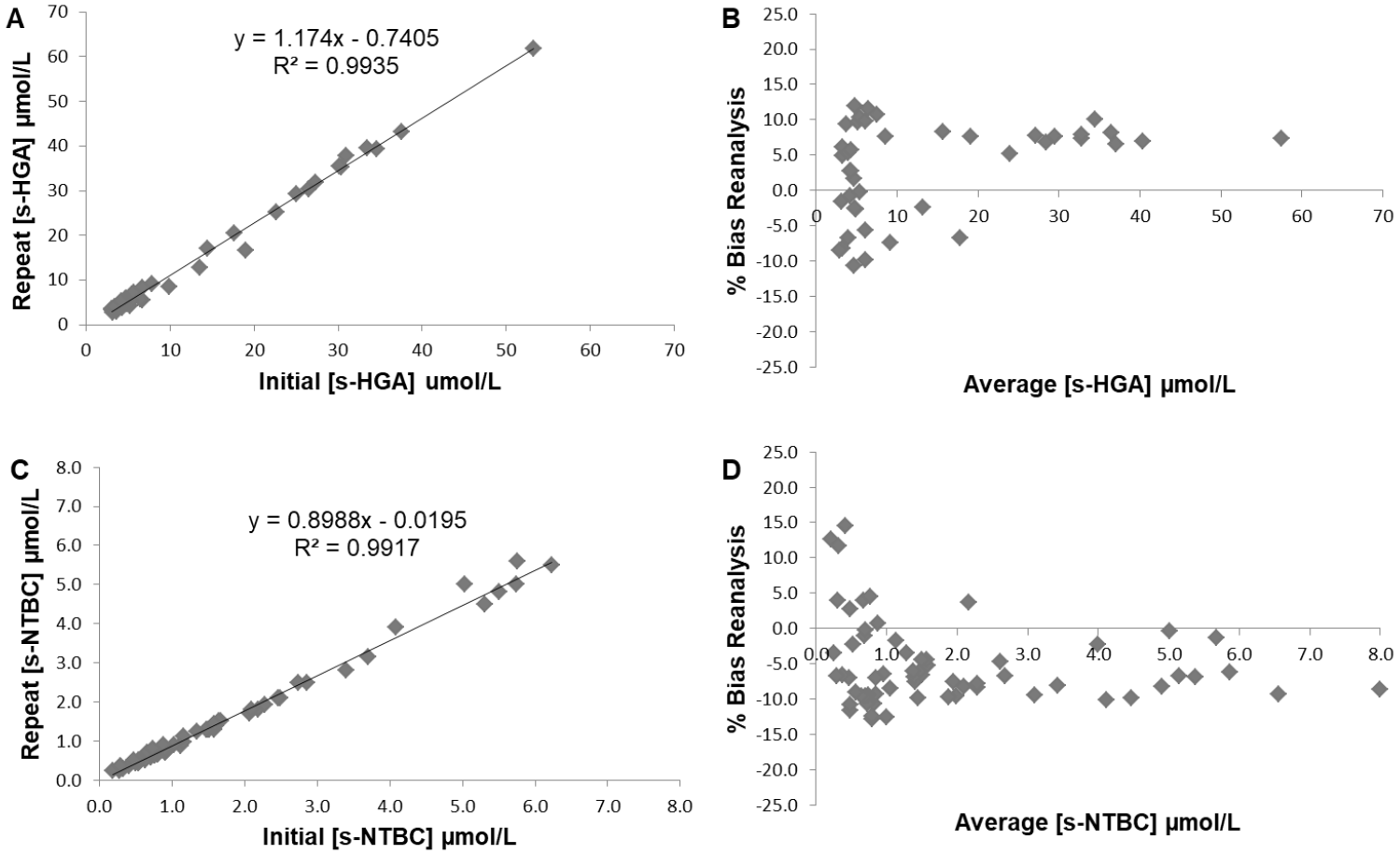

Figure 10.5: A and B s-HGA concentrations of pre-NTBC-treated patient samples over 5 years storage at -80°C. C and D s-NTBC in patient samples over 5 years storage at -80°C

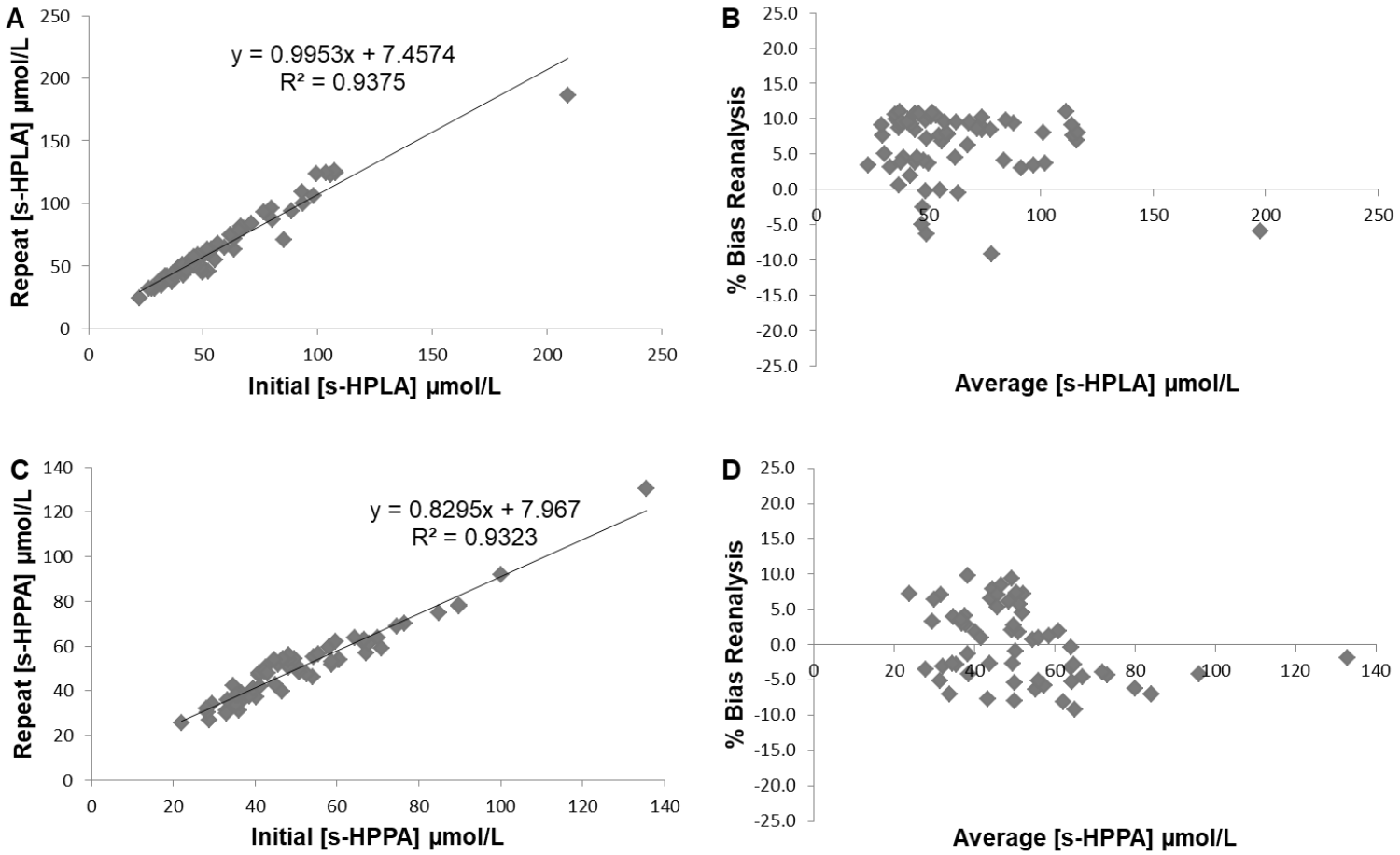

Figure 10.6: A and B s-HPLA concentrations of post-NTBC-treated patient samples over 5 years storage at -80°C. C and D s-HPPA reanalysis in patient samples after 5 years storage at -80°C

10.5 Discussion

Data described within this manuscript has demonstrated that although there are measurable differences in analyte concentrations upon reanalysis, they are not in large, down to sample degradation, but the postive bias for u-HGA, u-HPLA and u-HPPA is likely calibration driven. Similarly with serum analytes, s-TYR, s-PHE and s-HPLA which demonstrate an overall positive bias on reanalysis. Across the five year period, three sets of calibration standards were used which were modified and adjusted in scales once more concentration evidence was determined. Although the changes on reanalysis have been statistically deemed signficiant, careful review of the data is also encouraged. QCs were used in every analytical run, and to conform to trial protocols, they were included frequently (every ten samples or 10% of the run whichever was the greater number). The %CV and failure of QCs was monitored to ensure the run was valid. This was also adhered to during the reanalysis for continuity. The QC performance, calibration monitoring and assay performance gives us confidence that results reported across the duration of the clinical trials SONIA-1 and SONIA-2 were valid and robust and confirm the biomarker outcomes previously reported (Ranganath et al., 2016, 2020a). The key to ensuring a robust and scientifically sound outcome of any clinical trial not only depends on a thorough validation process but the assessment of long-term stability data.

Across the five years these samples were collected and stored, all pre-analytical conditions were regulated, including standardised sampling protocols and storage requirements; shipping frozen to our central laboratory for analysis and recorded storage temperatures for the duration of the trial. High quality samples ensure a high-quality outcome if all other procedures are followed. Delays in blood and plasma processing can have a large impact, seen routinely in clinical laboratories with ACTH, PTH, lactate, ammonia (Gifford et al., 2018) and at the initial stage of the assays, HGA was the major concern as there is minimal published data, and the reduction in u-HGA upon commencing NTBC, was a primary outcome of the clinical trial (Ranganath et al., 2016). In addition, long-term storage is not always assessed or reported and is becoming more essential for the increasing

interest in metabolomics where confidence in biological or pathological changes needs to be supported by robustness in sample integrity.

From an analytical stance, laboratory standard operating procedures (SOP's) and training competencies ensure staff performing analysis are consistent in their approach and that validation of analysis is robust. Analytical variables and bias of assay performance could be due to changes in calibration standards, operator dependency, temperature of the laboratory, change in column lot numbers, solvent and reagent purity and lot number variance, poor or major maintenance of the mass spectrometer and deterioration of calibration standards. All of these variables can be mitigated against and it is important that processes are in place to detect changes, i.e. QC and ion suppression monitoring, robust calibration procedures and training competencies along with highly competent analytical and clinical scientists who can accept or reject assays based on their expertise.

From a clinical laboratory perspective, participation in external quality assurance (EQA) schemes are important for monitoring long-term performance and comparative performance with other laboratories. However, not all analytes have such schemes and alternative methods of assurance are a requirement for assay accreditation under UKAS 15189, which may include sample exchange or sample re-assay to maintain batch to batch consistency.

For the purpose of the assays reported, serum TYR and PHE are part of the quantitative amino acid scheme (ERNDIM, European Research Network for evaluation and improvement of screening Diagnosis and treatment of Inherited disorders of Metabolism) and performance was within the schemes criteria for the duration of the clinical trials. Urine TYR and PHE were assayed on a sample exchange basis. However, no other laboratories in the UK measure quantitative HGA in serum and urine; and although serum NTBC is quantitated in patients with HT-1 they are of an order of magnitude higher and therefore quantitative comparison wasn't possible as it would require significant assay adjustment.

Assays dynamically change so commutability across time is essential and this study was performed using three refreshes of calibration standards due to the assigned 18 month shelf-life (prepared in-house, Hughes et al., 2014, 2015). Ranges were adapted with time as knowledge of metabolite changes were gained (Ranganath et al., 2016; Milan et al., 2017) without clinically affecting the trial outcomes.

Previous studies have identified that amino acids, some side chains and peptide bonds are susceptible to non-enzymatic hydrolysis. Haid et al., (2018) identified changes to amino acids particularly an increase in glutamine, arginine, glycine, phenylalanine and tryptophan. The positive bias in phenylalanine, although not clinically significant was also seen by Kamlage et al., (2018). Prolonged incubation (6hr versus 30 min) of blood at room temperature affected 24% of 225 serum metabolites tested with additional storage of 24hr resulting in statistically significant increases (14% analytes) and decreased (7%) of analytes tested. In particular those affected were amino acids with increases potentially due to continuous activity of proteases and peptidases.

Therefore all serum samples were crashed with perchloric acid in this study and frozen immediately, limiting any protease and peptidase activity. NTBC demonstrated a slight negative bias upon reanalysis. It exists in its molecular form at low pH whereas in higher pH solutions, the anionic form dominates; the existence of two tautomeric forms renders differing stability characteristics. NTBC is not stable in low pH solutions and a 30% decrease has been seen at 37^{o}C over 24hr (Dumas et al., 2017; Barchanska et al., 2019). This was also witnessed with storage at 4°C over one month (Prieto et al., 2011). However, the defined and prescriptive sample collection, separation and storage at -80°C prior to analysis and also over the five years of SONIA-2 ensured that all samples were and are treated equally, minimising changes due to pre-analytical conditions.

Chapter 11

General Discussion

11.1 Concluding discussion

This thesis has described the development and validation of several liquid chromatography mass spectrometry (LC-MS/MS) methods for the measurement of tyrosine (TYR), phenylalanine (PHE), homogentisic acid (HGA), hydroxyphenyllactate (HPLA), hydroxyphenylpyruvate (HPPA), nitisinone (NTBC) and creatinine. The methodology is novel, simple, robust and analytically sound. The LC-MS/MS methods have been developed with respective internal standards (IS) in both urine and serum/plasma matrices and the influence of the matrix determined in all cases. All in-house developed methods require constant updating, monitoring and stability checks to ensure that there is no drift or deterioration in calibration standard or quality control (QC) performance.

Before any of the above was established the main questions laid out before me was “What do I need to measure?” “Why?” and “How?”. This was going to be a challenge in that a lot of disorders already have established laboratory methods which may require modifying or improving. However, for AKU, there were none, in fact, apart from the drug NTBC it was not clear regarding what metabolites apart from HGA would be useful to analyse and at what concentration range to establish method parameters for, both pre- and post-treatment.

Researching the literature, there were methods from the early 1970’s onwards with varying degrees of complexity and run-times that could measure TYR, HGA and even NTBC (table 1.3). But there lay the problem, complexity and time, and in the case of NTBC, nowhere near the desired concentration. Further investigation into AKU and the TYR pathway suggested that I would most likely need to investigate other metabolites such as HPPA, HPLA and PHE.

With effectively a blank canvas in front of me, I set about deciding the “How”. To an analytical purist, that answer was straightforward. It could only be the ‘gold standard’ LC-MS/MS. With prior knowledge of the chemical structures for all our compounds of interest, I knew they would be ionisable either in the

positive or negative mode and therefore this instrumentation was the way forward. In conjunction with the proactivity of Agilent, our laboratory acquired a 6490 Triple Quadrupole LC/MS system with autosampler. The choice of LC-MS/MS played an integral part and after several discussions and field visits, Agilent was selected due to its superior ability to positive-negative switch during runs and its ease, but robustness for tuning and ion selection. The choice of column for separation again required an analytical knowledge coupled with experience within this field.

The next challenge was to determine if all the selected compounds with respective IS's could be solubilised, ionised, and measured in a mass spectrometer on the same chromatographic run in a 'routine-friendly' assay; a feat not achieved before. Previous methods (table 1.3) had all shown their complexities and so to achieve a successful clinical trial measuring thousands of samples, as well as maintaining a routine patient service; "complex" was not a pleasant or laboratory method-friendly word. As with all innovative and novel methods, especially if they are to be successful and 'routine' then the following considerations must always be put on the table:

1. Cost
2. Suitable equipment
3. Availability of reagents, including IS's
4. Assay complexity
5. Robustness of assay, i.e., accuracy, precision, stability, IQC and EQA
6. Recognised validation guidelines to be followed
7. Patient groups to determine reference and therapeutic ranges

Any one of the above points, if not adhered to with care and attention could have been a potential 'fly in the ointment' not only for this, but any method that required developing from scratch.

Once a method structure had been formulated adhering to the guidelines above, could the initial platform of machine and assay validation begin.

LC-MS/MS is the only internationally acceptable method for analysis of clinical trial samples and the only analytical procedure that could fulfil the objectives set out before us. Armed with a 6490 Agilent LC-MS/MS, international financial backing, an array of available and cost-effective reagents and a plan, could the initial painstaking validation work begin. Optimisation of the LC-MS/MS and assay validation has been covered extensively throughout this thesis, resulting in novel methods which after several more refinements were able to robustly quantitate all the major compounds in the TYR pathway associated with AKU (chapters 2, 3, 4 and 5).

This novel method developed has therefore provided the most comprehensive sets of data to be obtained on the TYR pathway, in AKU patients, and has not only enabled our group to successfully identify and monitor patients on NTBC at the National AKU Centre (NAC) in Liverpool, but to provide analytical capability for two large scale international clinical trials. SONIA-1 and SONIA-2 (Suitability of NTBC in AKU 1 and 2) samples from all of the participating trial sites were analysed at Liverpool using these methods and ultimately have provided the largest ever data set on metabolite changes in AKU patients on NTBC. This data has been key in supporting and obtaining licensing of NTBC for AKU patients, late 2020.

With these methods at our disposal, routine analysis of our NAC patients (who are administered 2 mg doses of NTBC; chapters 7 and 8) could begin as well as those initially for the SONIA-1 clinical trial (chapter 9), the success of which meant a progression from a dose-dependent study to a 4 year trial (SONIA-2) looking at the long-term effects of a 10 mg dose of NTBC on AKU patients both clinically and metabolically (Ranganath et al., 2020a).

Our paper on stability (publication pending, chapter 10) ensured there was not going to be a loss of efficacy or integrity with the method if we set an 18 month calibrator and QC shelf-life, especially as they are all in-house materials. For those compounds that were not able to be monitored via an EQA scheme (all but serum TYR and PHE) we analyse previous samples

and sample swap urine TYR and PHE as comparison data (with Alder Hey Hospital, Liverpool, every three months). All this, including a UKAS assessment ensured our in-house assays remain fit for purpose and fully accredited.

The SONIA-1 trial primary outcome was to determine whether there was a dose-dependency for NTBC in the successful depression of HGA levels in both urine and serum to a level seen in control individuals. This was a 4 week 'blind' trial using varying doses (0, 1, 2, 4 and 8 mg) of NTBC on AKU patients. Patient concentrations of HGA were successfully and increasingly reduced according to the elevating doses of NTBC administered. The information gathered from this initial trial enabled us to decide on a concentration of 10 mg to be administered to all the patients in the 4 year SONIA-2 trial.

In the meantime, our 'in-house' NAC patients continued on the lower dose of 2 mg for their long-term ongoing treatment. Additional data in chapter 8 post publication of the two-year data (Milan et al., 2017) has evidenced the longitudinal metabolite profile of AKU patients over 8 years which is the longest to date. The data has also confirmed the robustness of the LC-MS/MS methodologies with consistent biochemical data across the visits.

The sensitive LC-MS/MS assays and development of the ultra-low HGA assay (chapter 5) enabled studies into concentrations in health. A non-AKU individual will have a s-HGA concentration <0.1 µmol/L (Hughes et al., unpublished, chapter 5) and a u-HGA concentration <2.92 µmol/L (Davison et al., 2015) with s-TYR 30-87 µmol/L and u-TYR 14-147 µmol/24hr (Davison et al., 2015). Untreated AKU patients have s-HGA concentrations around 37 µmol/L and u-HGA 31,644 (± 5,533) µmol/24hr (Milan et al., 2017). The concentrations then start to significantly change once treatment begins depending on the dose of NTBC administered. This was particularly noticeable in the SONIA-1 dose-dependent clinical trial: s-HGA was suppressed to <3.1 µmol/L or non-detectable and u-HGA dropped to

155 ± 55 µmol/24hr at a dose of 8 mg NTBC. At this dose of NTBC, the consequent effects showed a steep rise in the metabolites u-HPLA (mean 13,853 µmol/24hr, u-HPPA (mean 16,623 µmol/24hr, u-TYR (mean 1,727 µmol/24hr) and s-TYR (mean 856 µmol/L) (Milan et al., 2019).

With the view that a higher NTBC dose in SONIA-2 would lead to most s-HGA concentrations being unmeasurable, the ultra-low s-HGA assay was developed with the view that 'normalisation' of s-HGA was ideal to reduce the detrimental effects of circulating HGA and to reduce or prevent further pigment formation. Data from SONIA-2 is not included within this thesis but was published in the Lancet (Ranganath et al., 2020a).

What is worth noting is the unexpected discrepancy in the total metabolite pools pre- and post-NTBC treatment (Milan et al, 2019). Making an assumption that 60% of the body is water within which matrix metabolites are distributed, there showed a larger total pool post-NTBC than pre-NTBC. A plausible explanation for the marked increase in TYR metabolite flux post-NTBC is that these metabolites were previously being directed down the HGA–benzoquinone pathway to form the ochronotic pigment, but with the NTBC block in place, then a surplus of TYR is circulating around in the bloodstream being unmetabolized or shunting off into other pathways.

As of writing, the drug NTBC has now been successfully licenced to treat AKU patients and due to the success of the SONIA trials, the NAC will be increasing the NTBC dose from 2 mg to 10 mg for all AKU patients which will effectively suppress HGA to the levels seen in SONIA-2. Neither feat would have been achievable without the robust methodologies I have developed and described within this thesis. More patients will now be able to receive NTBC and with further studies determining the optimal age to commence treatment, there is extra potential to reduce the damaging and debilitating effects of AKU on the body. As with most progress, these is always a caution, and that is the hypertyrosinaemia generated from blocking the pathway with NTBC. Future treatments and dietary modifications will target

this aspect to reduce any potential side-effects and monitoring of s-TYR is key to determining the effect of such interventions.

Future work will focus on near-patient or point of care testing for an initial diagnosis and could involve a urine dipstick, or an at-home test kit. In addition, s-TYR monitoring using at-home test kits will also feature. With identification of AKU patients, knowledge of the interference of HGA on routine clinical assays is essential from a laboratory education stance as the effect, particularly on the enzymatic creatinine assay is significant (chapter 6). HGA is not as stable, unless processed as described within this thesis however, a positive AKU urine sample is glaringly obvious, even after a week (Taylor et al., 2018). Once diagnosed, and the patient is beginning treatment, then regular point of care TYR finger prick samples can be taken and posted to the laboratory for analysis and continued monitoring. Future TYR modifying therapy outside of dietary intervention may need to focus on enzyme replacement therapy particularly when the NTBC dose for the NAC patients is increased. Development of a pill/capsule with a coated shell will be required or delivery by parenteral route, to evade the denaturing effects of stomach acid and in conjunction with NTBC will provide the ideal treatment.

Other work, particularly in the area of metabolomics which uses a time of flight (TOF) LC-MS/MS has the power to identify fold increases or decreases in biomarkers as a consequence of disorder status and treatment. This work involves significant ‘mining’ of data to identify any compounds that may play an obvious or subtle role in AKU. Although this can be useful, it is a semi-qualitative technique and translation into a quantitative approach will still need LC-MS/MS.

In conclusion, this thesis has been a direct patient-focused drive from several individuals. Robert Gregorys’ determination that he was not going to be “just another statistic” and the proactivity of the medical professor who he confided in Professor LR Ranganath. My part was to assemble from scratch and validate to strict analytical guidelines, robust, fit for purpose methods that could not only measure all the major metabolites associated with AKU in

both urine, plasma and serum but to be able to measure the drug NTBC and the metabolite profile pre- and post-treatment. The goals were to treat and monitor our NAC patients while international clinical trials were gathering sufficient data enabling NTBC to gain a licence for treating this debilitating disorder. All this information has enabled both analysts and clinicians alike to gain an invaluable knowledge of this disorder, not only referencing concentrations of the causitive compound, HGA, but a comprehensive compendium of the major metabolites post-NTBC treatment (HPPA and HPLA) as well as TYR, both in urine and serum.

I can honestly say, we have succeeded in our quest and may this be a catalyst for not only more inborn errors of metabolism but all diseases and disorders especially those that are overshadowed by the more frequent in occurrence and receive the attention and financial backing; as the ambition of the NHS was for “everyone to use it” and this research has fittingly demonstrated “every patient matters”.

Chapter 12

Bibliography

Ahmad S, Teckman JH, Lueder GT (2002)
Corneal opacities associated with NTBC treatment.
Am J Ophthalmol 134: 266-68.

Akbaba AI, Ozgül RK, Dursun A (2020)
Presentation of 14 alkaptonuria patients from Turkey.
J Pediatr Endocrinol Metab 33(2): 289-94

Akesson B, Forslin K, Wollheim F (1987)
Analysis of homogentisic acid in body fluids by high performance liquid chromatography.
J Chromatogr. 413: 233-36.

Akeda, K, Kasai, Y, Kawakita, E et al., (2008)
Thoracic Myelopathy with Alkaptonuria.
Spine 33(2): 62-5

Aktuglu-Zeybek AC, Zubarioglu T (2017)
Nitisinone: A review.
Orphan Drugs: Research and Reviews 7: 25-35.

Alajoulin OA, Alsbou MS, Ja'afreh SO et al., (2015)
Spontaneous Achilles tendon rupture in alkaptonuria.
Saudi Med J. 36(12): 1486-89

Albrecht, H (1902)
Ueber ochronose.
Z. Heil. path. Anat. 23: 366

Al-sbou M, Mwafi N (2012)
Nine cases of Alkaptonuria in one family in southern Jordan.
Rheumatol Int. 32: 621-25

Anikster Y, Nyhan WL, Gahl WA (1998)
Nitisinone and Alkaptonuria.
Am J Hum Genet. 63(3): 920-21

Annesley TM (2003).
Ion suppression in mass spectrometry.
Clin Chem. 49(7): 1041-44

Arnoux JP (2015)
Old treatments for new insights and strategies: proposed management in adults and children with alkaptonuria.
J Inherit Metab Dis. 38: 791-96

Ashorn M, Pitkanen S, Salo MK, et al., (2006)
Current strategies for the treatment of hereditary tyrosinaemia type 1.
Paediatr Drugs 8: 47-54

Baca E, Kural A, Ziroglu N et al., (2019)
Alkaptonuria: Spontaneous Achilles tendon rupture: Case report.
Joint Dis and Rel Surg. 30(3): 325-28

Barchanska H, Rola R, Szczepankiewicz W et al., (2019)
LC-MS/MS study of the degradation processes of nitisinone and its by-products.
Pharm Biomed Anal. 171: 15-21

Bartlett DC, Lloyd C, McKiernan PJ, et al. (2014)
Early nitisinone treatment reduces the need for liver transplantation in children with tyrosinaemia type 1 and improves post-transplant renal function.
J Inherit Metab Dis. 37(5): 745-52

Beadle GW and Tatum EL (1941)
Genetic Control of Biochemical Reactions in Neurospora.
Proc Natl Acad Sci. 15;27(11): 499-506

Bielenstein M, Astner L, Ekberg S (1999)
Determination of 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione in plasma by direct injection into a coupled column liquid chromatographic system.
J Chromatogr B Biomed Sci Apply. 730: 177-82

Bluefarb SM (1958)
Alkaptonuria and ochronosis.
Q Bull Northwest Univ Med Sch. 32(2): 101-5

Boedeker C (1859)
Ueber das Alcapton; ein neuer Beitrag zur Frage: Welche Stoffe des Hams kannen Kupferreduction bewirken?
Ztschr f rat Med. 7: 130

Bory C, Boulieu R, Chantin C et al., (1990)
Homogentisic acid determine in biological fluids by HPLC.
Clin Chem. 35(2): 321-22

Braconi D, Millucci L, Bernardini G et al., (2015)
Oxidative stress and mechanisms of ochronosis in alkaptonuria.
Free Radic Biol Med. 88:70-80

Buhrman, DL, Price PI, Rudewicz PJ (1996)
Quantitation of SR 27417 in Human Plasma Using Electrospray Liquid Chromatography-Tandem Mass Spectrometry:A Study of Ion Suppression.
J Am Soc Mass Spectrom. 7: 1099-1105

Bunch DR, El-Khoury JM, Gabler J et al., (2014)
Do deuterium labelled internal standards correct for matrix effects in LC-MS/MS assays? A case study using plasma free metanephrine and normetanephrine.
Clin Chem Acta. 729: 4-5

C62-A: Liquid chromatography-mass spectrometry methods:approved guideline.
Clin Lab Stds Inst. 34(16): 1-71

Cansever MS, Aktuglu-Zeybek AC, Erim FB (2010)
Determination of NTBC in serum samples from patients with hereditary tyrosinemia type 1 by capillary electrophoresis.
Talanta. 15: 1846-48

Capuano F, Angeloni E, Roscitano A et al., (2014)
Blackish Pigmentation of the Aorta in Patient with Alkaptonuria and Heyde's Syndrome.
Aorta 2(2): 74-6

Cox TF, Ranganath L (2011)
A quantitative assessment of alkaptonuria.
J Inherit Metab Dis. 34: 1153-62

Cox T, Psarelli EE, Taylor S et al., (2019)
Subclinical ochronosis features in alkaptonuria: a cross-sectional study.
BMJ Innov. 0:1-10

Crawhall JC, Mamer O, Tjoa S et al., (1971)
Urinary Phenolic Acids In Tyrosinemia. Identification And Quantitation By Gas Chromatography Mass Spectrometry.
Clin. Chim. Acta. 34: 47-54

Curtis SL, Roberts NB, Ranganath LR (2014)
Interferences of homogentisic acid (HGA) on routine clinical chemistry assays in serum and urine and the implications for biochemical monitoring of patients with alkaptonuria.
Clin Biochem. 47: 640-47

Curtis SL, Roberts NB, Milan AM et al., (2019)
Interference of hydroxyphenylpyruvic acid, hydroxyphenyllactic acid and tyrosine on routine serum and urine clinical chemistry assays; implications for biochemical monitoring of patients with alkaptonuria treated with nitisinone.
Clin Biochem. 71: 24-30

Daugherty NA, Hammond KB, Osberg IM (1978)
Bilirubin interference with the kinetic Jaffé method for serum creatinine.
Clin Chem. 24(2): 392-93

Davison AS, Milan AM and Dutton JJ (2013)
Potential problems with using deuterated internal standards for liquid chromatography-tandem mass spectrometry.
Ann Clin Biochem. 50: 274

Davison AS, Milan AM, Hughes AT et al., (2015)
Serum concentrations and urinary excretion of tyrosine and homogentisic acid in normal subjects.
Clin Chem Lab Med. 53: e81-e83

Davison AS, Milan AM, Gallagher JA, et al., (2016)
Acute fatal metabolic complications in alkaptonuria.
J Inherit Metab Dis. 39(2): 203-10

Davison AS, Hughes AT, Milan AM et al., (2020)
Alkaptonuria – Many questions answered, further challenges beckon.
Ann Clin Biochem. 57(2): 106-20

Davit-Spraul A, Romdhane H, Poggi-Bach J. (2012)
Simple and fast quantification of nitisinone (NTBC) using liquid chromatography tandem mass spectrometry method in plasma of tyrosinemia type 1 patients.
J Chromatogr Sci. 50: 446-49

Dawson, W. R. (1929)
A Note on the Egyptian Mummies in the Castle Museum, Norwich.
J Egyp Archaeol. 15(3/4): 186-90

Delanaye P, Cavalier E, Pottel H (2017)
Serum Creatinine: Not So Simple!
Nephron 136: 302-8

Delanghe JR, Speeckaert MM (2011)
Creatinine determination according to Jaffe—what does it stand for?
NDT Plus 4: 83-6

Deutsch JC, Santhosh-Kumar CR. (1996)
Quantitation of homogentisic acid in normal human plasma.
J Chromatogr B, Biomed Appl. 677: 147-51

Deutsch JC (1997)
Determination of p-hydroxyphenylpyruvate, p-hydroxyphenyllactate and tyrosine in normal human plasma by gas chromatography-mass spectrometry isotope-dilution assay.
J of Chromatog. B 690: 1-6

Dumas E, Giraudo M, Goujon E et al., (2017)
Fate and ecotoxicology impact of new generation herbicides from the triketone family: An overview to assess the environmental risks.
J Hazard Mater. 325: 136-56

Ellis MK, Whitfield AC, Gownas LA, et al., (1995)
Inhibition of 4-hydroxyphenylypyruvate dioxygenase by 2-(2-nitro-4-trifluoromethylbenzoyl)-cyclohexane-1,3-dione and 2-(2-chloro-4-methanesulfonylbenzoyl)-cyclohexane-1,3-dione.
Toxicol Appl Pharmacol 133:12-19

EMA Guidelines (2011). https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-bioanalytical-method-validation_en.pdf

FDA Bioanalytical Method Validation
https://www.fda.gov/media/70858/download

Fernandez-Canon JM, Granadino B, De Bernabe DB et al., (1996)
The molecular basis of alkaptonuria.
Nat Gen. 14:19–24

Fernandez-Canon JM, Penalva MA. (1997)
Spectrophotometic determination of homogentisate using Aspergillus nidulans homogentisate dioxygenase.
Anal Biochem. 245(2): 218-21

Felitsyn NM, Henderson GN, James MO et al., (2004)
Liquid chromatography-tandem mass spectrometry method for the simulataneous determination of δ-ALA, tyrosine and Creatinine in biological fluids.
Clin Chim Acta. 350(1-2): 219-30

Gallagher JA, Ranganath LR, Zatkova A. (2013)
Alkaptonuria. Chapter in Brenner's Encyclopaedia of Genetics, Academic Press, San Diego: 2nd Edition. p71-5

Gallagher JA, Dillon JP, Sireau N, et al., (2016)
Alkaptonuria: An example of a "fundamental disease" – A rare disease with important lessons for more common disorders.
Semin Cell Dev Biol. 52: 53-7

Garrod AE. (1908)
The Croonian lectures on inborn erros of metabolism. Lecture II. Alkpatonuria.
The Lancet 2(1): 73-9

Garrod AE (2002)
The incidence of alkaptonuria: a study in chemical individuality.
1902 [classical article].
Yale J Biol Med. 75: 221-31

Gifford JL, Nguyen WNT, de Koning L et al., (2018)
Stabilising specimens for routine ammonia testing in the clinical laboratory.
Clin Chem Acta. 478: 37-43

Gissen P, Preece MA, Willshaw HA et al., (2003)
Ophthalmic follow-up of patients with tyrosinaemia type I on NTBC.
J Inherit Metab Dis. 26: 13-16

Goicoechea De Jorge E, Lorda I, Gallardo ME et al., (2002)
Alkaptonuria in the Dominican Republic: identification of the founder AKU mutation and further evidence of mutation hot spots in the HGO gene.
J Med Genet. 39: E40

Haid M, Muschet C, Wahl S et al., (2018)
Long-Term Stability of Human Plasma Metabolites during Storage at -80 °C.
J Proteome Res. 17: 203-11

Helliwell TR, Gallagher JA, Ranganath L (2008)
Alkaptonuria--a review of surgical and autopsy pathology.
Histopath. 53: 503-12

Herebian D, Spiekerkotter U, Lamshoft M, et al. (2009)
Liquid chromatography tandem mass spectrometry method for the quantitation of NTBC 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione in plasma of tyrosinemia type 1 patients.
J Chromatogr B Analyt Technol Biomed Life Sci. 15: 1453-59

Hiroyoshi J, Saito A, Panthee N et al., (2013)
Aortic valve replacement for aortic stenosis caused by alkaptonuria.
Ann Thorac Surg. 95(3): 1076-79

Holme E, Lindstedt S. (1998)
Tyrosinaemia type I and NTBC (2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione).
J Inherit Metab Dis. 21:507-17

Holme E, Lindstedt S (2000)
Non-transplant treatment of tyrosinemia.
Clinics in Liver Disease 4 (4): 805-14

Honour JW. (2011)
Development and validation of a quantitative assay based on tandem mass spectrometry.
Clin Biochem. 48: 97-111

Hoste L, Deiteren K, Pottel H et al., (2015)
Routine serum creatinine measurements: how well do we perform?
BMC Nephr. 16(21): 1-9

Hsu WY, Chen CM, Tsai FJ, et al., (2013)
Simultaneous detection of diagnostic biomarkers of alkaptonuria, ornithine carbamoyltransferase deficiency and neuroblastoma disease by high-performance liquid chromatography/tandem mass spectrometry.
Clin Chim Acta. 420: 140-45

Hughes AT, Milan AM, Christensen P et al., (2014)
Urine homogentisic acid and tyrosine: simultaneous analysis by liquid chromatography tandem mass spectrometry.
J Chromatogr B Analyt Technol Biomed Life Sci. 963: 106-12

Hughes AT, Milan AM, Davison AS et al., (2015)
Serum markers in alkaptonuria: simultaneous analysis of homogentisic acid, tyrosine and nitisinone by liquid chromatography tandem mass spectrometry.
Ann Clin Biochem. 52(5): 597-605

Hughes JH, Wilson PJM, Sutherland H et al., (2020)
Dietary restriction of tyrosine and phenylalanine lowers tyrosinaemia associated with nitisinone therapy of alkaptonuria.
J Inherit Metab Dis. 43(2): 259-68

Huskova R, Chrastina P, Adam T et al., (2004)
Determination of creatinine in urine by tandem mass spectrometry.
Clinica Chimica Acta. 350: 99-106

Introne WJ, Phornphutkul C, Bernardini I et al., (2002)
Exacerbation of the ochronosis of alkaptonuria due to renal insufficiency and improvement after renal transplantation.
Mol Genet Metab. 77: 136-42

Introne WJ, Perry MB, Troendle J et al., (2011)
A 3-year randomized therapeutic trial of nitisinone in Alkaptonuria.
Mol Genet Metab. 103: 307-14

Introne WJ, Gahl WA (2003, updated 2016)
Alkaptonuria.
Gene Reviews 1-14

Jacomelli G, Micheli V, Bernardini G, et al., (2016)
Quick Diagnosis of Alkaptonuria by Homogentisic Acid Determination in Urine Paper Spots.
JIMD Rep. 31: 51-6

Jaffe M (1886)
Ueber den Niederschlag, welchen Pikrinsäure in normalem Harnerzeugt und Über eine neue Reaction des Kreatinins.
Z Physiol Chem 10: 391-400

Jebaraj I, Rao A (2006)
Achilles tendon enthesopathy in ochronosis.
J Postgrad Med. 52: 47-8

Kamlage B, Neuber S, Bethan B et al., (2018)
Impact of prolonged blood incubation and extended serum storage at room temperature on the human serum metabolome.
Metabolites 8: 6

Kavana M, Moran GR (2003)
Interaction of (4-hydroxyphenyl)pyruvate dioxygenase with the specific inhibitor 2-(2-nitro-4-(trifluoromethyly)benzoyl]-1,3-cyclohexanedione.
Biochemistry 42: 10238-45

Keenan CM, Preston AJ, Sutherland H et al., (2015)
Nitisinone Arrests but Does Not Reverse Ochronosis in Alkaptonuric mice.
JIMD Reports 24: 45-50

Keller JM, Macaular W, Nercessian OA et al., (2005)
New developments in ochronosis: review of the literature.
Rheumatol Int. 25(2): 81-5

Khedr M, Judd S, Briggs MC et al., (2018)
Asymptomatic Corneal Keratopathy Secondary to Hypertyrosinaemia Following Low Dose Nitisinone and a Literature Review of Tyrosine Keratopathy in Alkaptonuria.
JIMD Reports 40: 31-7

Kisa PT, Gunduz M, Dorum S et al., (2021)
Alkaptonuria in Turkey: Clinical and molecular characteristics of 66 patients.
Eur J Med Genet. 64: 104197

La Du BN, Zannoni VG, Laster L et al., (1958)
The nature of the defect in tyrosine metabolism in alkaptonuria.
J Biol Chem. 230(1): 251-60

La Marca G, Malvagia S, Materazzi S, et al., (2012)
LC-MS/MS method for simultaneous determination on a dried blood spot of multiple analytes relevant for treatment monitoring in patients with tyrosinemia type 1.
Anal Chem. 84: 1184-88

Lee SL and Stenn FF (1978)
Characterization of mummy bone ochronotic pigment.
JAMA 240(2): 136-38

Lindstedt S, Holme E, Lock EA et al., (1992)
Treatment of hereditary tyrosinaemia type I by inhibition of 4-hydroxyphenyl pyruvate dioxygenase.
The Lancet 340: 813-17

Lock EA, Ellis MK, Gaskin P et al., (1998)
From toxicological problem to therapeutic use: the discovery of the mode of action of 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC), its toxicology and development as a drug.
J Inherit Metab Dis. 21(5): 498-506

Lock E, Ranganath LR, Timmis O. (2014)
The role of nitisinone in tyrosine pathway disorders.
Curr Rheumatol Rep. 16: 457-64

Lock EA (2017)
From Weed Killer to Wonder Drug.
Adv Exp Med Biol. 959: 175-85

Loken PR, Magera MJ, Introne W et al., (2010)
Homogentisic acid interference in routine urine creatinine determination.
Mol Gen and Metab. 100: 103-4

Lusitanus (1649)
Praxis Medica Admiranda.
Lib, III, cap. 134: 4

Lustberg J, Schulman JD, Seegmiller JE. (1971)
The preparation and identification of various adducts of oxidized homogentisic acid and the development of a new sensitive colorimetric assay for homogentisic acid.
Clin Chim Acta. 35: 325-32

Lynch JJ, Van Vleet TR, Mittelstadt SW et al., (2017)
Potential functional and pathological side effects related to off-target pharmacological activity.
J Pharmacol Toxicol Methods 87: 108-26

Lynch KL (2016)
CLSI C62-A: A New Standard for Clinical Mass Spectrometry
Clin Chem. 62(1): 24-9

Marcet AJC (1822)
Account of a singular variety of urine, which turned black soon after being discharged; with some particulars respecting its chemical properties.
Roy Med Chir Soc Glasgow 12: 37-43

Markus AA, Patrick J, Swinkels DW, et al., (2001)
New technique for diagnosis and monitoring of alcaptonuria: quantification of homogentisic acid in urine with mid-infrared spectrometry.
Anal Chim Acta. 429: 287-92

Martin J, Batkoff B (1987)
Homogentisic acid autoxidation and oxygen radial generation: implications for the etiology of alkaptonuric arthritis.
Free Radic Biol Med. 3(4): 241-50

McKiernan PJ. (2006)
Nitisinone in the treatment of hereditary tyrosinaemia type 1.
Drugs 66: 743-50

McKiernan PJ (2013)
Nitisinone for the treatment of hereditary tyrosinemia type I.
Expert Opin Orphan Drugs. 1: 491-97

Michaeley WJ, Kratz GW. (1986)
Certain 2-(2-substituted benzyl)-1,3-cyclohexanediones.
European Patent Application 0135191

Milan AM, Hughes AT, Davison AS et al., (2017)
The effect of nitisinone on homogentisic acid and tyrosine: A 2-year survey of patients attending the National Alkaptonuria Centre, Liverpool.
Ann Clin Biochem. 54: 323-30

Milan AM, Hughes AT, Davison AS et al., (2019)
Quantification of the flux of tyrosine pathway metabolites during nitisinone treatment of Alkaptonuria.
Sci Rep. 11;9(1): 100-24

Milch RA (1960)
Studies of alcaptonuria: inheritance of 47 cases in eight highly inter-related Dominican kindreds.
Am J Hum Genet. 12: 76-85

Mistry JB, Bukhari M and Taylor AM (2013)
Alkaptonuria.
Rare Diseases 1(1): 1-7

Montagutelli X, Lalouette A, Coudé M, et al., (1994)
AKU, a mutation of the mouse homologous to human alkaptonuria, maps to chromosome 16.
Genomics 19: 9-11

Mueller MN, Sorensen LB, Stranddjord N et al., (1965)
Alkaptonuria and Ochronotic Arthropathy.
Med Clin of N Am. 49(1): 101-15

Murray KK, Boyd RK, Eberlin MN et al., (2013)
Definitions of terms relating to mass spectrometry (IUPAC recommendations 2013).
Pure Appl Chem. 85: 1515-1609

Myers GL, Miller WG, Coresh J et al., (2006)
Recommendations for Improving Serum Creatinine Measurement: A Report from the Laboratory Working Group of the National Kidney Disease Education Program
Clin Chem 52:15-18

Nakahara T, Ishida J, Yamaguchi M et al., (1990)
Determination of a-Keto Acids Including Phenylpyruvic Acid in Human Plasma by High-Performance Liquid Chromatography with Chemiluminescence Detection.
Analytic. Biochem. 190: 309-13

Nemethova M, Radvanszky J, Kadasi L, et al. (2016)
Twelve novel HGD gene variants identified in 99 alkaptonuria patients: focus on 'black bone disease' in Italy.
Eur J Hum Genet. 24: 66-72

O'Brien WM, La Du NB and Bunim JJ (1963)
Biochemical, pathological and clinical aspects of alkaptonuria, ochronosis and ochronotic arthropathy.
Am J Med. 34: 813-38

Olsson B, Cox TF, Psarelli EE et al., (2015)
Relationship Between Serum Concentrations of Nitisinone and Its Effect on Homogentisic Acid and Tyrosine in Patients with Alkaptonuria.
JIMD Rep. 24: 21-7

Osler W (1904)
Ochronosis: The pigmentation of cartilages, sclerotic and skin in alkaptonuria.
The Lancet 1-8

Owen LJ, Wear JE, Keevil BG (2006)
Validation of a liquid chromatography tandem mass spectrometry assay for serum creatinine and comparison with enzymatic and Jaffe methods.
Ann Clin Biochem. 43: 118-23

Owen LJ, Keevil B (2012)
Testosterone measurement by liquid chromatography tandem mass spectrometry: the importance of internal standard choice.
Ann Clin Biochem. 49: 600-2

Oztekin N, Balta GS, Cansever MŞ. (2018)
Determination of homogentisic acid in urine for diagnosis of alcaptonuria: Capillary electrophoretic method optimization using experimental design.
Biomed Chromatogr. 32: e4216

Pauwels S, Cassiman D, Vermeersch P (2012)
Evaluation of the interference by homogentisic acid and other organic acids on the enzymatic and Jaffé method creatinine assay.
Clin Chem Lab Med. 50(4): 749-50

Peake M, Whiting M (2006)
Measurement of Serum Creatinine – Current Status and Future Goals.
Clin Biochem Rev. 27: 173-84

Pettit SJ, Fisher M, Gallagher JA et al., (2011)
Cardiovascular manifestations of Alkaptonuria.
J Inherit Metab Dis. 34: 1177-81

Phocas E, Andriotakis C, Matsas B et al., (1963)
Alkaptonuria and ochronotic arthritis.
Clin Radiol. 14(2): 175-77

Phornphutkul C, Introne WJ, Perry MB et al., (2002)
Natural History of Alkaptonuria.
N Eng J Med. 347: 2111-21

Preston AJ, Keenan CM, Sutherland H et al., (2014)
Ochronotic osteoarthropathy in a mouse model of alkaptonuria, and its inhibition by nitisinone.
Ann Rheum Dis. 73: 284-89

Prieto JA, Andrade F, Lage S et al., (2011)
Comparison of plasma and dry blood spots as samples for the determination of nitisinone (NTBC) by high-performance liquid chromatography-tandem mass spectrometry. Study of the stability of the samples at different temperatures.
J Chromatogr B. 879: 671-76

Ranganath LR, Cox TF (2011)
Natural history of alkaptonuria revisited: analyses based on scoring systems.
J Inherit Metab Dis. 34: 1141-51

Ranganath LR, Jarvis JC, Gallagher JA (2013)
Recent advances in management of alkaptonuria.
J Clin Pathol. 66: 367-73

Ranganath LR, EE Psarelli, TF Cox, et al., (2015)
Diagnostic tools and strategies for assessing disease progression in Alkaptonuria.
Expert Opinion on Orphan Drugs 3(6): 705-17

Ranganath LR, Milan AM, Hughes AT et al. (2016)
Suitability Of Nitisinone In Alkaptonuria-1 (SONIA-1): an international, multicentre, randomised, open-label, no-treatment controlled, parallel-group, dose-response study to investigate the effect of once daily nitisinone on 24-h urinary homogentisic acid excretion in patients with alkaptonuria after 4 weeks of treatment.
Ann Rheum Dis. 75(2): 362-67

Ranganath LR, Khedr M, Milan AM et al., (2018)
Nitisinone arrests ochronosis and decreases rate of progression of Alkaptonuria: Evaluation of the effect of nitisinone in the United Kingdom National Alkaptonuria Centre.
Mol Genet Metab. 125: 127-34

Ranganath LR, Psarelli EE, Arnoux JB et al., (2020a)
Efficacy and safety of once-daily nitisinone for patients with alkaptonuria (SONIA 2): an international, multicentre, open-label, randomised controlled trial.
The Lancet; diabetes-endocrinology 8: 762-72

Ranganath LR, Milan AM, Hughes AT, et al., (2020b)
Homogentisic acid is not only eliminated by glomerular filtration and tubular secretion but also produced in the kidney in alkaptonuria.
J Inherit Metab Dis. 43(4): 737-47

Roberts NB, Curtis SA, Milan AM, et al., (2015)
The pigment in alkaptonuria relationship to melanin and other coloured substances: A review of metabolism, composition and chemical analysis.
JIMD Rep. 24: 51-66

Russo PA, Mitchell GA, Tanguay RM (2001)
Tyrosinemia: A Review.
Pediatric and Developmental Pathology 4: 212-21

Sacks S. (1951)
Alkaptonuric arthritis; report of a case.
J Bone Joint Surg Br. 33: 407-14

Sakthivel S, Zatkova A, Nemethova M, et al. (2014)
Mutation screening of the HGD gene identifies a novel alkaptonuria mutation with significant founder effect and high prevalence.
Ann Hum Genet. 78: 155-64

Salazar JH (2014)
Overview of Urea and Creatinine.
Lab Med. 45(1): 19-20

Sass-Kortsak A, Ficici S, Paunier L et al. (1967)
Secondary Metabolic Derangements in Patients with Tyrosyluria.
Canad. Med. Ass. J. 97: 1079-83
Scribonius GA (1584)
De Inspectione Urinarum.
Lemgo, Germany, p.50. 2.

Schenck (1609)
Urine nigra in sanis quibusdam. Observationes Medicae.
Lib. III. Frankfort, p.558. 3.

Sealock RR, Galdston M, Steele JM (1940)
Administration of Ascorbic Acid to an Alkaptonuric Patient.
Exp Biol Med. 44: 580-83

Seegmiller JE, Zannoni VG, Laster BN et al., (1961)
An enzymatic spectrophotometric method for the determination of homogentisic acid in plasma and urine.
J. Biol Chem. 236: 774-77

Sharma A, Alvarez A, Nazif T (2021)
Tale of a Black Heart!
Ann of Thor Surg. doi: doi.org/10.1016/j.athoracsur.2021.05.063

Simon G, Zorab PA (1960)
The radiographic changes in alkaptonuric arthritis: A report on three cases (one an egyptian mummy).
Brit. J. Radiol. 34: 384-86

Sloboda N, Wiedemann A, Mertenb M et al., (2019)
Efficacy of low dose nitisinone in the management of alkaptonuria.
Mol Genet Metab. 127: 184-90

Spaapen LJM, Ketting D, Wadman SK et al., (1987)
Urinary D-4-Hydroxyphenyllactate, D-Phenyllactate and D-2-Hydroxyisocaproate, Abnormalities of Bacterial Origin.
J. Inher. Metab. Dis. 10: 383-90

Spiekerkoetter U, Couce ML, Das AM, et al., (2021)
Long-term safety and outcomes in hereditary tyrosinaemia type 1 with nitisinone treatment: a 15-year non-interventional, multicentre study.
The Lancet Diabetes Endocrinol. 9: 427-35

Srsen S, Müller CR, Fregin A et al., (2002)
Alkaptonuria in Slovakia: thirty-two years of research on phenotype and genotype.
Mol Genet Metab. 75: 353-59

Stenn FF, Milgram JW, Lee SL, Weigand RJ (1977)
Biochemical identification of homogentisic acid pigment in an ochronotic egyptian mummy.
Veis A. Science. 197(4303): 566-68

Stenn FF, Milgram JW, Lee SL et al., (1979)
Biochemical Discovery of Homogentisic Acid Pigment in an Ochronotic Egyptian Mummy.
Henry Ford Hosp Med Journal. 27(1): 44-9

Stewart RM, Briggs MC, Jarvis JC, et al. (2014)
Reversible keratopathy due to hypertyrosinaemia following intermittent low-dose nitisinone in alkaptonuria: A case report.
JIMD Rep. 17: 1-6

Suwannarat P, O'Brien K, Perry MB et al., (2005)
Use of nitisinone in patients with alkaptonuria.
Metabolism 54(6): 719-28

Taylor AM, Preston AJ, Paulk NK, et al., (2012)
Ochronosis in a murine model of alkaptonuria is synonymous to that in the human condition.
Osteoarthritis Cartilage 20: 880-86

Taylor JM, Hughes AT, Milan AM et al., (2018)
Evaluation of the Mitra microsampling device for use with key urinary metabolites in patients with Alkaptonuria.
Bioanalysis 10: 1919-32

Thakur S, Markman P, Cullen H. (2013)
Choice of valve prosthesis in a rare clinical condition: aortic stenosis due to alkaptonuria.
Hear Lung Circ. 22(10): 870-72

Tinti L, Taylor AM, Santucci A, et al., (2011)
Development of an in vitro model to investigate joint ochronosis in alkaptonuria.
Rheumatology 50: 271-77

Titus GP, Mueller HA, Burgner J et al., (2000)
Crystal structure of human homogentisate dioxygenase.
Nat Struct Biol. 7: 542-46

van Deemter JJ, Zuiderweg FJ, Klinkenberg A (1956)
Longitudinal diffusion and resistance to mass transfer as causes of non ideality in chromatography.
Chem. Eng. Sci. 5: 271-89

Virchow R (1866)
Ein Fall von allgemeiner Ochronose der Knorpel und knorpelähnlichen Theile.
Archiv für Pathologische Anatomie und Physiologie und für Klinische Medicin. 37: 212-19

Wei-Yi H, Ching-Ming C, Fuu-Jen T et al., (2013)
Simultaneous detection of diagnostic biomarkers of alkaptonuria, ornithine carbamoyltransferase deficiency and neuroblastoma disease by high-performance liquid chromatography/tandem mass spectrometry.
Clin Chem Acta. 420: 140-45

Wells C and. Maxwell BM (1962)
Alkaptonuria in an Egyptian mummy.
The Br J of Radiol. 35(418): 679-82

Wolff JA, Barshop B, Nyhan WL et al., (1989)
Effects of ascorbic acid in alkaptonuria: alterations in benzoquinone acetic acid and an ontogenic effect in infancy.
Pediatr Res. 26: 140-44

Wolkow M, Baumann E (1891)
Ueber das Wesen der Alkaptonurie. Z.
Phys Chem. 15: 228-85

Yamaguchi S, Koda N and Ohashi T. (1986)
Diagnosis of alkaptonuria by NMR urinalysis: rapid qualitative and quantitative analysis of homogentisic acid.
Tohoku J Exp Med. 50: 227-8

Zannoni VG, Lomtevas N, Goldfinger S (1969)
Oxidation of homogentisic acid to ochronotic pigment in connective tissue.
Biochim Biophys Acta. 177(1): 94-105

Zatkova A. (2011)
An update on molecular genetics of Alkaptonuria (AKU).
J Inherit Metab Dis. 34(6): 1127-36

Zatkova A, Ranganath L, Kadasi L (2020)
Alkaptonuria: Current perspectives.
Appl Clin Genet. 13: 37-47

Zhao Y, Liu G, Angeles A et al., (2016)
A validated LC-MS/MS method for the quantitative measurement of creatinine as an endogenous biomarker in human plasma.
Bioanalysis 8: 1997-2005

Zoutendam PH, Bruntlett CS, Kissinger PT. (1976).
Determination of homogentisic acid in serum and urine by liquid chromatography with amperometric detection.
Anal Chem. 48: 2200-2202

Websites accessed:

Chemicalize: https://chemicalize.com

HGD mutation database: http://hgddatabase.cvtisr.sk/

Metlin: https://metlin.scripps.edu/landing_page.php?pgcontent=mainPage

https://www.ncbi.nlm.nih.gov/gene

https://www.ncbi.nlm.nih.gov/omim

https://enzyme.expasy.org

https://akusociety.org/

https://www.genome.gov

Sci-Hub: https://sci-hub.hkvisa.net/

Chapter 13

Appendix 1

Student name and number: Andrew Thomas Hughes – 930446923

Supervisors: Professor JA Gallagher, Dr AM Milan and Professor LR Ranganath

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Chapter 2

Journal of Chromatography B

Article: Urine homogentisic acid and tyrosine: Simultaneous analysis by liquid chromatography tandem mass spectrometry. 2014, 963:106-112

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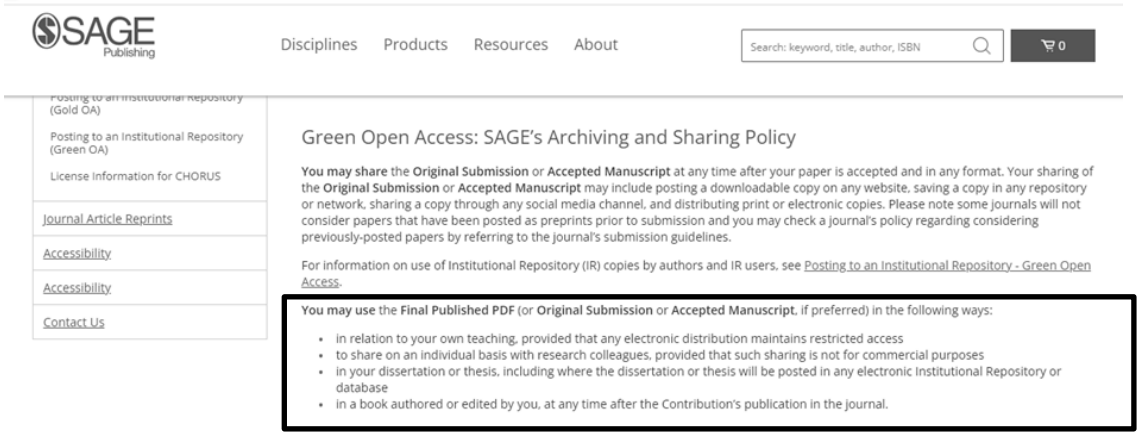
Chapter 3

Annals of Clinical Biochemistry

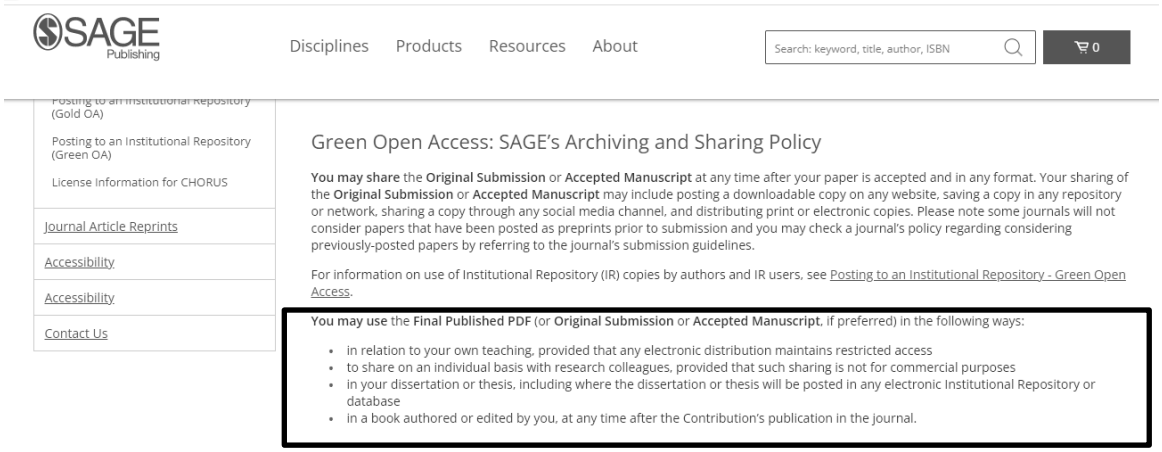
Article: Serum markers in alkaptonuria: simultaneous analysis of homogentisic acid, tyrosine and nitisinone by liquid chromatography tandem mass spectrometry. 2015, 52:597-605.



Chapter 7

Annals of Clinical Biochemistry

Article: The effect of nitisinone on homogentisic acid and tyrosine: a two-year survey of patients attending the National Alkaptonuria Centre, Liverpool.



Chapter 9

Annals of Rheumatic Diseases

Article: Suitability of Nitisinone in Alkaptonuria 1 (SONIA 1): an international, multicentre, randomised, open-label, no-treatment controlled, parallel group, dose-response study to investigate the effect of once daily nitisinone on 24hr urinary homogentisic acid excretion in patients with alkaptonuria after 4 weeks of treatment.

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When anyone asks me “What is the meaning of life?”

My answer is always “Make a positive difference”.

Only your achievements and contributions will be recognised and remembered. Nobody remembers a manager, only a leader and achiever. Status means nothing. We all return to the cosmic dust.

The universe will not hand out a second chance….

AT Hughes (2021)