Inhibitory actions of dietary and pharmaceutical components on steroid glucuronidation

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A thesis submitted in partial fulfilment of the requirements of Kingston University for the degree of Doctor of Philosophy

Kingston University, School of Life Sciences

I declare that the work reputed in this thesis is entirely my own and has been carried out at Kingston University, UK

This thesis has not been submitted, in whole or in part, for any other degree at this or any other University.

Carl Jenkinson

Abstract

Anabolic steroid abuse remains the most potent and frequent form of doping in elite sports, originally utilising synthetic potent anabolic steroids. However, advances in highly sensitive mass spectrometry-based urine drug screens for synthetic anabolic steroids has led to exploits in the illicit use of the natural anabolic steroid testosterone, which is difficult to distinguish between the endogenous form of testosterone. Glucuronidation, performed by UDP glucuronosyltransferases (UGTs), is a key process for inactivation of anabolic steroids before excretion. The key enzyme involved in testosterone glucuronidation is UGT2B17, in contrast to epitestosterone which is mainly glucuronidated by UGT2B7. Changes in the regulation of these enzymes could alter excreted concentrations of these steroids and affect urinalysis, performed to determine testosterone doping in sport. Literature reports reveal alterations to testosterone glucuronidation through inhibition and pharmacogentic variations of UGT2B17, along with the interaction of epitestosterone on testosterone glucuronidation. This study aims to investigate the role of compounds commonly found in dietary substances such as teas and red wine samples, along with pharmaceuticals on UGT mediated testosterone and epitestosterone glucuronidation. The interaction of epitestosterone and stanozolol on testosterone glucuronidation was also investigated.

HPLC and LC-MS/MS analysis were used to monitor levels of testosterone and epitestosterone glucuronidation following UGT supersome and microsome based assays *in vitro*. A rat model was used to investigate the role of diclofenac and stanozolol on excreted testosterone and and epitestosterone. An LC-MS/MS method capable of measuring 0.125 ng/mL testosterone and 0.250 ng/mL epitestosterone (deconjugated) in rat blood and urine was optimised and validated.

Dietary green and white teas along with red wine inhibit UGT2B17 testosterone glucuronidation and UGT2B7 epitestosterone glucuronidation. The inhibitory activities of constituent catechin and phenolic compounds against these enzymes have been determined. In an *in vivo* study, stanozolol reduced the long term excretion levels of testosterone, coupled to increased epitestosterone excretion and reduced T/E ratios. On the other hand diclofenac did not appear to alter excreted testosterone and epitestosterone levels, apart from having a short term effect on T/E levels.

This study demonstrates the role of dietary, pharmaceutical and steroid interactions on UGT testosterone and epitestosterone glucuronidation, along with an evaluation of the consequences linked with enhanced anabolic steroid levels and anti-doping regulation.

Acknowledgements

I would firstly like to thank my director of studies Professor Declan Naughton for his support, guidance and advice throughout this project. The help and encouragement he has given to me throughout this project will forever be appreciated. I would also like to express my gratitude to my supervisor Professor Andrea Petroczi for her help, support and advice during this project, particularly with data analysis and interpretation of results. Thank you also to my supervisor Dr James Barker for his help and providing mass spectrometry advice.

I would like to thank Dr Nawed Deshmukh and Dr Iltaf Shah, their advice and initial training on LC-MS/MS is greatly acknowledged. In addition I would like to thank all of the technical team in the School of Life Science and Chemistry and Pharmacy, particularly Dr Julian Swinden for his assistance with the LC-MS/MS. I am also grateful to all the researchers in the Life Science postgraduate office that have supported me throughout this project. Claire Ryall, Saira Khan, Niousha Yarandi, Naomi Ikeda and Lauren Mulcahy are particularly acknowledged for their support during this project.

Finally, I thank my family, in particular my parents Richard and Patricia Jenkinson, for their encouragement and support throughout this project.

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List of abbreviations

AAS	Anabolic androgenic steroids
ANOVA	Analysis of variance
C	Catechin
CG	Catechin gallate
CYP	Cytochrome P450
DAD	Diode array
del/del	deletion/deletion
DHT	Dihydrotestosterone
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
(+)EC	(+)Epicatechin
(-)EC	(-)Epicatechin
ECG	Epicatechin gallate
EG	Epitestosterone glucuronide
EGC	Epigallocatechin
EGCG	Epigallocatechin-3-gallate
EPO	Erythropoietin
ESI	Electrospray ionisation
FDA	Food and drug administration
FSH	Follicle-stimulating hormone
GC	Gallocatechin
GC-MS	Gas chromatography mass spectrometry
GT	Green tea
HIV	Human immunodeficiency virus
HPLC	High Performance Liquid Chromatography
ins/del	insertion/deletion

ins/ins	insertion/insertion
iP	Intraperitoneal
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LH	Luteinising hormone
LLE	Liquid-liquid extraction
LOD	Limit of detection
LLOQ	Lower limit of quantification
MRM	Multiple reaction monitoring
mRNA	Messenger ribonucleic acid
NSAID	Non steroidal anti-inflammatory drug
PTFE	Polytetrafluoroethylene
QC	Quality control
r^2	Regression/determination coefficient
RNA	Ribonucleic acid
RSD	Relative standard deviation
SEM	Standard error of the mean
SRM	Selective reaction monitoring
T/E	Testosterone/epitestosterone
T/LH	Testosterone/luteinising hormone
TG	Testosterone glucuronide
UDPGA	Uridine diphosphate glucuronic acid
UGT	UDP-glucuronosyltansferase
UPLC	Ultra performance liquid chromatography
USDA	United States Department of Agriculture
UV	Ultra violate
V	Voltage
v/v	volume/volume
WADA	Wold Anti-Doping Agency

WTB	White tea beard
WTL	White tea leaf
WTP	White tea powder

Chapter 1. Introduction

1.1 Anabolic steroids

Anabolic androgenic steroids (AAS) are compounds that are structurally similar to the natural anabolic steroid testosterone (Graham *et al.*, 2008; Shahidi, 2001). The synthetic derivatives of testosterone are usually designed to enhance the anabolic effects such as promoting synthesis of proteins and muscle growth (Mottram and George, 2000). Anabolic steroids have commonly been associated with misuses in society such as performance enhancing in sport (Kicman, 2008). Active endogenous steroids are produced from endocrine tissues (Figure 1.1), such as the gonads and adrenals that can then be moved into circulation to exert their effects on target areas (Luu-The, 2013).

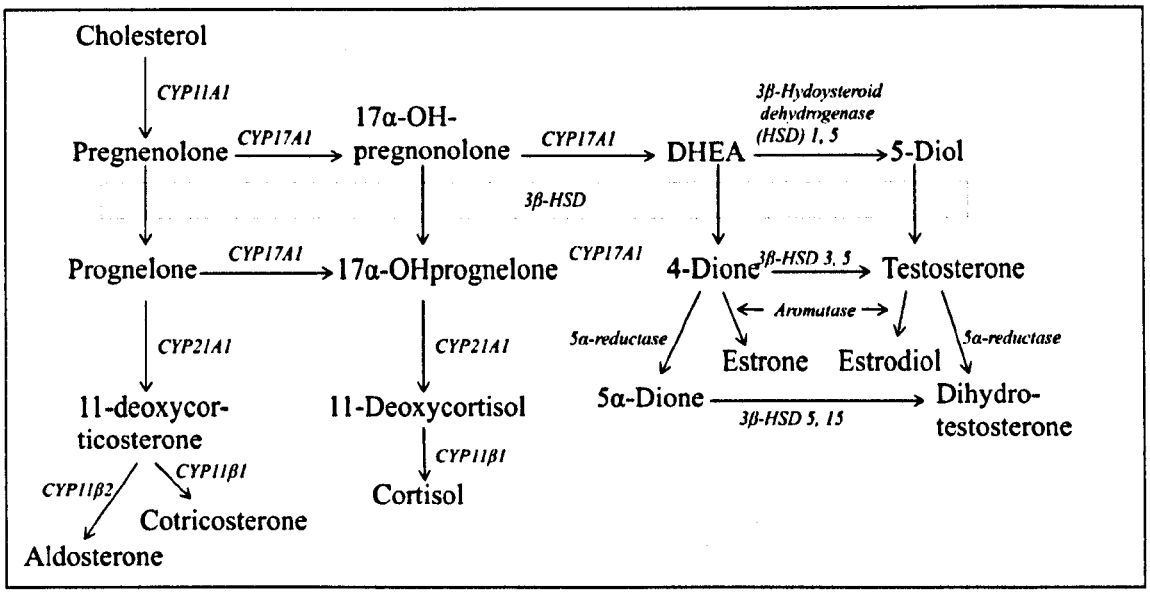


Figure 1.1 Steroid metabolic pathway adapted from Luu-The (2013).

A number of synthetic steroids that have different modes of action have been developed to avoid the rapid metabolism that occurs with orally administered testosterone. Modified

testosterone reduces metabolism of the molecule, enhancing the duration of anabolic effect, whilst having enhanced affinity and activity at androgen receptors. Structural modifications of testosterone occur by alkylation at the 17-α-position with a methyl or ethyl group to produce orally active steroids. Another approach is the esterification of testosterone at the 17-β-position that has been shown to prolong the effectiveness of the steroid (Hartgens and Kuipers, 2004; Kopera, 1993). Stanozolol is an example of a synthetic steroid that is a derivative of naturally produced testosterone (Pozo *et al.*, 2009). One of the structural modifications of stanozolol is the attachement of a 17α-methyl group. The 17α methy groups prevent first pass metabolism in the liver that would deactivate the steroid (Gao *et al.*, 2005) along with improving oral activity (Kicman, 2008).

1.1.1 Positive effects of anabolic steroids

AAS facilitate the increase in muscle growth through stimulation of protein synthesis. Initial binding of AAS to androgen receptors in skeletal muscles forms a steroid receptor complex that interacts with DNA and RNA to promote muscle synthesis (Hartgens and Kuipers, 2004; Shahidi, 2001; Kadi, 2008). As well as enhancing muscle growth AAS have other pharmacological actions that can aid in improving athletic performance. An example of this is the anti-glucocorticoid effects of AAS by acting as competitive antagonists at glucocorticoid receptors. Glucocorticoids are released by a number of conditions including responses to strenuous exercise and various diseases. Increased levels of circulating glucocorticoids result in muscle atrophy through decreasing rates of protein synthesis and increasing protein breakdown (Hickson *et al.*, 1990; Kicman, 2008). Therefore, AAS can reduce muscle protein breakdown though limiting the impact of glucocorticoids at receptors.

An additional benefit of AAS is promoting the production of erythropoietin (EPO) (Hartgens and Kuipers, 2004), which regulates red blood cell production. Haemoglobin in red blood cells transports oxygen from the lungs to areas around the body including skeletal muscles. Through enhancing EPO production, AAS can aid in the production of red blood cells for increased transport of oxygen that will have an impact on athletic performance (Gaudard *et al.*, 2003; Shahidi, 2001; Kicman, 2008). AAS use can also have behavioural effects through stimulation of androgens to the brain. This gives a feeling of euphoria and increased aggression along with changes to mood and behaviour (Maravelias *et al.*, 2005).

1.1.2 Adverse effects of anabolic steroids

Along with the anabolic activity of AAS there are also androgenic effects that cause side effects. These side effects can be severe and irreversible depending on the type of AAS and length of use. It is therefore ideal for an AAS to have maximum anabolic effects whilst having minimal androgenic effects (Kicman, 2008). The ratio of anabolic effect and androgenic effects varies between synthetic AAS, however no AAS has been found to be completely anabolic without androgenic effects (Kuhn, 2002). Stanozolol is a synthetic steroid, modified to have enhanced anabolic activity whilst having reduced androgenic effects in comparison to testosterone (Evans, 2004; Helfman and Falanga, 1995).

The use of AAS in males decreases endogenous testosterone and decreased spermatogenesis production through a reduction in the levels of luteinising hormone (LH) and follicle-stimulating hormones (FSH). Reduction in LH and FSH can also lead to

testicular atrophy, this is usually reversed following the withdrawal of AAS, however reduced sperm count and density can remain abnormal for up to 6 months following withdrawal (Maravelias *et al.*, 2005; Dohle *et al.*, 2003; Kicman, 2008). In females AAS can lead to menstrual abnormalities along with masculinisation characteristics. Some of these effects include acne, deepening of the voice and hirsutism. Some of these effects are irreversible even following on from the withdrawal of AAS (Maravelias *et al.*, 2005).

Hepatic effects have been associated with the use of 17-alpha alkylated steroids through elevation of liver enzymes such as aspartate aminotransferase, alanine aminotransferase, and lactate dehydrogenase (Maravelias *et al.*, 2005; Kicman, 2008). Tumours in the liver have been linked to AAS use (Maravelias *et al.*, 2005; Soe *et al.*, 1994), along with cholestatic jaundice which can occur occasionally with AAS use, although this tends to resolve within 3 months of AAS withdrawal (Maravelias *et al.*, 2005).

A number of cardiovascular effects are associated with AAS with adverse effects on lipid profiles in serum. Changes to cholesterol levels with steroid use increases risks of atherosclerotic heart disease. The reversibility of any adverse cholesterol change is dependent on the duration and extent of AAS use (Maravelias *et al.*, 2005; Glazer, 1991). Hypertension has also been reported in athletes taking AAS over prolonged periods (Maravelias *et al.*, 2005; Ferenchick, 1990), as well as causing irreversible myocardium changes including ventricular hypertrophy (Maravelias *et al.*, 2005; Urhausen *et al.*, 2004).

Adverse renal effects have occurred in athletes through extensive use of AAS. Several reported cases have been made of athletes with Wilm's tumour, which is usually uncommon in humans (Joyce, 1991; Maravelias *et al.*, 2005). Other renal effects

associated with the use of AAS include acute renal failure and injury, nephropathy and glomerulonephritis (Winnett *et al.*, 2011; Herlitz *et al.*, 2010).

Other effects that have been associated with AAS use include effects on endocrine levels including decreases in thyroid hormones (Maravelias *et al.*, 2005; Shahidi, 2001). AAS can alter phychological states and lead to behavioural changes such as aggressiveness, depression and changes in mood (Maravelias *et al.*, 2005; Bahrke *et al.*, 1990; Clark and Henderson, 2003). However, testosterone gel has also been shown to demonstrate antidepressant effects (Pope *et al.*, 2003)

1.1.3 Testosterone

Testosterone is a naturally occurring steroid in the body synthesised from cholesterol (Wynn, 1975; Saudan *et al.*, 2006). The hormone is secreted mainly in the testes of males but also secreted in the adrenal gland, allowing testosterone to be secreted in females (Sten *et al.*, 2009a). Production of testosterone in males is on average between 6-10 mg per day with around 1% of this excreted in urine daily. The concentration of excreted testosterone increases after administration. However, owing to a short half life of testosterone, excreted concentrations fall back to normal reference range values in a short space of time following a spike in excretion (Catlin *et al.*, 1997). Testosterone can undergo a number of metabolic processes; in reproductive tissues the enzyme 5α-reductase converts testosterone as a prohormone to the androgren dihydrotestosterone (DHT). The metabolic process of testosterone alters in other tissues where testosterone is converted to oestrogen or oeastradiol (Kicman, 2008).

Testosterone regulates a number of physiological processes in the body such as protein metabolism in muscle, regulation of lipid levels and bone metabolism (Saudan *et al.*, 2006; Wilson and Griffin, 1980). Owing to its endocrine role, synthetic testosterone is used in a number of clinical applications. Testosterone replacement therapy has been used for condition such as hypogonadism in order to restore circulating testosterone levels (Kapoor *et al.*, 2006). Testosterone can also be used as part of treatment in psychological conditions for enhancing changes in mood. However uses of testosterone in treatment can incur side-effects such as those discussed above for AAS as well as stimulating prostate cancer (Margo and Winn, 2006).

Synthetic forms of testosterone are available which alter the endogenous structure slightly. Exogenous testosterone contains less C^{13} profile than the endogenous form, as endogenous forms are produced from cholesterol in the body, giving endogenous forms a higher C^{13}/C^{12} ratio (Sottas *et al.*, 2010; Saudan *et al.*, 2006; De La Torre *et al.*, 2001). The short term effects of testosterone enanthate has been shown at doses of 300 mg per week which increased muscle strength and power in comparison to placebo with effects still being observed after three weeks (Rane and Ekstrom, 2012; Rogerson *et al.*, 2007).

1.1.4 Epitestosterone

Epitestosterone is a naturally occurring 17α epimer of testosterone (Figure 1.2) and is excreted in the urine at similar concentrations to testosterone (Aguilera *et al.*, 2002) or slightly lower concentrations (Starka, 2003). Epitestosterone does not have the anabolic androgen role of testosterone and the physiological role of epitestosterone is unclear (Starka, 2003). Like testosterone, epitestosterone is also produced in the testes in males

and in the ovaries of females (Aguilera *et al.*, 2002; Dehennin, 1993; Acevedo and Corral-Gallardo, 1965).

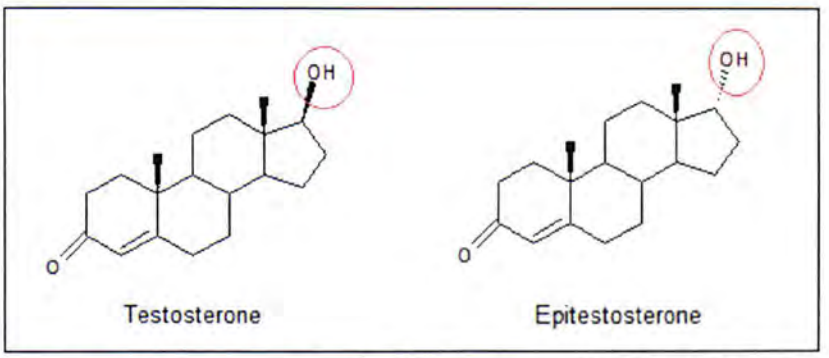


Figure 1.2 Structures of testosterone and epitestosterone, the 17α epimer of testosterone is highlighted. Structures drawn in Chemsketch version 11.02.

Epitestosterone is excreted mainly as epitestosterone glucuronide (Starka, 2003; De Nicola *et al.*, 1966) as well as epitestosterone sulfate (Starka, 2003; Korenman *et al.*, 1964). The urinary excretion of epitestosterone in males ranges from 200-500 nMol per day, whilst in females it is 80-500 nMol per day (Starka, 2003). Epitestosterone glucuronide has been reported to increase after intravenous injection of a large dosage testosterone (Starka, 2003), (Tamm *et al.*, 1966). It has also been shown that after long term administration of testosterone in males there was a reduction in urinary epitestosterone glucuronide and sulfate (Starka, 2003; Dehenin and Matsumoto, 1993).

1.2 Use of anabolic agents in sport and doping tests measurements

The use of steroid administration such as testosterone is a practice taken by some athletes to enhancing performance in power and endurance sports (http://www.wada-ama.org/Documents/Resources/Testing-Figures/WADA-2011-Laboratory-Testing-

Figures.pdf). Performance enhancement could be in the form of increasing performance, increasing muscle mass and improving recovery (Evans, 2004; Graham *et al.*, 2008).

During the time of the Moscow Olympic Games in 1980, analytical methods to detect testosterone and synthetic steroids started to improve owing to improved detection by gas chromatography-mass spectrometry (GC-MS). This led to changes in steroid doping from synthetic forms to endogenous steroids such as testosterone as the detection of this steroid has been difficult owing to the variation in metabolism and excretion of this compound between individuals (Van De Kerkhof *et al.*, 2000). In addition to this, attempts to estimate the misuse of AAS in society has been difficult as AAS use is often concealed (Rane and Ekstrom, 2012). Typical drug regime patterns of testosterone dosages have been shown to range between 250-3200 mg over 4-12 week cycles (Evans, 2004).

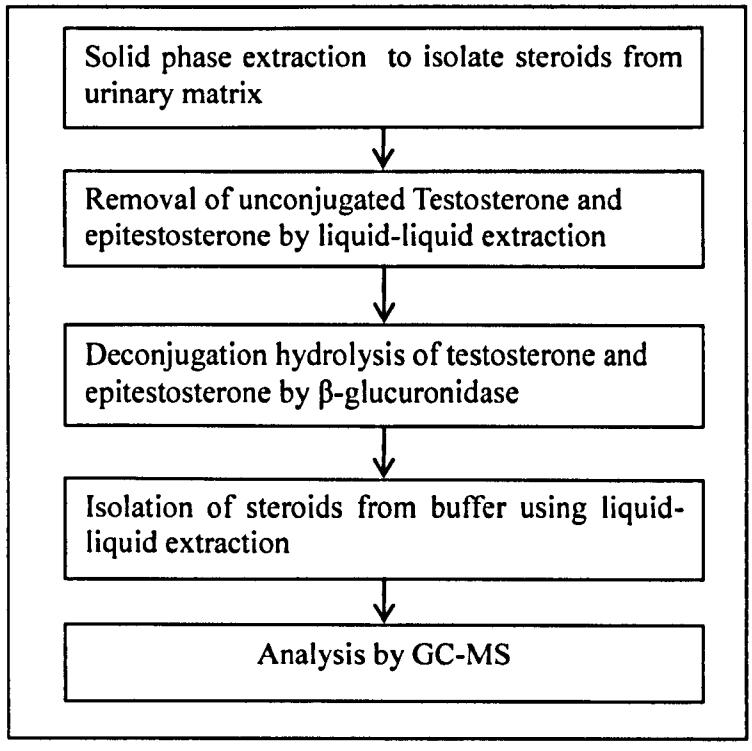


Figure 1.3 Procedure for sample preparation and analysis in doping laboratories (Van De Kerkhof et al., 2000).

Owing to their performance enhancing characteristics, the use of testosterone and other anabolic steroids is currently banned in and out of sporting competition. Monitoring of testosterone and other anabolic steroids in sport is controlled by the World Anti Doping Agency (WADA) and national bodies (http://www.wada-ama.org/en/anti-doping-community/nados/list-of-nados/). The current protocol of analysis of testosterone and epitestosterone concentrations in urine is outlined in Figure 1.3. One of the purposes of WADA is to communicate substances that are currently banned in sport. The WADA 2014 list of prohibited substances (http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-Prohibited-list/2014/WADA-prohibited-list-2014-EN.pdf) shows numerous AAS including testosterone, along with other stimulant agents are banned in and out of competition. Based on the figures of the WADA 2012 laboratory statistics (http://www.wada-ama.org/Documents/Resources/Testing-Figures/WADA-2012-Anti-Doping-Testing-
Figures-Report-EN.pdf), anabolic agents had the highest number of adverse and atypical findings from samples tested that accounted for 50.6% of all banned substance adverse and atypical findings. Elevated testosterone glucuronide to epitestosterone glucuronide (T/E) ratios accounted for 55.5% of the atypical findings within the anabolic agent's class.

The conventional method of detecting testosterone doping is by using a measurement of the T/E ratio in urine (Rane and Ekstrom, 2012). Urinary testosterone and epitestosterone concentrations are measured by gas chromatography tandem mass spectrometry (GC/MS) after deconjuation of the glucuronide metabolised compound by β-glucuronidase enzyme hydrolysis (Sottas *et al.*, 2010; Van De Kerkhof *et al.*, 2000). Originally the T/E ratio cut off mark was set at 6 or above to indicate a suspicious sample that required follow up tests,

however in 2005 the cut off ratio was reduced to 4 to in order to improve the sensitivity in detecting testosterone misuse (Mareck *et al.*, 2010).

The method of using T/E measurements in detecting testosterone doping in sport is justified as there is variation between an individual's serum testosterone concentrations that can range from 2.7-10.7 ng/dL (blood index). This would lead to alterations in excreted urinary testosterone between individuals with or without additional testosterone intake. Testosterone alone would not be effective in determining testosterone doping as no set standard could be set for a population. Testosterone intake increases the levels of testosterone metabolites in urine, whilst the excreted levels of epitestosterone remains unaltered. The T/E method would therefore be effective in measuring changes to testosterone levels using epitestosterone as the marker to base changes in testosterone levels on (Saudan *et al.*, 2006). Testosterone is not metabolised to epitestosterone (Catlin *et al.*, 1997) and it has also been shown that testosterone administration lowers the concentration of epitestosterone which could enhance T/E further by increasing testosterone whilst reducing epitestosterone (Catlin *et al.*, 1997; Dehennin and Matsumoto, 1993). However the accuracy of this method is dependent on the idea that testosterone and epitestosterone would be metabolised at the same rate at all times and no external or inter-individual factors could alter the rate of metabolism of either steroid (Starka, 2003).

The use of epitestosterone is listed on the WADA list of banned substances (http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-Prohibited-list/2014/WADA-prohibited-list-2014-EN.pdf). This is owing to the masking properties of epitestosterone in lowering the T/E ratio as opposed to having an enhanced anabolic effect like testosterone. Atypical findings are required to be reported when urinary

epitestosterone exceeds 200 ng/mL. However it has previously been found difficult to prove epitestosterone administration unless urinary values exceed 1000 ng/mL owing to excretion variations within individuals (Aguilera *et al.*, 2002). WADA 2011 laboratory statistics (http://www.wada-ama.org/Documents/Resources/Testing-Figures/WADA-2011-Laboratory-Testing-Figures.pdf) shows only three occurrences (0.1% of adverse findings within anabolic agents), were elevated epitestosterone was identified as an adverse finding. The WADA 2012 statistics did not reveal any adverse findings of elevated epitestosterone (http://www.wada-ama.org/Documents/Resources/Testing-Figures/WADA-2012-Anti-Doping-Testing-Figures-Report-EN.pdf). Several reports have highlighted potential false positive T/E ratios that have been above the cut off value of 4 owing to reduced epitestosterone as opposed to elevated testosterone (Raynaud *et al.*, 1992). Reports from athlete samples have indicated that the common T/E ratios are around 1, however this can range from less than 0.2 to greater than 6 (Figure 1.4). It has also been shown that T/E ratios within individuals can remain consistent for months and years (Catlin *et al.*, 1997).

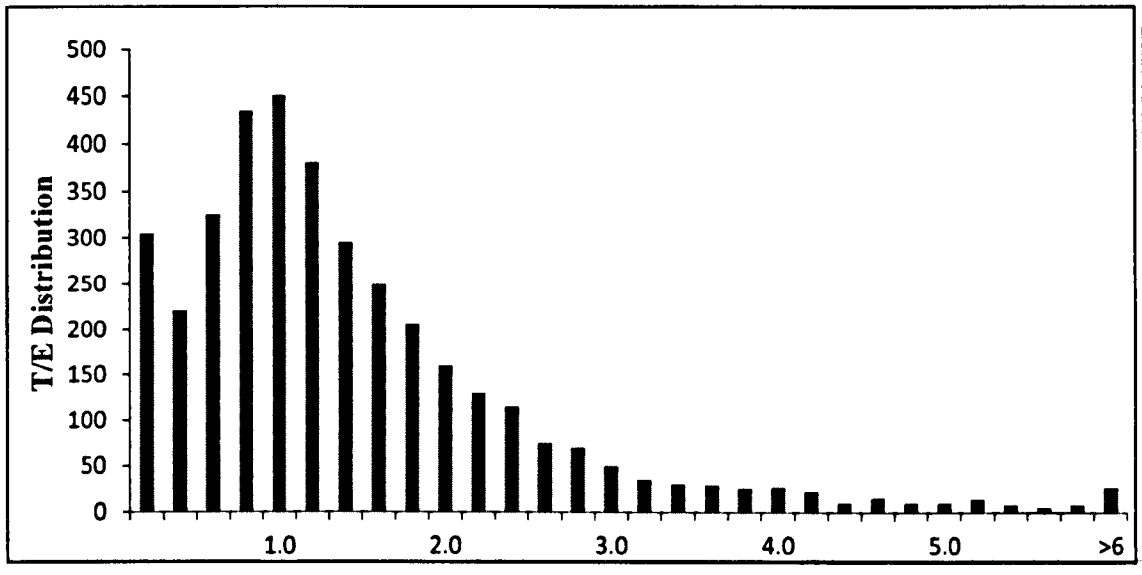


Figure 1.4 Urinary T/E ratio distribution from the urine samples of 3710 football players. Ratios in the >6 group indicate any T/E ratios measuring 6 or above. The three highest ratios were 30, 36 and 57 (Catlin *et al.*, 1997).

There are a number of other synthetic AAS that are banned in and out of sporting competition owing to their anabolic effect. This includes the synthetic steroid stanozolol (http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-Prohibited-list/2013/WADA-Prohibited-List-2013-EN.pdf). Stanozolol and the metabolite 3'-hydroxystanozolol are structurally different from other AAS which can make it difficult to detect, these compounds are also often excreted in urine at very low concentrations (Deshmukh *et al.*, 2012b; Mateus-Avois *et al.*, 2005). 3'-Hydroxystanozolol has been shown to be deconjugated by synthetic β-glucuronidase hydrolysis in urine (Schanzer, 1996), this metabolite is then used as marker in detecting stanozolol misuse (Deshmukh *et al.* 2012; http://www.wada-ama.org/documents/world_anti-doping_program/wadp-is-laboratories/wada_td2010mrplv1.0_minimum%20required%20performance%20levels_sept%2001%202010_en.pdf).

Numerous internal and external factors have been reported that can alter T/E ratios. Ethanol consumption (2.0 g/kg) in females increases urinary testostesterone whilst epitestosterone excretion remained low. Female urinary T/E ratio increased 277% in the whole population studied after 14 hours. In the same study alcohol did not have any significant effects on testosterone or urinary T/E levels in males (Van De Kerkhof *et al.*, 2000).

Endocrine factors can also have an impact on T/E ratios. This includes some endocrinological diseases with hirsutism being one of the best known examples for altering excretions of testosterone and epitestosterone (Van De Kerkhof *et al.*, 2000). Studies have concluded contradicting urinary excretion levels that include increases in both testosterone and epitestosterone excretion giving values above references range (Pal, 1979) whilst

another study (De Nicola *et al.*, 1966) reported greater increases in epitestosterone excretion leading to decreased T/E ratios around 10 times lower than original values.

Cardiac rhythm patterns are associated with varying T/E ratios, levels were changed by 30% or less in males (Mareck-Engelk *et al.*, 1995a), whilst this is thought to be more significantly altered in females (Mareck-Engelk *et al.*, 1995b; Van De Kerkhof *et al.*, 2000). Contradictory reports have been produced on the effects of exercise on testosterone and epitestosterone levels. No significant changes to T/E ratios in samples taken daily before and after exercise were reported during cycling exercises (Van De Kerkhof *et al.*, 2000).

Pharmaceutical alterations to T/E have also been discussed with oral contraceptives resulting in unstable T/E ratios which were usually elevated by suppressed excretion of epitestosterone. Probenecid, used in the treatment of gout, decreases steroid excretion through inhibiting active transport mechanisms and reducing excretion by up to 20%, this does not affect ratio levels as both testosterone and epitestosterone are reduced by the same amounts (Van De Kerkhof *et al.*, 2000).

Owing to these limitations associated with measuring urinary testosterone and epitestosterone levels using the T/E measurement, other methods have been suggested that may have a more accurate reflection of testosterone use. Bayesian analysis and genotype approaches to T/E ratio cut off values would provide a more accurate approach to monitoring T/E ratios. This would involve having individual T/E cut off values based on phase II metabolising enzyme genotypes and an individual's longitudinal steroid profile (Schulze *et al.*, 2009; Sottas *et al.*, 2007; Sottas *et al.*, 2008). Other ratio markers have

been suggested as an alternative to using epitestosterone. This includes using the testosterone/luteinising hormone (T/LH) ratio (Saudan *et al.*, 2006; Kicman *et al.*, 1990). The sensitivity of using LH for a marker of testosterone use has already been determined (Kicman *et al.*, 1990; Perry *et al.*, 1997). Once testosterone is administered the ratio of T/LH will be increased owing to increased testosterone excretion and the suppression of secretion of gonadotrophins. This method has the additional advantages of detecting epitestosterone administration along with testosterone (Van De Kerkhof *et al.*, 2000).

1.3 UGT mediated glucuronidation of steroids

Phase I and phase II metabolic reactions occur to AAS for modification to inactivate these compounds and for urinary excretion (Kuuranne *et al.*, 2003). Through Phase I catalysis reactions the steroid becomes more polar and inactivates the anabolic effects of steroids by reduction (Schanzer, 1996).

Phase II reactions occur by UDP-glucuronosyltansferase (UGT) enzymes, membrane bound located on the endoplasmic reticulum. UGTs are subdivided into sub 1 and 2 subfamilies and further subdivided to 2A and 2B groups (Kuuranne *et al.*, 2003). The UGT2B enzymes are considered to be the most active groups in steroid glucuronidation and inactivation (Turgeon *et al.*, 2001). Virtually all drug classes are considered to be substrates for UGTs and these compounds are metabolised by these enzymes as part of phase II metabolism (Guillemette, 2003). UGT enzymes grouping and pharmacogenomic classes are shown in Figure 1.5.

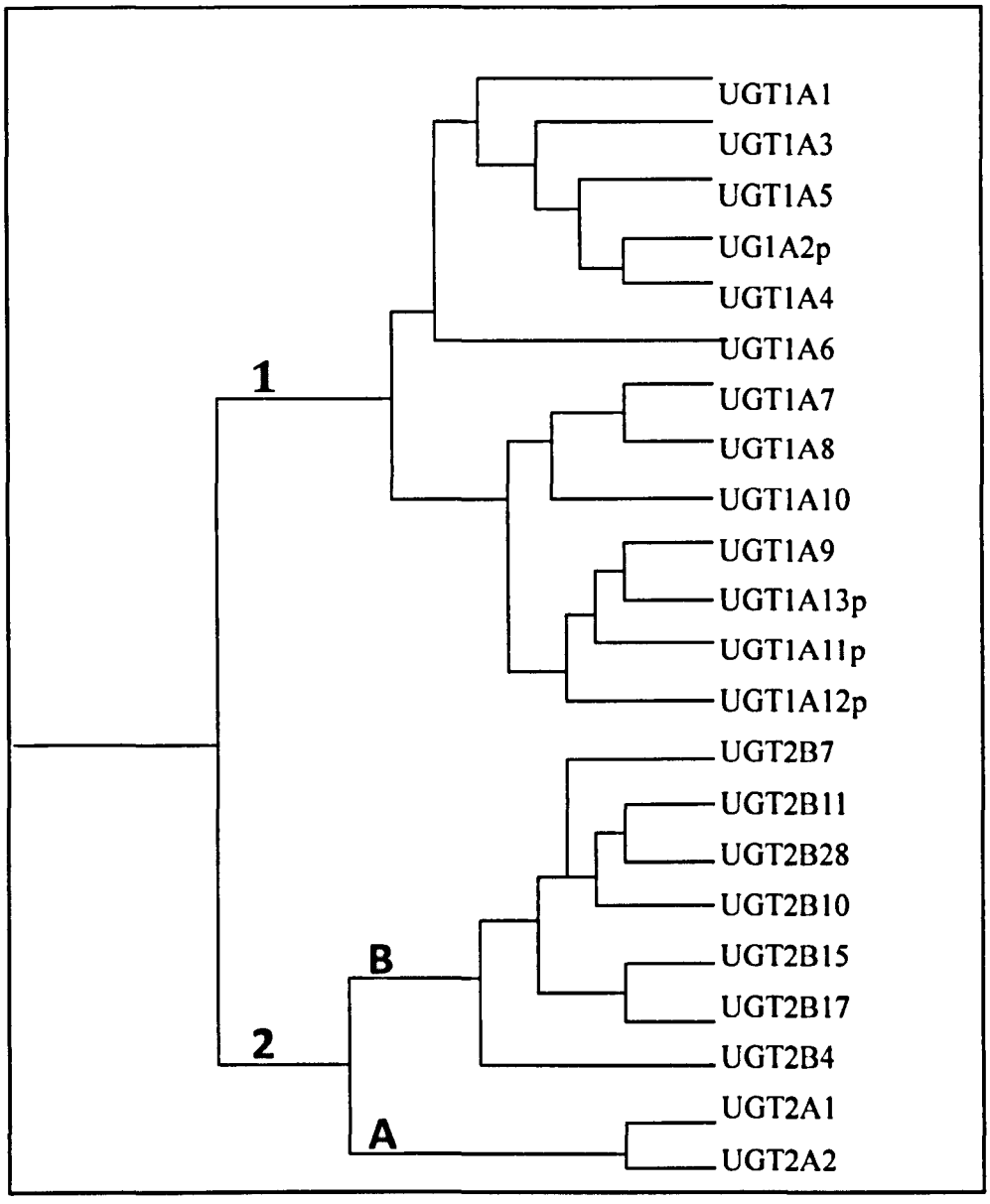


Figure 1.5 Phylogenic tree showing the groupings of different UGT isoenzymes. Adapted from Guillemette (2003).

Phase II metabolic reactions involve glucuronidation, catalysed by UGT enzymes. Glucuronidation involves the conjugation of glucuronic acid moiety from Uridine diphosphate glucuronic acid (UDPGA) to aglycones. This is usually the final step in deactivation before excretion (Radominska-Pandya *et al.*, 1999). The energy in the bond between UDP and glucuronic acid produces the energy for the bond between the substrate

and glucuronic acid (Fisher *et al.*, 2001; Wells *et al.*, 2004). The formation of testosterone glucuronide by the addition of glucuronic acid to testosterone is displayed in Figure 1.6. Glucuronidation of compounds increases solubility aiding the excretion in urine or bile (Sten *et al.*, 2009a). Glucuronidation of AAS prevents the interaction of these compounds with receptors and allows elimination of the polar molecule (Guillemette *et al.*, 1997). The major site of glucuronidation is the liver, where most UGT enzymes are located (Kuuranne *et al.*, 2003), although these enzymes are also found in other tissues including the prostate. Figure 1.7 shows the structural change in testosterone following the glucuronidation and the role of UGT2B enzymes in the glucuronidation pathways of AAS. Glucuronidation occurs at the hydroxile group of the androgen. The role of phase I and phase II enzymes in the inactivation of androgens in displayed in Figure 1.8.

β-Glucuronidase catalyses a reversible reaction by hydrolysis of the glycosidic bond (Ho, 1995; Lampe *et al.*, 2002). β-Glucuronidase is found in the bacteria in the gastrointestinal tract and found in the urine (Skar *et al.*, 1988). The rate of deconjugation by β-glucuronidase is much slower than glucuronidation by UGTs meaning there is still greater composition of glucuronidated compounds excreted in urine (Bock and Kohle, 2009).

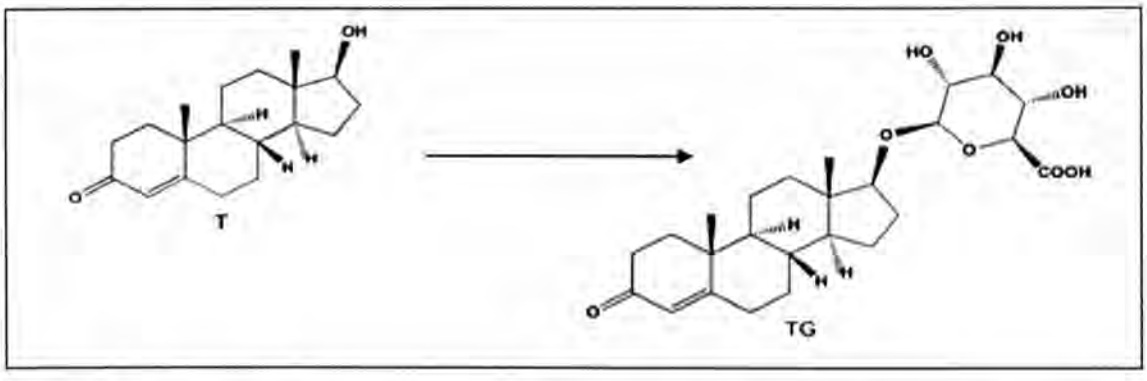


Figure 1.6 Addition of glucuronic acid to testosterone to form testosterone gucuronide by UGT isoenzymes with substrate specificity for testosterone. Adapted from Ambrosini (2012).

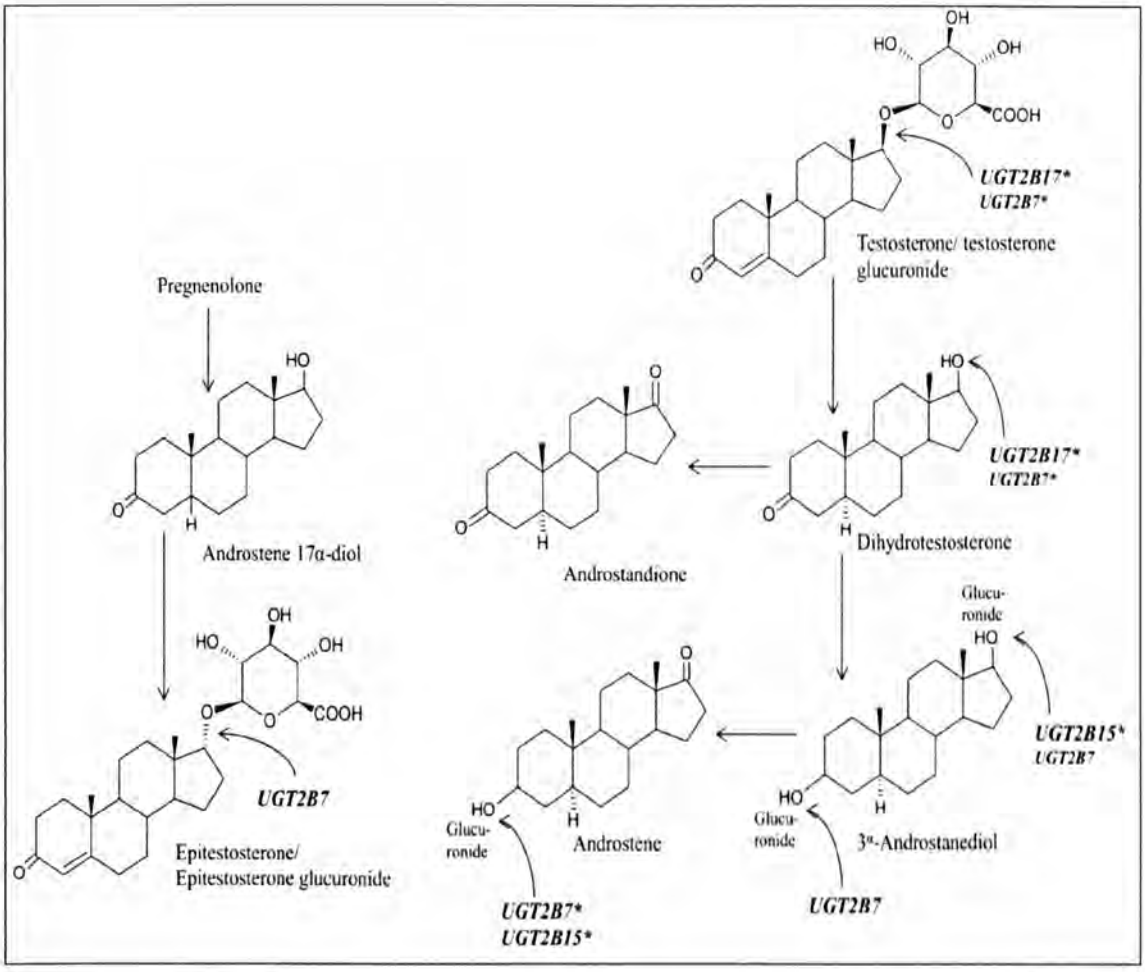


Figure 1.7 Role of UGT enzymes in glucuronidation of several androgens and the metabolic pathway for the conversion of androgens to form testosterone and epitestosterone. Smaller letters for UGT enzymes indicates minor activity towards the androgen. * indicates altered activity of the UGT enzyme for that androgen that is dependent on pharmacogenetic variation of the UGT. Adapted from Rane and Ekstrom (2012). Structures drawn in Chemsketch version 11.02.

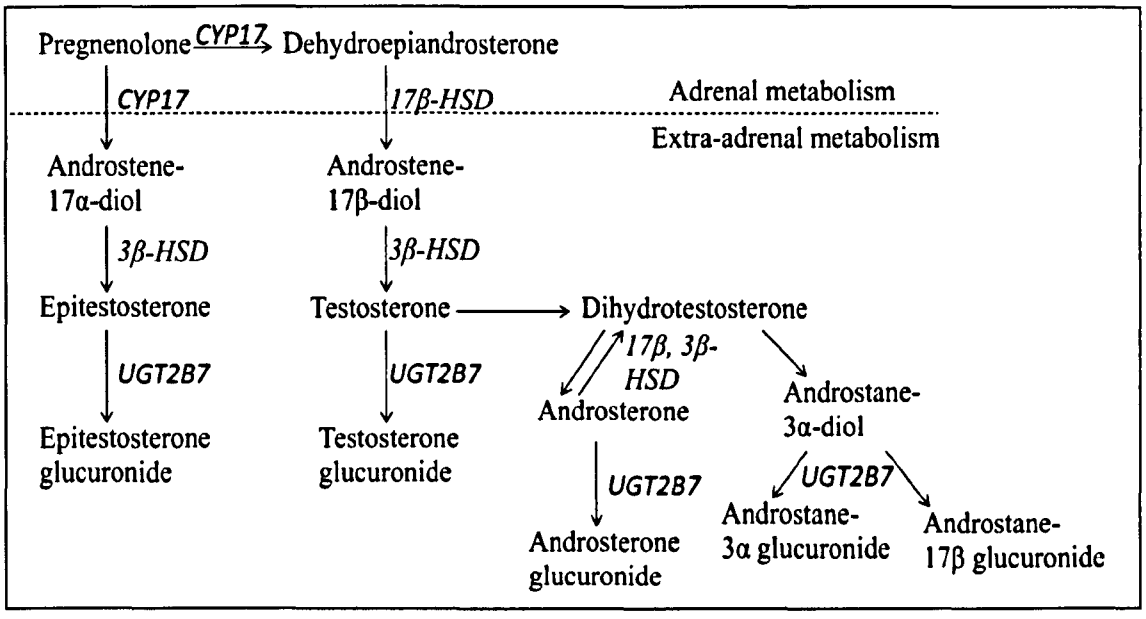


Figure 1.8 Metabolic pathways for the inactivation of androgens by glucuronidation, including the glucuronidation pathway of epitestosterone, testosterone. Adapted from Rana and Erkstrom (2012) and Swanson *et al.*, (2007).

The major metabolite of stanozolol is 3'-hydroxystanozolol which is excreted in the urine as glucuronidated conjugates (Pozo *et al.*, 2009; Deshmukh *et al.*, 2012b). Whilst stanozolol and 3–hydroxystanozolol are both glucuronidated compounds there are no reports to suggest which UGT enzyme are involved in the glucuronidation of these compounds.

1.3.1 UGT2B17

UGT2B17 is the most active UGT enzyme screened in testosterone glucuronidation, this is followed by UGT1A1 as the next most active which has around half the activity of UGT2B17. Epitestosterone is not glucuronidated by UGT2B17, although it does interfere with UGT2B17 testosterone glucuronidation at high concentrations (Sten *et al.*, 2009a).

The expression of UGT2B17 mRNA in the liver is 2.7% of all UGT mRNA expression (Izukawa *et al.*, 2009).

UGT2B17 shares 95% homology with UGT2B15. However, the sites of conjugation are slightly altered between these enzymes, UGT2B17 conjugates C19-steroid molecules at the 3α- and 17β-OH positions whereas UGT2B15 is only active at conjugation at the 17β-OH, therefore having different specificity of androgens (Turgeon *et al.*, 2003).

1.3.2 UGT2B7

UGT2B7 is the most active enzyme in epitestosterone glucuronidation (Sten *et al.*, 2009a). This enzyme has also been shown to glucuronidate testosterone at a very low rate at 1.5% of the rate of epitestosterone glucuronidation (Sten *et al.*, 2009a). UGT2B7 also glucuronidates a number of additional substances including morphine (Coffman *et al.*, 1997) and other pharmaceuticals including the antiretroviral drugs efavirenz and ziduvodine (Belanger *et al.*, 2009). UGT2B7 has a wide specificity for steroid classes including glucuronidation of 5α reduced steroids including mineralocorticoids, glucocorticoids, progestins and androgens as well as phase I β-reduced C19 and C21 steroids (Belanger *et al.*, 2003).

1.4 Glucuronidation studies and steroid analysis

A number of reported *in vitro* assays demonstrate the measurement of glucuronidation rates of testosterone and glucuronidation screening of compounds. Glucuronidation analyses, including testosterone have been performed using human and rat liver

microsomes and recombinant UGT isoenzymes (Sten *et al.*, 2009a; Sten *et al.*, 2009b; Turgeon *et al.*, 2003; Kuuranne *et al.*, 2003). Preparations of recombinant UGT expression include HK293 cells transfected with UGT2B17 cDNA, expression of UGT in baculovirus-infected insect cells and as commercial UGT supersomes (BD supersomes). Testosterone substrate concentrations generally range from 5-150 μM with microsomal and UGT protein concentrations ranging from 0.1 to 1 mg/mL (Sten *et al.*, 2009a; Sten *et al.*, 2009b; Turgeon *et al.*, 2003; Kuuranne *et al.*, 2003). Testosterone and epitestosterone glucuronidation have been observed after incubation with UGT2B17 and UGT2B7 respectively, at 37°C after 15 minutes (Sten *et al.*, 2009a).

Quantitative analysis of testosterone and epitestosterone has included the analytical techniques HPLC and LC-MS/MS. Common mobile phases for HPLC analysis of testosterone and epitestosterone include acetonitrile and water (He *et al.*, 2005) and methonal and acetonitrile (Ng and Yuen, 2003). Reported HPLC methods have limits of detection of between 1 and 10 ng/mL (He *et al.*, 2005; Gonzalo-Lumbreras *et al.*, 2003). Water and acetonitrile are generally the mobile phases used for LC-MS/MS methods to separate testosterone and epitestosterone. The limits of detection for testosterone and epitestosterone using LC-MS/MS analysis are reported to be 0.1 ng/mL with analysis ranging up to 100 ng/mL (Draisci *et al.*, 2000; Hauser *et al.*, 2008).

Rat studies involving the use of diclofenac have used oral and injection routes of administration. Oral doses have been used at 15 mg/kg over 7 days (Sallustio and Holbrook, 2001), whilst oral dosages at 100, 200 and 300 mg/kg were used at lethal toxic ranges (Hickey *et al.*, 2001). Administration of diclofenac was administered by intraperitoneal injection (30 mg/kg/day) for 3 consecutive days (Seitz *et al.*, 1998).

Intravenous injection has been used as another technique for administration of stanozolol in rats at concentrations as high as 7.5 mg/kg (Clark *et al.*, 1998).

1.5 Alterations to testosterone glucuronidation

Changes to glucuronidation activities of UGTs towards testosterone and other AAS can occur from various different sources. Inter-individual glucuronidation rates can vary owing to genetic polymorphisms associated with UGTs, particularly those associated with AAS metabolism (Lampe *et al.*, 2000; Rane and Ekstrom, 2012). Other factors affecting steroid glucuronidation are agents that can directly influence the metabolic activity of UGT enzyme such as inhibitors. Compounds that can directly target the enzyme, affecting the active site or another site on the enzyme affecting its function could have short term implications on the metabolic rate.

These alterations in the metabolism of steroids could have a number of implications. It has been reported that pharmacogenetic variations in UGT2B17 can alter steroid metabolite levels including altering T/E ratios between individuals depending on the expressed UGT2B17 genotype (Schulze *et al.*, 2008b). Variation in steroid levels owing to pharmacogenetic variations could also have implications on clinical conditions such as prostate cancer (Lampe *et al.*, 2000). Inhibiting UGT glucuronidation activity could have short term implications on AAS glucuronide concentrations and elevating circulating levels (Sten *et al.*, 2009b).

1.5.1 NSAIDs, xenobiotic compounds and phenolic compounds

Diclofenac and ibuprofen are non steroidal anti inflammatory drugs (NSAIDs) that are commonly used in the treatment of pain and inflammation. It has been demonstrated *in vitro* that both diclofenac and ibuprofen are glucuronidated by UGT enzymes. These compounds competitively inhibited UGT2B15 and UGT2B17 glucuronidation of testosterone (Sten *et al.*, 2009b).

A number of xenobiotic and phenolic compounds have been glucuronidated by UGT2B17 following screening assays. Several of the compounds screened, including the phenolics 1-naphthol and p-nitrophenol, were glucuronidated by UGT2B17. The amount of glucuronide formation varied between the types of compounds (Turgeon *et al.*, 2003). It has yet to be shown whether these compounds conjugated by UGT2B17 have any effects on testosterone glucuronidation activity with this enzyme.

Epirubicin and zidovudine are antiretroviral drugs that are glucuronidated by UGT2B7 (Belanger *et al.*, 2009), the most active UGT enzyme in the glucuronidation of epitestosterone (Sten *et al.*, 2009a). It has also been demonstrated that the combination of epirubicin and ziduvudine can lead to drug-drug interactions leading to competitive inhibition between the two drugs when one of the drug concentrations is increased (Belanger *et al.*, 2009).

1.5.2 Pharmacogenetic variations

UGT2B17 polymorphisms contribute up to 66% of variations in T/E ratio. A deletion polymorphism in UGT2B17 has been identified, with the absence of UGT2B17 in some

individuals as a result of having this polymorphism. All individuals contained copies of the low rate testosterone glucuronidating UGT2B15, regardless of the presence of UGT2B17. Genotype of UGT2B17 is categorised as insertion/insertion (ins/ins), insertion/deletion (ins/del), deletion/deletion (del/del) (Wilson *et al.*, 2004).

Deletion in UGT2B17 genotype has been associated with low or negligible testosterone excretion in individuals following the administration of testosterone (Rane and Ekstrom, 2012; Schulze *et al.*, 2008b). Excreted levels of epitestosterone are consistent regardless of the UGT2B17 genotype (Schulze *et al.*, 2008b; Juul *et al.*, 2009). These genotypes have therefore impacted urinary T/E values within individuals. Following the administration of testosterone, T/E ratios rose rapidly to almost 100 within individuals that have the ins/ins genotype and 50 in the ins/del genotype. However, there was only a very small increase in T/E of less than 5 with individuals that had the del/del genotype (Rane and Ekstrom, 2012; Schulze *et al.*, 2008b). Urinary T/E ratios are naturally lower in individuals with the del/del genotype (Juul *et al.*, 2009). Serum testosterone concentrations were higher in homozygous deletions of UGT2B17 compared to those with increased copy numbers (Yang *et al.*, 2008). Subtle differences were observed in serum testosterone levels based on the UGT2B17 genotype following testosterone undecanoate substitution therapy in hypogonadal men (Bang *et al.*, 2013).

Differences in the distribution of UGT2B17 polymorphisms have been shown amongst ethnic groups. The del/del genotype has been demonstrated to be seven times more prevalent in Korean asian men (66.7%) then in Swedish caucasian men (9.3%). The distributions in genotype were correlated by Swedish caucasian men excreting more testosterone and the T/E ratio was significantly higher than compared to Korean asian men

(Jakobsson *et al.*, 2006). Variation in UGT2B17 copy number has been associated with increased susceptibility for osteoporosis where expression in UGT2B17 gene copy number correlated with characteristics associated with osteoporosis owing to changes in bone density (Yang *et al.*, 2008).

Another polymorphism of UGT2B17 has also been characterised that is based around the promoter region which affected glucuronidation rates (Hu *et al.*, 2010). UGT2B17 carriers of this polymorphism may reduce further, the amount of excreted testosterone that occurs along with the deletion polymorphisms of UGT2B17 (Rane and Ekstrom, 2012). This would be due to the already reduced expression of UGT2B17 being further altered by an additional polymorphism at the promoter region.

Genetic variations in other androgen phase II glucuronidating enzymes have been identified. A genetic polymorphism (H268Y) in the most active epitestosterone glucuronidating enzyme, UGT2B7 does not affect epitestosterone glucuronidation activity (Rane and Ekstrom, 2012; Swanson *et al.*, 2007).

Cytochrome P450 (CYP)17 phase I metabolising enzyme has a polymorphism that is associated with increased urinary epitestosterone glucuronides. The polymorphism was in the CYP17 promotor region (A/2 or A2 A^{-1}/2) which had the higher urinary epitestosterone concentrations. The A1/A1 polymorphism of this enzyme had higher T/E ratios due to less urinary epitestosterone excreted (Rane and Ekstrom, 2012; Schulze *et al.*, 2008a).

1.5.3 Clinical implications of altering testosterone glucuronidation

The impact of altering circulating and excreting testosterone through inhibition and pharmacogenetic variations of UGT2B17 could have several significant clinical implications. A number of studies have been highlighted in a recent review (Thind *et al.* 2013), that shows increased risks of prostate cancer based on altered UGT2B17 function as a result of pharmacogenetic variations. This is due to increased circulating active testostosterone and dihydrotestosterone in the prostate region. However there are conflicting reports as to the severity of these polymorphisms can have on prostate cancer. The effects of any inhibition of UGT2B17 inhibition on prostate cancer is unknown, however if UGT2B17 could alter glucuroidation rates similar to those of polymorphisms, this could also impact on prostate cancer risk.

Conditions such as hypogonadism that involve testosterone deficiencies and testosterone replacement therapy could also be affected from inhibition of testosterone glucuronidation activity. A recent study (Bang *et al.*, 2013) showed subtle changes to serum testosterone levels following the administration of testosterone undecanoate therapy in hypogonadal men with different UGT2B17 polymorphism, particularly due to the lower excretion of testosterone with individuals having the deletion carriers of UGT2B17. The inhibition of UGT2B17 could contribute to reducing the levels of testosterone excretion, this could have implications in testosterone replacement dosages and increasing the bioavailability of testosterone in hypogonadal individuals.

1.6 Dietary substances and compounds

1.6.1 Tea flavonoids and catechins

Tea is a beverage consumed worldwide, derived from the species *Camellia sinensis*. There are various types of tea produced that are determined by the manufacturing process, displayed in Figure 1.9. The type of tea determines compound composition including levels of phenolic compounds depending on the manufacturing process (Hilal and Engelhardt, 2007).

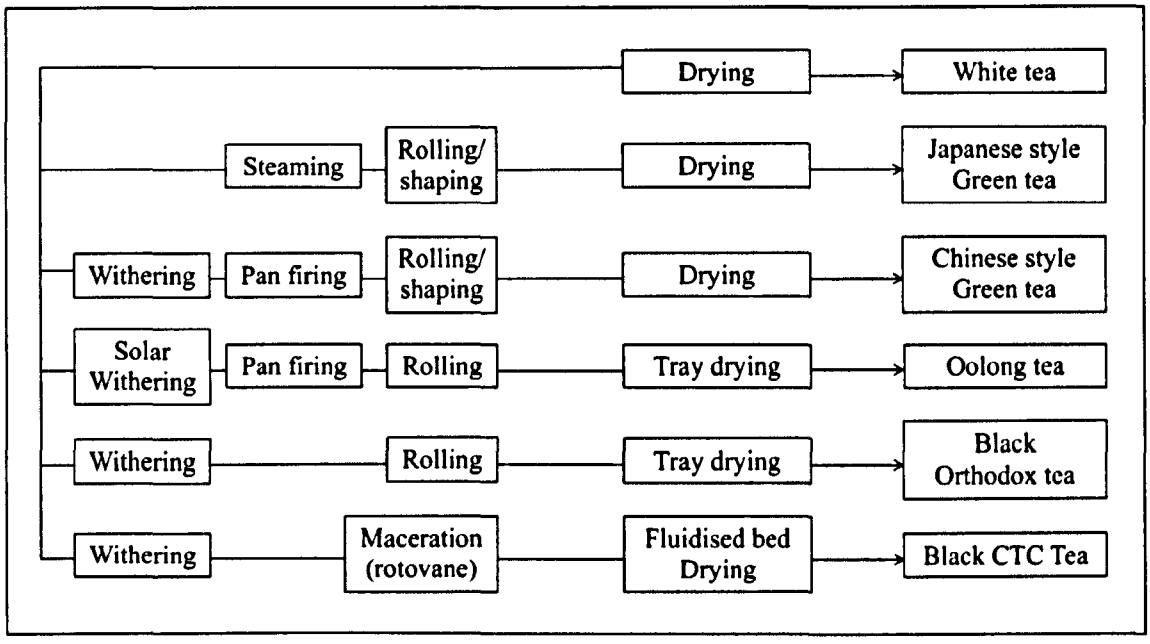


Figure 1.9 Processes involved in tea manufacturing and the different types of tea manufactured. Adapted from Hilal and Engelhardt, 2007.

Polyphenols that are commonly found in teas are secondary metabolites from plants and are used in physiological roles such as plant regulation and providing defence systems. The major polyphenols found in tea are flavonoids, in particular the flavonols (flavan-3-

ols), known as catechins (Wang and Ho, 2009). Flavonoids including catechins are capable of altering the activity of a number of enzymes in plants, animals and humans (Rusak *et al.*, 2008; Di Carlo *et al.*, 1999). Flavonols are found in a number of dietary substances in addition to teas including vegetables and fruits as well as other beverages such as red wine (Scalbert and Williamson, 2000). An increase in the consumption of green tea has been associated with increased plasma and urine concentrations of epigallocatechin gallate (EGCG), epigallocatechin (EGC) and epicatechin (EC). These catechin concentrations increased rapidly over two hours and gradually returned to original values after 8 hours (Yang *et al.*, 1998).

Catechins can make up to 30% composition of the dry weight of some green and white teas. Tea catechins are found mainly in green and white teas with very low concentrations in black teas owing to growing conditions and steps in processing (Hilal and Engelhardt, 2007). The most common catachins found in teas are catechin (C), EC, epicatechin gallate (ECG) and EGCG. Other isomers of catechins are found in smaller amounts, including gallocatechin (GC), epigallocatechin (EGC) and catechin gallate (CG). The structures of these catechins are shown in Figure 1.10. The composition of some of the common catechin compounds and quercetin found in a sample of green tea and black tea are shown in Table 1.1. The green tea had a greater composition of catechin compounds compared the sample of black tea.

Table 1.1 Concentrations of catechins and quercetin in a sample of green and black tea. Information taken from United States Department of Agriculture (USDA) database for the flavonoid content of selected foods (2003).

Flavonoid	Green tea brewed (mg/100g)	Black tea brewed prepared with tap water (mg/100g)
(-)-Epicatechin	8.47	2.33
(-)-Epicatechin 3-gallate	20.95	0.01
(-)-Epigallo catechin	17.08	0.17
(-)-Epigallocatechin 3-gallate	82.89	11.43
(+)-Catechin	2.73	1.52
Quercetin	2.69	2.07

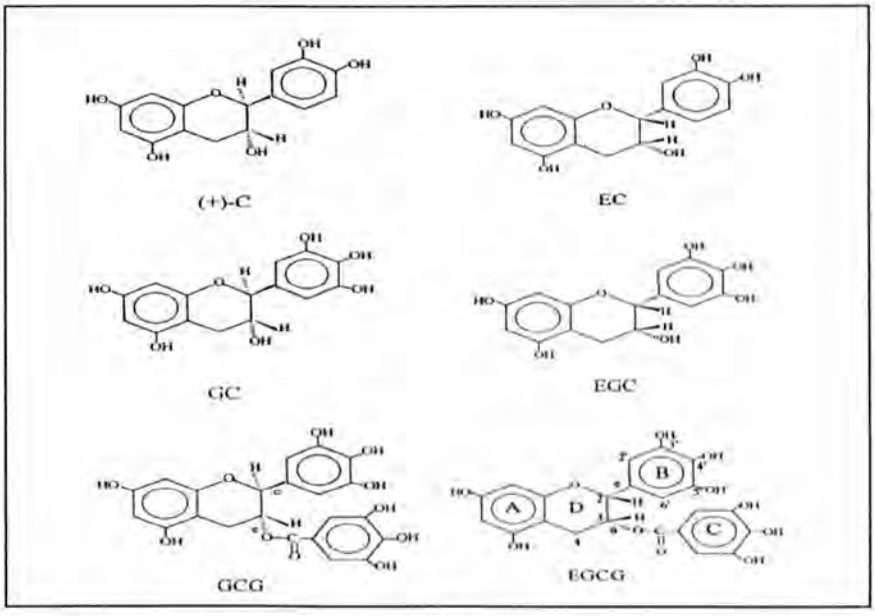


Figure 1.10 Catechin structures (taken from Guo *et al.*, 1999).

There are a number of therapeutic actions associated with catechins, some of these therapeutic effects are a result of their antioxidant properties. This includes anticancer activity and protection from various cancers as well as cardiovascular and neurodegenerative diseases (Zaveri, 2006; Mukhtar and Ahmad, 2000; Mandel *et al.*,

2006). Other therapeutic activities of catechins include antithrombotic (Kang *et al.*, 1999), antiviral (Song *et al.*, 2005) and antimicrobial effects (Taylor *et al.*, 2005).

Catechins have been shown to alter the metabolic activity of enzymes through inhibition. ECG and EGCG inhibit the activity of collagenase (Makimura *et al.*, 1993). Different catechin isomers had varying inhibitory action on DNA methyl transferase, in this case the gallic acid moiety was important in the high affinity inhibitory action (Lee *et al.*, 2005).

Oral administration of green tea extracts interfered with sex hormones in rats. It was shown that there was increased plasma testosterone along with elevation of other hormones including LH and FSH over 8 weeks (Satoh *et al.*, 2002). On a short term basis, following the intraperitoneal (iP) administration of EGCG in rats, hormone levels in serum including testosterone dropped over 7 days, whilst the administration of EC, EGC and ECG had no short term effects on hormone levels (Kao *et al.*, 2000).

UGT enzymes have shown glucuronidation activity towards catechins through studies involving human liver microsomes and individual UGTs. Increased activity of UGT in rats has been shown following long term ingestion of green tea. It has been postulated that green tea promotes the inactivation of chemical carcinogens through increased UGT activity towards these substrates, making them inactive and facilitating excretion (Crespy *et al.*, 2004). Human UGTs including UGT1A1, 1A3, 1A8 and 1A9 had glucuronidation activity against EGCG. EGC was also glucuronidated by these enzymes but at a lower rate (Lu *et al.*, 2003). Catechin and EC have been found to be glucuronidated in rat small intestine. The major metabolites that transferred across the intestinal epithelium were glucuronidated and O-methylated metabolites (Kuhnle *et al.*, 2000). Analysis by liquid

chromatography tandem mass spectrometry (LC-MS/MS) indicated EC and EGC are excreted as free fraction, O-methylated and glucuronidated compounds in human, rat and mouse urine (Li *et al.*, 2001). Following the consumption of red wine (120 mL), plasma samples revealed metabolites of catechins present including methylated, sulphated and glucuronide conjugates. These concentrations peaked after one hour and remained in plasma for over 8 hours. Following red wine consumption (120 mL), total catechin concentration in plasma peaked at 90 nMol/L after one hour, following this total catechin gradually decreased over an 8 hour period (Donovan *et al.*, 2002).

1.6.2 Red wine and phenolic compounds

Organic substances are the main constituents of red wine. This includes substances such as sugars, acids, salts, alcohols (including ethanol), flavonoids and polyphenols. The concentration of flavonoids and polyphenols in red wine is usually less than a total of 1 mg/mL. Red wine is also composed of inorganic substances including salts and metal ions. These compounds are present at lower concentrations in the range of 0.1-10 μg/mL (Grindlay *et al.*, 2011).

Red wine consists of a number of phenolic compounds that are subcategorised based on their structure. The structures of key phenolic compounds found in red wine are shown in Figure 1.11. Red wine consumption significantly increases concentrations of phenolic compounds in plasma (Caccetta *et al.*, 2000), and urine (Duthie *et al.*, 1998), this includes increases in urinary excretion of catechin metabolites following red wine consumption (Donovan *et al.*, 2002).

Non-flavonoids are structures which have single aromatic ring with at least one hydroxyl group. Examples of non-flavonoids include hydroxyl-benzoic acids (e.g. gallic and vanillic acid) and hydroxyl-cinnamic acids (e.g. caffeic and chlorogenic acid). Polyphenols that include catechin, have a structure that consists of multiple phenol rings (Waterhouse, 2002).

Polyphenolic compounds include flavonoids that consist of a flavone benzo-gamma-pyrone structure (Cao *et al.*, 1997; Havsteen, 2002). The major classes of flavonoids include flavan-3-ols (e.g. catechin isomers), flavonols (e.g. quercetin), flavones (e.g. luteolin), flavanones (e.g. naringenin) and cyanins (e.g. tannins) (Havsteen, 2002; Arct and Pytkowska, 2008). Phenolic compounds and flavonoids are found in numerous other foods including fruits and vegetables (Yao *et al.*, 2004).

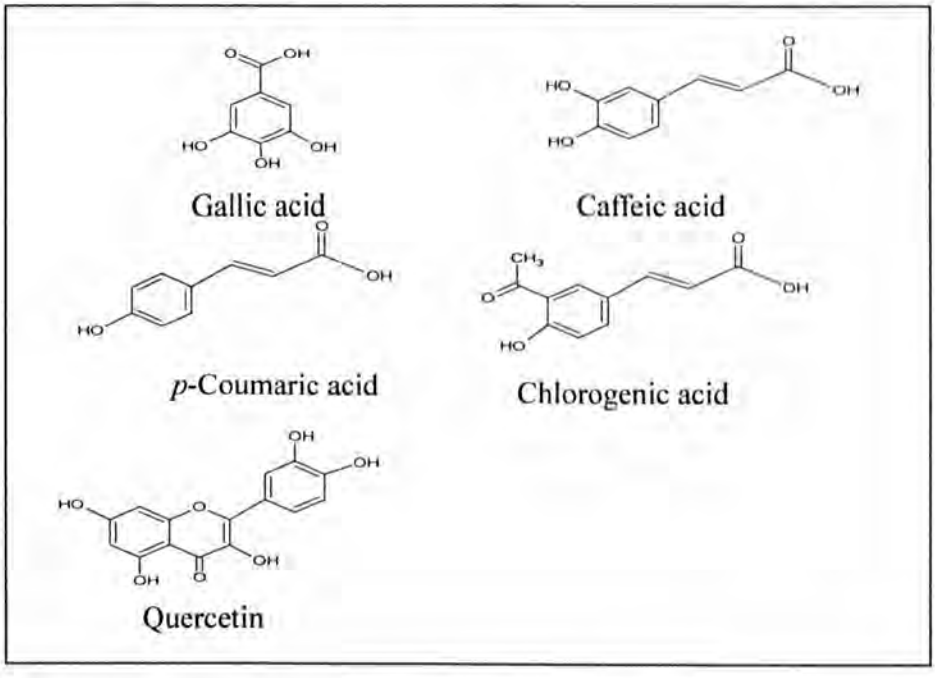


Figure 1.11 Structure of common phenolic compounds in red wine used in this study. Structures drawn in Chemsketch version 11.02.

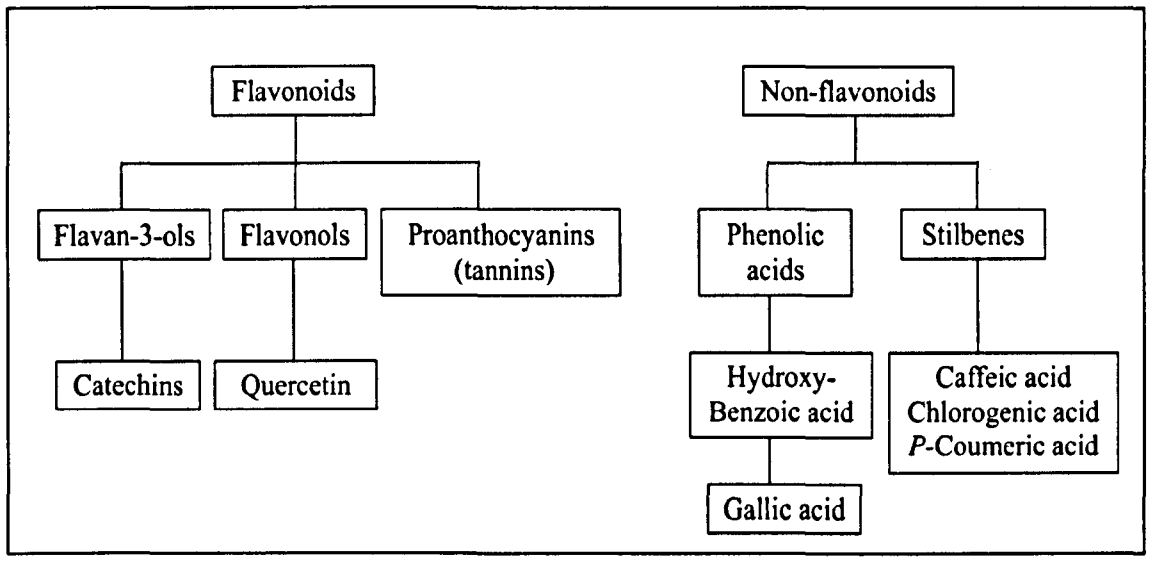


Figure 1.12 Phenolic compounds found in red wine used in this study (Waterhouse, 2002).

A number of health benefits have been associated with phenols in red wine that are similar to those mentioned above on the health benefits of catechins in teas. Polyphenols in red wine act as antioxidants through free radical scavenging and metal ion chelation (Prochazkova *et al.*, 2011). This antioxidant effect reduces the damage caused from enhanced numbers of free radicals to human cells, reducing the accumulated damage from free radicals that can lead to the onset common diseases in major organs throughout the body (Yoo *et al.*, 2010). Free radical scavenging occurs through the OH group on the phenolic compound, this involves donating an electron that stabilises free radicals through resonance (Fraga *et al.*, 2010; Yoo *et al.*, 2010).

The interactions of red wine phenolic compounds with specific enzymes play an important role in their health benefits. Phenolic compounds act as inhibitors with enzyme groups that have purines as substrates such as kinases, ATPases and reverse transcriptase. Inhibition also occurs with enzymes that have NADPH as cofactors including aldose reductase, lactic dehydrogenase and 11β-hydroxysteroid dehydorgenase (Fraga *et al.*, 2010). Flavonoid

compounds have anti-inflammatory activity through inhibition of the enzyme involved in prostaglandin synthesis, prostaglandin cyclooxygenase (Wang *et al.*, 2011).

Quercetin is present in fruits and particularly fruit beverages such as grape juices along with being found at high concentration in green and black teas ranging between 1.0-2.3 mg/100 mL (Aherne and O'brien, 2002). Quercetin is usually most abundant in red wine with concentrations ranging up to 8.84 mg/L in samples (Fang *et al.*, 2007). The metabolites of quercetin found in plasma include quercetin glucuronide moieties and sulphates (Manach *et al.*, 2004; Day *et al.*, 2001).

1.7 Conclusions

From the literature it can be seen that UGT enzymes play an important role in the inactivation and elimination of steroids, with particular emphasis on UGT2B17 being the most active enzyme in testosterone glucuronidation and UGT2B7 is the most active in epitestosterone glucuronidation. The regulation of excreted concentrations of testosterone and epitestosterone is important in providing an accurate account of steroid levels and any external administration of testosterone owing to the urinary T/E ratio used by WADA to detect testosterone abuse in sport. There is little evidence on the role common compounds such as those present in commonly consumed dietary substances could interact and inhibit UGT enzymes and therefore interfere with AAS glucuronidation.

This project explores the roles of certain dietary and pharmaceutical compounds on the effects of testosterone and epitestosterone glucuronidation. A number of potential inhibitors to be analysed include catechin and flavonoid compounds that have been

reported to be glucuronidated by UGTs and act as inhibitors with a variety of enzyme types. From a doping point of view, these results could highlight the implications on the current T/E ratio doping test if there are any alterations to glucuronidation rates following the addition of these commonly consumed substances. This thesis explores the interaction of epitestosterone on UGT2B17 testosterone glucuronidation at ng/mL concentrations as well as how glucuronidation rates vary between male and female liver microsome samples.

1.8 Aims and objectives

The aim of this study was to determine the inhibitory effects, if any, of dietary and pharmaceutical components on the glucuronidation of steroids *in vitro*. This aim had particular emphasis on determining inhibitory action of specific dietary components including teas and red wine on testosterone and epitestosterone glucuronidation. The main aim of this study is sub-categorised which are outlined in the following chapters.

Chapter 2 – Assay and analysis methods

The aim of the chapter is to describe the methods and validation procedures used for the studies in the proceding chapters.

- Enzyme assay method and sample preparation.
- HPLC method development and validation for the analysis of steroids and dietary samples.
- Extraction methods for analysis of steroids by LC-MS/MS.

Chapter 3 – Testosterone glucuronidation inhibition studies

The aim of this chapter is to determine the inhibitory action, if any, the dietary components and compounds have on UGT2B17 mediated testosterone glucuronidation.

- Determine testosterone glucuronidation activity of UGT2B17 supersomes and pooled human liver microsomes.
- Perform the screening assays of dietary substances for inhibitory activity on testosterone glucuronidation to assess if any of the samples significantly inhibit UGT2B17 activity.
- Perform HPLC analysis of dietary samples to determine key catechin and phenolic compounds present in these samples.
- Identify active compounds inhibiting UGT2B17 and perform kinetic analysis to determine expressed inhibition.

Chapter 4 – Dietary and pharmaceutical inhibition of epitestosterone glucuronidation and the role of epitestosterone on testosterone glucuronidation.

The aim of this chapter is to assess any inhibition that dietary components and compounds have on UGT2B7 mediated epitestosterone glucuronidation, particularly focussing on foods and compounds that inhibited the activity of UGT2B17. Another aim was to monitor the interaction of epitestosterone on the glucuronidation of testosterone by UGT2B17 at ng/mL concentrations using LC-MS/MS.

- Determine the rate of epitestosterone glucuronidation using UGT2B7 supersomes.
- Identify if epitestosterone interferes with testosterone glucuronidation at ng/mL concentration levels.

- Determine if there are differences in testosterone glucuronidation with male and female pooled microsomes and the changes that occur by adding epitestosterone at increasing concentrations.
- Analyse the inhibitory effects of compounds from results obtained in chapter 3, on UGT2B7 epitestosterone glucuronidation.
- Perform inhibitory studies on two antiretroviral pharmaceuticals to determine if these compounds inhibit epitestosterone glucuronidation by UGT2B7.

Chapter 5 – Effects of diclofenac and stanozolol administration on urinary testosterone and epitestosterone excretion in rats and implications on T/E ratios.

The aim of this chapter is to determine the changes in testosterone and epitestosterone concentrations in rat urine, following the administration of diclofenac and stanozolol.

- Perform validation of a method developed for the determination of testosterone and epitestosterone in rat blood and urine using LC-MS/MS to determine accuracy, precisions, extraction recovery and the optimum deconjugation conditions for β-glucuronidase hydrolysis.
- Analysis of testosterone and epitestosterone in rat samples through the duration of study to monitor the effects of diclofenac on testosterone and epitestosterone levels in rat urine.
- Examine the glucuronidation activity of microsomes of each of the rats following the termination of the study by performing testosterone glucuronidation assays *in vitro*.
- Monitor the effects stanozolol and 3'-hydroxystanozolol have on UGT2B17 testosterone glucuronidation *in vitro*.

Chapter 6 – Discussion and future work.

The aim of this chapter is to describe the results obtained in the studies from this project and their implications.

- General discussion and limitations of the study.
- Conclusions and future work.

1.9 Summary of study

A summary of the project design, processes and flow of the study, along with subject areas influenced by this project is shown in Figure 1.13.

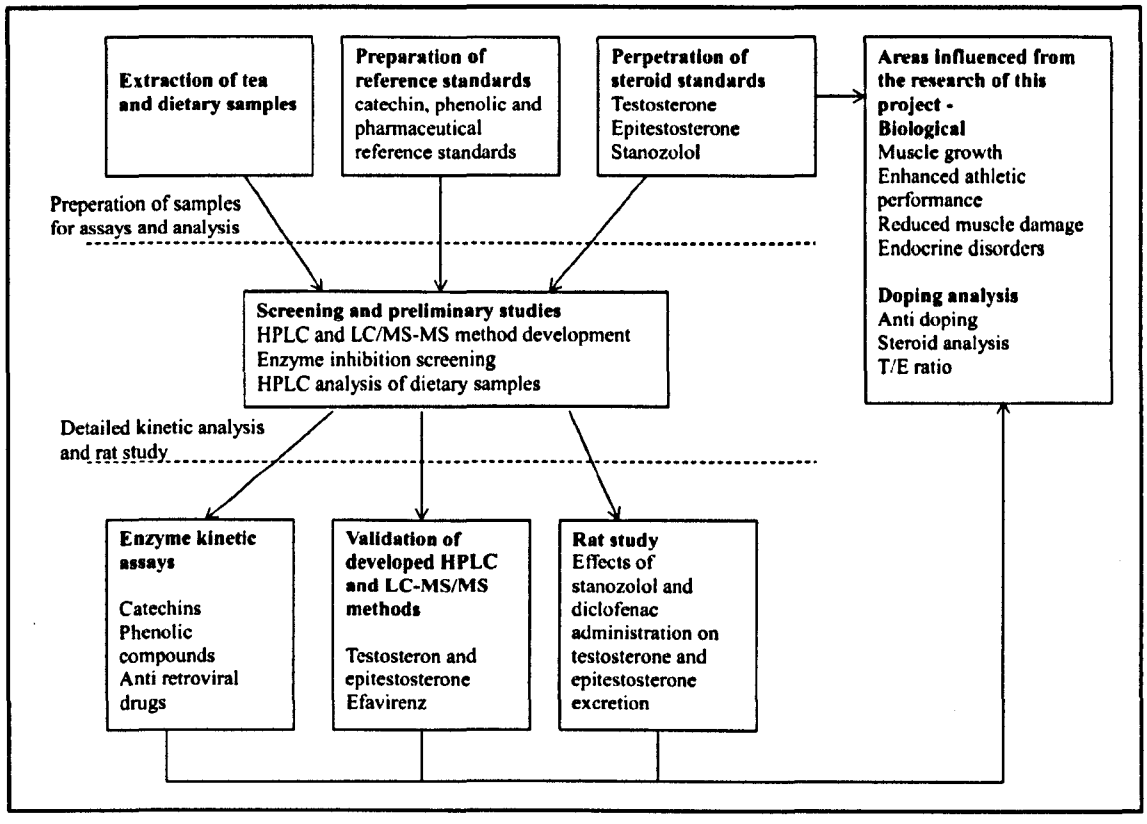


Figure 1.13 Flow chart summarising the experimental design and stages in this project along with the properties of anabolic steroids.

Chapter 2. Materials and methods

This chapter describes the materials and details the method used throughout this project in chapters 3-5. The chemicals, solvents, phenolic standards and consumables were purchased from Sigma Aldrich (Poole, UK), Fisher Scientific (Loughborough, UK), LGC Standards (Teddington, UK), Agilent Technologies (Stockport, UK), BD Biosciences (Oxford, UK), Roche Diagnostics (Burgess Hill, UK), Millipore (Watford, UK), Sera Laboratories (Haywards Heath, UK) and Nacalai USA, Inc. (San Diego, USA). The chemicals, solvents and phenolic standards used were either analytical or HPLC grade, along with LCMS grade water and acetonitrile.

2.1 Chemicals, reagents and instrumentation

Table 2.1 Chemicals used throughout project.

Chemicals	Item number/size	Grade	Source
Japanese Sencha Green Tea	-	-	Waitrose, London, UK
White Yunnan Leaf tea, China (White tea leaves)	-	-	Fortnum & Mason, London, UK
Silver dragon beard white tea, China (White tea beard)	-	-	Fortnum & Mason, London, UK
Botany white tea (White tea powder)	VBD-67430	Bio reagent	BotanyCare, France.
JP Chenet cabernet-syrah red wine	-	-	Sainsbury's, London, UK
Organic cacao beans	-	-	Waitrose, London, UK
Madascan black cacao block	-	-	Waitrose, London, UK
Chocolate confectionary reference standard	LGC7016/15 g	Analytical	LGC Standard
UGT2B17 supersomes	456437/ 0.5 mL	Bio reagent	BD Biosciences
UGT2B7 supersomes	456427 0.5 mL	Bio reagent	BD Biosciences
Pooled human microsomes	452161/0.5 mL	Bio reagent	BD Biosciences
Pooled human male microsomes	452172/0.5 mL	Bio reagent	BD Biosciences
Pooled human female	452183/ 0.5 mL	Bio reagent	BD Biosciences

microsomes			
UGT reaction mixture solution A	451300/2mL	Bio reagent	BD Biosciences
UGT reaction mixture solution B	451320/5 mL	Bio reagent	BD Biosciences
UDPGA	U6751/100 mg	Analytical	Sigma Aldrich
Tris-buffer	T1378/100 g	Analytical	Sigma Aldrich
Magnesium chloride	M8266/100 g	Analytical	Sigma Aldrich
Alamethecin	A4665/5 mg	HPLC	Sigma Aldrich
Pooled rat urine, 4xcharcoal stripped.	USD-909-DHC	Bio reagent	Sera laboratories
Pooled male rat plasma, 4xcharcoal stripped, lithium herapin.	PLHSDM-909-DHC & PLHSDM-909-XHC/4x10mL.	Bio reagent	Sera laboratories
Male brown norway rat whole blood, lithium herapin.	BLHNBIM-909-V-29894/ 6x100 µL	Bio reagent	Sera laboratories
β-glucuronidase	03707598001/5 mL	Bio reagent	Roche Diagnostics
Sodium hydroxide	306576/25 g	Analytical	Sigma Aldrich
Hydrochloric acid			
Orthophosphoric acid	O/0450/PB17/2.50 L	Reagent	Fisher Scientific
Sodium hydrogen phosphate dibasic heptahydrate	431478/50 g	Analytical	Sigma Aldrich
Sodium phosphate monobasic dihydrate	71505/250 g	Analytical	Sigma Aldrich
Reference standards			
(-)Gallocatechin	G6657-1 mg	HPLC	Sigma Aldrich
Caffeine	27600-100 g	HPLC	Sigma Aldrich
(-)Epigallocatechin from green tea	E3769-5 mg	HPLC	Sigma Aldrich
(-)Epicatechin gallate from green tea	E3893-10 mg	HPLC	Sigma Aldrich
(-)Catechin gallate from green tea	C0962-1 mg	HPLC	Sigma Aldrich
(-)Epigallocatechin gallate	E4143-50 mg	Analytical	Sigma Aldrich
(-)Epicatechin	E1753/1 g	HPLC	Sigma Aldrich
(+)Epicatechin	02573-34/10 mg	HPLC	Nacalai, USA
4-Ethylphenol	E44205/5 g	Analytical	Sigma Aldrich
Gallic acid	G7384/100 g	Analytical	Sigma Aldrich
Caffeic acid	C0625/2 g	HPLC	Sigma Aldrich
p-Coumaric acid	C9008/1 g	HPLC	Sigma Aldrich
Chlorogenic acid	C3878/250 g	Analytical	Sigma Aldrich
Quercetin	Q4951/10 g	HPLC	Sigma Aldrich
1-Naphthol	N1000/10 g	Analytical	Sigma Aldrich
Ziduvodine	ICRS1420/100 mg	Analytical	LGC Standard
Efavirenz	ICRS1411/100 mg	Analytical	LGC Standard
Diclofenac sodium salt	D6899-10G	Analytical	Sigma Aldrich
Ibuprofen	I4883/1G	GC	Sigma Aldrich
Testosterone	T1500/ 1 g	Analytical	Sigma Aldrich
Epitestosterone	E5878/ 100 mg	Analytical	Sigma Aldrich
Testosterone glucuronide	T-2000/5 mg	Analytical	Sigma Aldrich

Epitestosterone glucuronide	NMIAD603/1 mg	Analytical	LGC Standard
Stanozolol	NMIAD646/10 mg	Analytical	LGC Standard
3'Hydroxystanozolol	NMIAD577/ 1 mg	Analytical	LGC Standard
Stanozolol-D3 (0.1 mg/mL) in dimethoxyethane	CERS-910/1 mL	Analytical	LGC Standard
Testosterone-D3	NMIAD644/1 mg	Analytical	LGC Standard

Table 2.2 Solvents used.

Solvents	Grade	Source
Water	HPLC Chromasolv	Fisher Scientific
Water	LC-MS Chromasolv	Sigma Aldrich
Water	Deionised	Elga Process Water
Acetonitrile	HPLC Chromasolv	Fisher Scientific
Acetonitrile	LC-MS Chromasolv	Sigma Aldrich
Methanol	HPLC Chromasolv	Fisher Scientific
Ethanol	200 proof (absolute), for molecular biology.	Sigma Aldrich
Pentane	HPLC Chromasolv	Sigma Aldrich
Dimethyl sulphoxide (DMSO)	D/4120/PB08/500 L	Fisher Scientific

Table 2.3 Consumables used.

Consumables	Description	Source
Ascentis C18 column	25 cm x 4.6 mm, 5µm	Sigma Aldrich
Kromasil C18 column	25 cm x 4.6 mm, 5µm	Sigma Aldrich
Zorbax C18 column	2.1 mm x 150 mm, 1.8µm	Agilent Technologies
Ascentis guard cartridge	2 cm x 4 mm, 5µm	Sigma Aldrich
Inline column filter	Inline filter 2µm frits	Agilent Technologies
Syringe driven filter	0.45 µm sterile MEC	Millipore
Syringe driven filter	0.20 µm PTFE	Millipore

Table 2.4 Instrumentation

Instruments	Model	Source
HPLC	1260 Agilent infinity HPLC. Agilent 1260 Diode array VL+.	Agilent Technologies
LC-MS/MS	1260 Agilent infinity LC system. 6430 triple quadruple mass spectrometer.	Agilent Technologies
LC-MS/MS	Accela UPLC system. Triple Quadropole TSQTM mass spectrometer.	Thermoscientific

Centrifuge	5810R temperature controlled	Eppendorf
Microcentrifuge	Minispin	Eppendorf
Vortex mixer	IKA vortex genius 3	IKA
Sample concentrator	Techne Dri-block DB-3D	Fisher Scientific
pH meter	Hydrus 600 benchtop series	Fisher Scientific
Water bath	OLS 200	Grant Instruments

2.2 HPLC and LC-MS/MS instrumentation

Agilent HPLC along with Agilent and Thermoscientific LC-MS/MS systems were used for analysis in all the projects detailed in later chapters. Figure 2.1 displays images of the analytical machinery described and used in this study. During method validation, the sensitivity of testosterone and epitestosterone analysis was greater on the Agilent LC-MS/MS than the Thermoscientific LC-MS/MS. The LC-MS/MS used for the specific projects in this study was determined by the concentration range of testosterone and epitestosterone that would be analysed. The Agilent HPLC system consisted of a binary pump, degasser, autosampler and column heater, coupled with an Agilent 1260 diode array (DAD) detector VL+. The Agilent HPLC was controlled using Agilent Chemstation software.

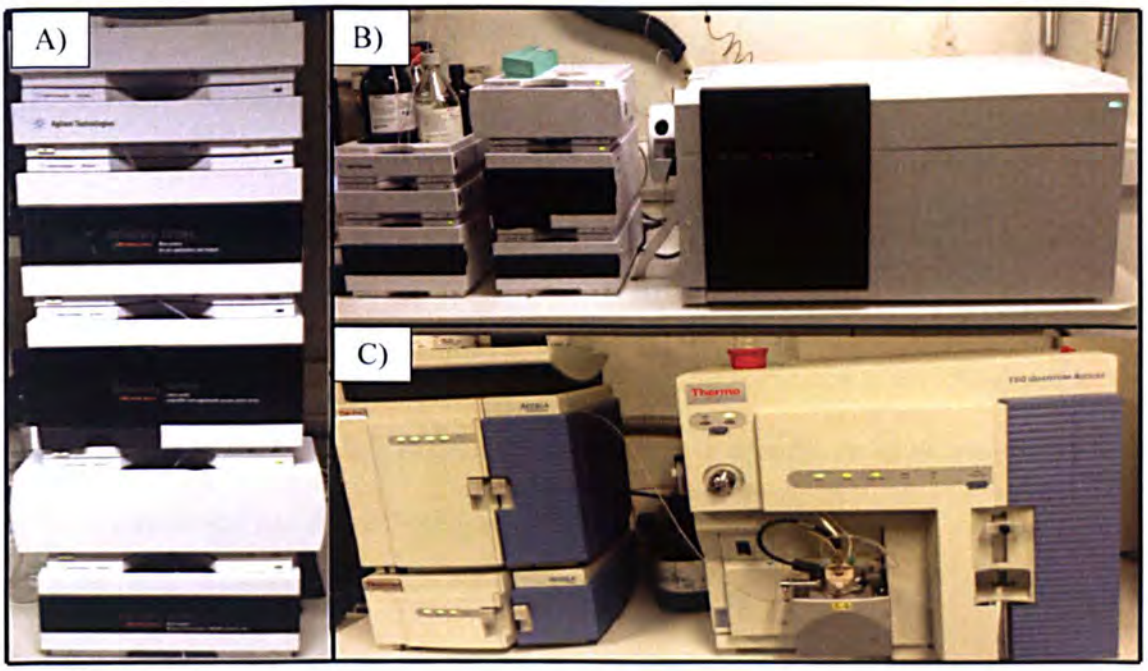


Figure 2.1 Analytical machinery used in this project. A) Agilent HPLC, B) Agilent LC/MS-MS, C) Thermoscientific LC-MS/MS.

The Agilent LC-MS/MS system was a 1260 Agilent infinity LC system consisting of a binary pump, degasser, temperature controlled autosampler and column heater. The LC system was coupled to a 6430 triple quadruple mass spectrometer. Optimisation of mass spectrometer parameters was performed using Masshunter optimizer software (version B.03.01). The LC-MS/MS system was controlled using Masshunter workstation software (LC/MS data acquisition, version B.03.01).

The Thermoscientific LC-MS/MS system was an Accela ultra performance liquid chromatography (UPLC) system consisting of a quaternary pump, degasser, column heater and temperature controlled autosampler. The UPLC system was coupled to a Triple Quadropole TSQTM mass spectrometer. The LC-MS/MS system was operated using Xcalibur version 2.0 software.

2.3 Dietary sample preparation and analysis by HPLC-DAD

2.3.1 Dietary samples

Tea extracts and the cacao beans for inhibition screening studies and catechin analysis by HPLC were prepared by manually grinding to fine particles using a pestle and mortar. The cacao block was grated manually to fine particles. 1.6 g of each sample was dissolved in 80 mL of boiling water and left for 5 minutes, stirring manually on occasions. The tea solution was cooled and filtered using a Millex 0.45 μM syringe filter.

Red wine for inhibition screening studies was added directly to reactions following filtration using a Millex 0.45 μM syringe filter. An evaporated red wine sample was prepared for further inhibition assays and analysis of phenolic compound in the sample by HPLC by evaporating 5 mL of red wine to dryness. The remaining dried residue was dissolved in 5 mL water; vortex mixed and filtered using a Millex 0.45 μM syringe filter.

Other dietary samples including fruit samples were prepared by initially washing in deionised water and removing stems manually. Samples were crushed manually, vortex mixed and centrifuged for 10 minutes at 3220 x g. The supernatants were removed and filtered using Millex 0.45 μM syringe filter. All samples were stored at 4 °C until use and aliquots where stored at -20 °C.

2.3.2 Analysis of dietary samples by HPLC analysis

HPLC analyses of tea, wine and dietary samples were performed to identify common catechins and phenolic compounds present in these samples. The samples injected were

prepared for analysis as described in section 2.3.1. Stock catechin and phenolic standards were dissolved in either water or ethanol, depending on solubility at the concentration of 1 mg/mL and stored at -20°C. Working standards for analysis were prepared at concentrations ranging from 10-200 µg/mL.

2.3.2.1 Catechin analysis

HPLC analysis was used to assess the catechin compounds present in tea and dietary samples, with a variety of mobile phases used with varying detector wavelengths based on previous methods researched (Wang *et al.*, 2000) as shown in Table 2.5.

Table 2.5 Mobile phases and detector UV wavelengths used to determine the optimum method for analysis of catechin compounds in teas and other dietary components.

Mobile phase	**Ratio**	**UV detection**
Methanol/water	20/80	210 nm
Methanol/water/orthophosphoric acid	20/79.9/0.1	210 nm and 280 nm
Acetonitrile/water	10/90	210 nm
Acetonitrile/water/orthophosphoric acid	10/89.9/0.1	210 nm

The optimal method was found to be a mobile phase of methanol/water/orthophosphoric acid (20/79.9/0.1) as previously described (Wang *et al.*, 2000). The flow rate was 1 mL/min with an injection volume of 20 µL and detection was measured at 210 nm. Catechin compounds were identified by comparing the retention times with catechin reference standards.

2.3.2.1 Analysis of phenol compounds

The determination of phenolic content in red wine was analysed as previously described (Seemungal *et al.*, 2011). The mobile phase was 0.1% orthophosphoric acid in water (A) and methanol (B). Separation of compounds was performed using a gradient method of these solvents, described in Table 2.6.

Table 2.6 HPLC mobile phase gradient for the analysis of phenol compounds in red wine.

HPLC run time (min)	**Solvent A (%) 0.1% orthophosphoric acid in water**	**Solvent B (%) Methanol**
0	80	20
10	60	40
20	50	50
30	45	55
50	35	65

The flow rate was 1 mL/min with a 10 µL injection volume with a UV detection of 280 nm. Phenolic compounds where identified by comparing the retention times with phenol standards. At the end of the run time a 10 minute equilibrium period was applied using the starting conditions before injecting the next sample.

2.4 Testosterone and epitestosterone glucuronidation assay and analysis by HPLC.

2.4.1 Assay

The glucuronidation assay for testosterone and epitestosterone was performed based on the method described in the data sheet for the human UGT2B17 supersomes. The UGT supersomes contained baculovirus cDNA engineered to express human UGT. Initial concentrations of testosterone and epitestosterone used varied throughout experiments. Testosterone was either dissolved in DMSO and added as 2% v/v of the final reaction or dissolved in acetonitrile and added at 1% v/v. Epitestosterone was dissolved in methanol and added as 1% v/v.

The standard reaction mixture contained 2 mM UDPGA, 25 μg/mL alamethecin, 8 mM magnesium chloride, 50 mM of a pH 7.5 Tris-HCl buffer and deionised water to make up total volume of 1 mL. Control samples containing just the enzyme and substrate reaction, contained the same standard mixture with the volume of water contributing to 66% of total reaction volume. This negative control is used to compare with other samples to determine inhibition. Another control sample was prepared that did not contain any of the co factor UDPGA. Diclofenac and ibuprofen were used as positive controls for UGT2B17 inhibition as these are known inhibitors of this enzyme (Sten *et al.*, 2009b). Samples were incubated at 37 °C for 5 minutes followed by the addition of UGT supersomes or human liver microsomes at a set concentration (0.05-0.2 mg/mL) to initiate the reaction. Samples remained incubated for the duration of the reaction followed by termination at a set time point, either by transferring aliquots of 100 μL from samples to 100 μL of ice cold acetonitrile or by adding 100 μL of ice cold acetonirtrile to samples and vortex mixed.

Samples were stored on wet ice prior to preparation for analysis. Samples were centrifuged at 10,000 g for 5 minutes. Aliquots of these samples were taken for analysis by HPLC (section 2.4.2) by injecting directly or for preparation for analysis by LC-MS/MS (section 2.4.3.2).

The preparation of samples following glucuronidation for analysis by LC-MS/MS was performed using liquid-liquid extraction (LLE) as previously described (Deshmukh *et al.*, 2010) that was slightly modified. An internal standard testosterone-D3 was added before centrifugation at 50 µL volume to give a concentration of 10 ng/mL. Following centrifugation, 400 µL aliquots were taken from samples and transferred to glass centrifuge tubes. LLE was performed by adding pentane and vortex mixed vigorously for 20 seconds, followed by centrifugation at 3,220 x g for 10 minutes at 4 °C. The pentane layer was transferred to a silanized vial and evaportated to dryness using a gentle stream of compressed air with the samples incubated at 40 °C. The samples were reconstituted with 200 µL of acetonitrile and filtered using a syringe driven 0.2 µM PTFE filter prior to injection for LC-MS/MS analysis.

2.4.2 Steroid HPLC analysis

Reverse phased HPLC was used for the analysis of testosterone and epitestosterone concentrations following termination of the glucuronidation reaction to determine unconjugated levels of these steroids. An initial method was developed for the analysis of testosterone dissolved in DMSO. Another reversed phase HPLC method adapted from He *et al.*, (2005a), for analysis of both testosterone and epitestosterone was used. Testosterone

and epitestosterone compounds were identified by running standards under the same conditions and determining retention times.

The HPLC method for analysis of testosterone in DMSO, separation was carried out using a 25 cm × 4.6 mm i.d., 5 μM Ascentis Supelco C18 column at 25 °C. The mobile phase was methanol and water (80:20). The flow rate was 1 mL/min with injection volume of 100 μL. Detection of testosterone 246 nm using a DAD detector.

Separation of testosterone and epitestosterone was carried out as previous described (He *et al.*, 2005), with slight modifications, using a 25 cm x 4.6 mm i.d., 5 μM Kromasil Supelco C18 column at 25 °C. The mobile phase was an isocratic gradient of water and acetonitrile (61:39) at a flow rate of 1 mL/min and an injection volume of 50 μL, detected at 245 nm.

2.4.3 LC-MS/MS analysis for the detection of testosterone and epitestosterone

Two LC-MS/MS based methods were used for analysis of testosterone and epitestosterone. These methods had differences in sensitivities, therefore the sample and concentration range analysed determined the analytical method for each experiment.

2.4.3.1 Method A (Agilent)

The method for detecting testosterone, epitestosterone and internal standard stanozolol D3 was based on a previous method (Deshmukh *et al.*, 2012a), with modifications. Separation of testosterone and epitestosterone was performed using a 2.1 x 150 mm x 1.8μM Agilent SB-C18 chromatography column with a 0.2 micron in line filter installed before the column to aid in preventing column blockage. The mobile phase was acetonitrile and

water with the gradient flow outline in Table 2.7. The flow rate was 250 μL/min. A needle wash of acetonitrile was used after each injection.

Table 2.7 LC mobile phase gradient conditions for the separation of testosterone and epitestosterone.

LC run time	Water %	Acetonitrile %
0	53	47
5	53	47
9	0	100
11	53	47
25	53	47

The mass spectrometer electrospray ionisation (ESI) source was operated in positive ion mode. Testosterone (m/z 289.3), and epitestosterone (m/z 289.3) and stanozolol D3 (m/z 332.3) protonated molecules $[M + H]^+$, were used as precursor ions for collision induced dissociation (CID) for MS-MS analysis. Multiple reaction monitoring (MRM) mode was used to monitor precursor ions. Table 2.8 outlines the MRM transitions, collision energy and retention times of testosterone, epitestosterone and stanozolol D3. Optimum ionisation conditions were; capillary voltage 4000 V, drying gas temperature 325 °C, drying gas flow rate 10 L/min, nebulising gas pressure 35 psi and fragmentor voltage of 100 V.

Table 2.8 Retention times, MRM transitions and collision energies of testosterone and epitestosterone.

Compounds	Retention Time (min)	MRM transitions	Collision energy (eV)
Testosterone	8.60	289.2 > 109	22
		289.2 > 97	26
Epitestosterone	10.58	289.2 > 109	21
		289.2 > 97	25
Stanozolol D3	11.64	332.2 > 81.2	50

2.4.3.2 Method B (Thermo Scientific)

Detection of testosterone, epitestosterone and internal standard testosterone D3 was as previously described (Deshmukh *et al.*, 2012a) with conditions altered slightly for optimisation. In this method the testosterone D3 was used as the internal standard instead of stanozolol D3. The mobile phase gradient was altered to aid the separation of testosterone and epitestosterone peaks and the MS parameters were optimised for this MS machine. The column used for separation was an Agilent SB-C18 chromatography column (2.1 x 150 mm x 1.8μM) purchased from Agilent Technologies UK Ltd. The column temperature was maintained at 45°C and the autosampler tray was set at 0 °C. The flow rate was 200 μL/min and the injection volume was 15 μL. The mobile phase was water and actonitrile using a gradient described in Table 2.9. The method was operated using selective reaction monitoring (SRM) mode with the parameters of testosterone, epitestosterone and testosterone D3, along with the collision energies and retention times are shown in Table 2.10.

Table 2.9 LC mobile phase gradient composition.

LC run time (min)	Water %	Acetonitrile %
0	53	47
1	53	47
5	53	47
9	0	100
11	0	100
12	53	47
15	53	47

Table 2.10 Retention times, MRM transitions and collision energies of testosterone and epitestosterone.

Compound	Retention time (min)	Precursor ions (m/z)	Product ions (m/z)	Collision energy (e/v)
Testosterone	7.3	289.2	97.2 109.1	20 24
Epitestosterone	8.3	289.2	97.2 109.1	20 24
Testosterone-D3	7.3	292	97.2 109.1	20 24

2.4.4 Efavirenz analysis by HPLC

Reverse phase HPLC was used for the analysis of efavirenz dissolved in methanol. The HPLC mobile phase and detection was based on an optimised method used for the data

sheet of this compound using a 25 cm x 4.6 mm i.d, 5 μM Kromasil Supelco C18 column at 25°C. The mobile phase was water and methanol (10:90) with a flow rate of 1 mL/min and a 20 μL injection volume and detected at 250 nm with a DAD detector.

2.4.5 Method validation

HPLC and LC-MS/MS methods were validated in accordance with Food and Drug Administration (FDA) guidelines to determine precision and accuracy, sensitivity, selectivity, linearity, limit of detection (LOD), lower limit of quantification (LLOQ), inter and intraday precision and percentage recovery (http://www.fda.gov/downloads/Drugs/.../ Guidances/ucm070107.pdf.\).

A negative control matrix was used for the development and validation of methods in each study. The control matrix used for all validation studies in the analysis of testosterone and epitestosterone in rat blood and urine was heavy (4x) charcoal stripped 0.2μ filtered Sprague Dawley male rat urine and plasma that had no quantified testosterone or epitestosterone present. The control matrix used to validate precision and accuracy of testosterone and epitestosterone following glucuronidation assays was a standard reaction mixture outlined in section 2.4.1, without UGT enzyme and UDPGA.

Calibration curves of known standards were prepared by spiking the matrix solution with analytes and internal standard. Quality controls (QC) were prepared in the same manner over four concentration ranges of the calibration curve. For LC-MS/MS samples, the concentration was determined by measuring the ratio of analyte to internal standard, by dividing the peak area of the analyte by the peak area of the internal standard. The calibration curve was plotted for testosterone and epitestosterone levels in plasma and

urine by using the analyte to internal standard ratio. The linearity of the calibration curve was determined using linear analysis. For HPLC samples the concentration was measured by measuring the area under the curve for the analyte.

Method accuracy and precision was measured using replicated QC samples (N=6, per concentration level) at four concentration levels along the calibration curve range and comparing these values with known concentration levels. Accuracy was measure based on FDA guidelines that state there should not be a greater deviation of 15% of QC samples compared to the actual concentration and the LLOQ QC should not deviate more than 20% from the actual LLOQ concentration (http://www.fda.gov/downloads/Drugs/.../ Guidances/ucm070107.pdf). The precision of the method was assessed by using the relative standard deviation (RSD) percentage of QC samples intra and inter-day over three consecutive days. Based on FDA guidelines, precision, intra and inter-day RSD percentage should not exceed 15% at each QC concentration level and the RSD percentage at LLOQ should not exceed 20% (http://www.fda.gov/downloads/Drugs/.../ Guidances/ucm070107.pdf).

To assess the extraction recovery of the liquid-liquid extraction method, blank matrix samples were spiked with known concentrations of testosterone and epitestosterone and stanozolol D3. The LLE extraction was carried out based on protocol described in section 2.5.2 and the samples where measured by LC-MS/MS. Extraction recovery was determined by comparing the peak areas of the analytes and internal standard, as a percentage of the peak areas of unextracted standard solutions of analytes and internal standard in acetonitrile (http://www.fda.gov/downloads/Drugs/.../Guidances/ucm070107 .pdf).

Method selectivity was assessed by analysing the matrix effect following extraction and spiking with known concentrations of testosterone, epitestosterone and stanozolol D3. The concentrations where then compared with the same concentrations of analyte and internal standard, unextracted and spiked in acetonitrile.

The deconjugation activity of β-glucuronidase was assessed by incubating the enzyme with standard concentrations ranging between 1-15 ng/mL of testosterone glucuronide and epitestosterone glucuronide along with 2 ng/mL internal standard stanozolol D3 in steroid free plasma and urine matrices. The samples were spiked with 50 µL β-glucuronidase and incubated for two hours at 50°C. The samples were then cooled on wet ice before extraction to prepare for LC-MS/MS analysis. The concentration of deconjugated testosterone and epitestosterone was compared with samples spiked at the same concentration ranges with testosterone and epitestosterone.

2.6 Rat serum and urine preparation and LC-MS/MS analytical method for testosterone and epitestosterone

2.6.1 Animals used in the project and study details.

The experiments were carried out in compliance with regulations on the housing and care of experimental animals and in harmony with the EC Council directives on laboratory animals (86/609/EEC). The rat study and sample collection was carried out at the department of Anatomy, Histology and Embryology, Semmelweis University, Budapest, Hungary by trained personnel; Dr. Gergely Zachár and Dr. Andrea D. Székely. Analysis of rat urine and microsome samples was carried out by the author.

Male brown Norway rats, weighing between 280-340 g where used in the project. Rats were split into four groups of six to investigate the effects of diclofenac and stanzozolol on testosterone and epitestosterone metabolism in rat urine. Rats were kept in a room temperature controlled environment with alternating 12 hour light and dark cycles. Food and water was freely available. Rats were administered with increasing diclofenac doses between 0-25 mg/kg, 6 times a week for 6 weeks. Stanozolol was injected as daily dosages at 5 mg/kg for 3 weeks. Further details on exact specifications on drug administration timelines and rat groups are discussed in chapter 5.

2.6.2 Serum and urine preparation, extraction and deconjugation for analysis by LC-MS/MS.

Sample preparation and extraction were performed as previously described (Deshmukh *et al.*, 2010), modified for urine and serum analysis. Samples were prepared by adding 100 µL of serum or urine to 1 mL of 0.2 M pH7 phosphate buffer. 50 µL of internal standard stanozolol D3 was added to give a concentration of 2 ng/mL. β-glucuronidase enzyme was used to deconjugate glucuronidated testosterone and epitestosterone in line with previous deconjugation experiments (Deshmukh *et al.*, 2012b; Leinonen *et al.*, 2006), modified for testosterone and epitestosterone in rat serum and urine. 50 µL of β-glucuronidase was added to solution and the samples were incubated for two hours at 50 °C. The samples were cooled on wet ice before extraction.

LLE was performed by the addition of 4 mL of pentane to samples in a glass centrifuge and vortex mixed for 20 seconds. Following this, samples were centrifuged at 3500 x g for

10 minutes at 4 °C. The organic layer was transferred to silanised vials and evaporated using a gentle stream of compressed air using a heating block to incubate the samples at 40 °C. Samples were reconstituted with 100 µL of acetonitrile and filtered for analysis by LC-MS/MS using a 0.2 micron PTFE membrane filter.

2.6.3 LC-MS/MS analysis of rat serum and urine

The LC-MS/MS analytical method described in section 2.4.3.1 was used for analysis of these samples.

2.7 Statistical analysis

Experimental data were measured either in duplicates or triplicates with the mean and standard error of mean (SEM) values calculated in Microsoft Excel. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 18. Independent sample two-tailed Student's t-test was used for comparing mean values between two groups. One way ANOVA was used was used to test significant difference between multiple groups using Tukey post hoc test when ANOVA showed significant difference. Data are presented as mean ± SEM. To test the significance of the reduction in glucuronidation activity, a one tailed, one sample t-test (Ha: activity <100%) was used with a significance level set at $p < 0.05$.

The statistical analysis in chapter 5 for measuring urinary steroid concentrations in groups of rats was performed using SPSS. One way ANOVA was used to analyse differences between groups. Partial eta-squared was used to determine the effect size of the groups

used, 0.01 was considered a small effect size, 0.06 a medium effect size and 0.14 a large effect size. Two-tailed Student's t-tests were used to analyse changes between concentrations within a group at different sample collection days. Cohen's d was used to determine the effect size were there was no significant difference.

Enzyme inhibition kinetic parameters Vmax and Km were determined using Lineweaver-Burk and Dixon plot analysis. Chemsketch version 11.02 software was used for drawing chemical structures.

Chapter 3. Interaction of dietary components and compounds on testosterone glucuronidation.

3.1 Introduction

UGT2B17 is the most active enzyme in testosterone glucuronidation, having a major role in the deactivation and excretion of testosterone. Other UGT enzymes glucuronidate testosterone at much lower rates than UGT2B17 (Sten *et al.*, 2009a). It would therefore be expected that inhibition of UGT2B17 would have the highest impact on testosterone glucuronidation. Inhibition of this enzyme could lead to changes in urinary excreted levels T/E ratios, as epitestosterone is not glucuronidated by UGT2B17 (Sten *et al.*, 2009a). The current method of detecting intake of testosterone by athletes in sporting competition is a urinary T/E ratio based on the concentration of testosterone glucuronide over epitestosterone glucuronide in urine (Sten *et al.*, 2009b). Alterations to the rates of glucuronidation of testosterone could alter excreted levels of testosterone, which could affect the T/E ratio.

Two NSAIDs, diclofenac and ibuprofen were found to competitively inhibit and reduce the rate of testosterone glucuronidation by UGT2B17 and UGT2B15 (Sten *et al.*, 2009b) *in vitro*. It had previously been shown that NSAIDs are glucuronidated by UGT enzymes in human liver microsomes (Kuehl *et al.*, 2005). This study demonstrated that combining the NSAIDs with testosterone as a substrate led to a reduced rate of glucuronidation. Apart from these NSAIDs, there is little knowledge on inhibitors of testosterone glucuronidation by UGT2B17, particularly relating to substances that are regularly consumed such as dietary foods.

UGT2B17 is involved in the glucuronidation of other AAS including androstane (Belanger *et al.*, 2003), as well as glucuronidating a number of phenolic and xenobiotic compounds. Examples of phenolic compounds that have been glucuronidated include 4-ethylphenol and 1-naphthol (Turgeon *et al.*, 2003). 1-Naphthol is a metabolite of the fertilisers carbaryl and naphthalene. 4-Ethylphenol is found in red wine as a way of producing distinct aromas (Pollnitz *et al.*, 2000). It has been reported that 1-naphthol is linked to reduced levels of urinary testosterone in males (Meeker *et al.*, 2006). 4-Ethylphenol can also be found in other dietary substances including grape juices, as a by product from the production of yeast (Barata *et al.*, 2006).

Dietary substances including fruits, vegetables, teas and wine have been shown to contain a number of different phenolic and flavonoid compounds (Cook and Samman, 1996). Catechins are flavonoid compounds that are found in a number of dietary components including teas (Yang *et al.*, 2009). UGTs from both 1A and 2B isosymes are involved in the glucuronidation of catechins with glucuronidated catechin identified excreted in urine (Lu *et al.*, 2003; Kuhnle *et al.*, 2000). Little is currently known about the interaction of phenolic and catechin compounds on the inhibition of substrates of UGT enzymes.

As testosterone is a key substrate of UGT2B17 and dietary samples have numerous phenolic compounds that have been glucuronidated by UGT2B17 and other UGT enzymes, it is postulated that the co-administration of testosterone and dietary substances could interfere with testosterone glucuronidation. To assess this hypothesis, the interaction of dietary substances and compounds including tea and red wine samples on testosterone glucuronidation by UGT2B17 has been studied.

3.2 Aims

The aims of this chapter are to investigate the inhibition of UGT2B17 testosterone glucuronidation by dietary samples and compounds. To meet this aim –

- A number of dietary samples, namely tea, wine and fruit samples to determine any effects on testosterone using glucuronidation activity; screened.
- HPLC analysis on dietary samples was performed to determine the presence of catechin compounds. Also perform a HPLC method on the red wine sample to confirm common red wine phenolic compounds are present.
- Individual catechin and phenolic compounds screened for inhibition of testosterone glucurionidation. Inhibitory activity of selected catechin and phenol compounds was investigated at different concentrations and initial testosterone concentrations to identify the type of inhibition being expressed.

3.3 Methods

The full methods are described in chapter two. Briefly:

- Solutions of dietary sample (1.6 g per 80 mL) were prepared and samples were added to testosterone glucuronidation assays at a set volume (2.3.1, 2.4.1).
- HPLC analysis was used to determine residual testosterone levels following the termination of glucuronidation reactions (2.4.2). HPLC analysis was also used to investigate catechin and phenolic composition of dietary samples (2.3.2).

Stock solutions of testosterone were prepared as 5 mg/mL solutions in DMSO and acetonitrile. These solutions were diluted to working standards and samples for standard curve and QC samples. These samples were stored in silanised vials at -20°C.

The HPLC method validation for testosterone analysis was performed by spiking testosterone dissolved in solvent, DMSO or acetonitrile, (1% v/v) into a 1 mL matrix solution consisting of 25 µg/mL alamethecin, 8 mM magnesium chloride, 50 mM of a pH 7.5 Tris-HCl buffer and deionised water.

3.4 Results

3.4.1 Method validation

Chromatograms of testosterone standards for the HPLC methods with mobile phase of water:methanol and water:acetonitrile are shown in Figure 3.1. The calibration curves, regression coefficients (r^2) and regression equations for testosterone used within the reference ranges of the experiments in this chapter are shown in Figure 3.2. The calibration curves showed good linearity within the reference ranges with r^2 values of 0.999. The inter- and intra-day accuracy and precision for both HPLC methods are shown in Table 3.1. Both accuracy and precision for both methods are within the limits set by FDA guidelines.

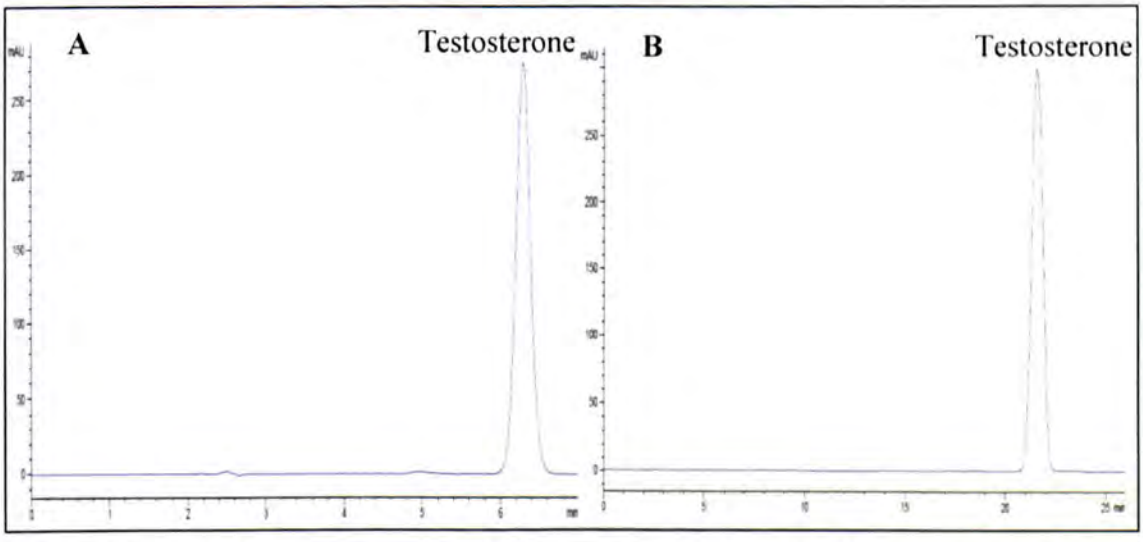


Figure 3.1 Typical chromatograms of testosterone for two HPLC methods used in this study; A- mobile phase water/methanol; B- mobile phase water/acetonitrile.

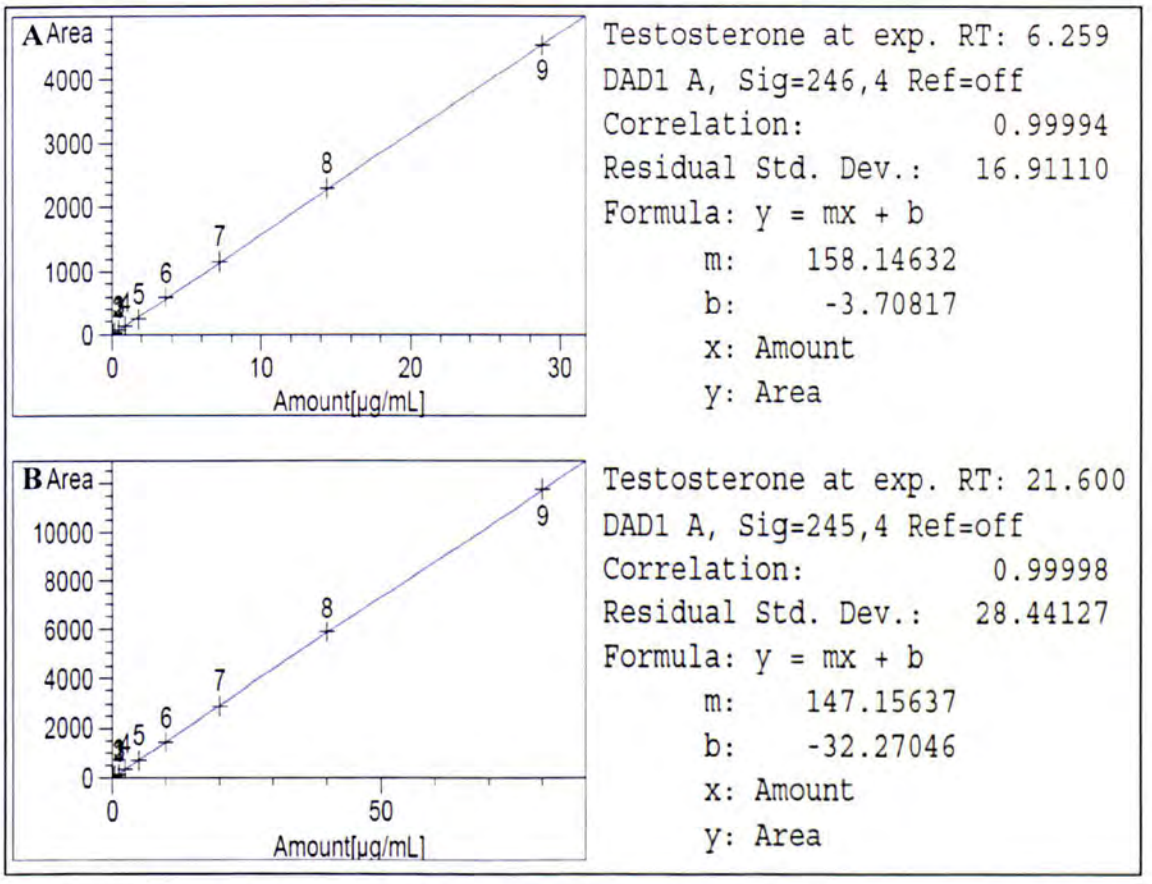


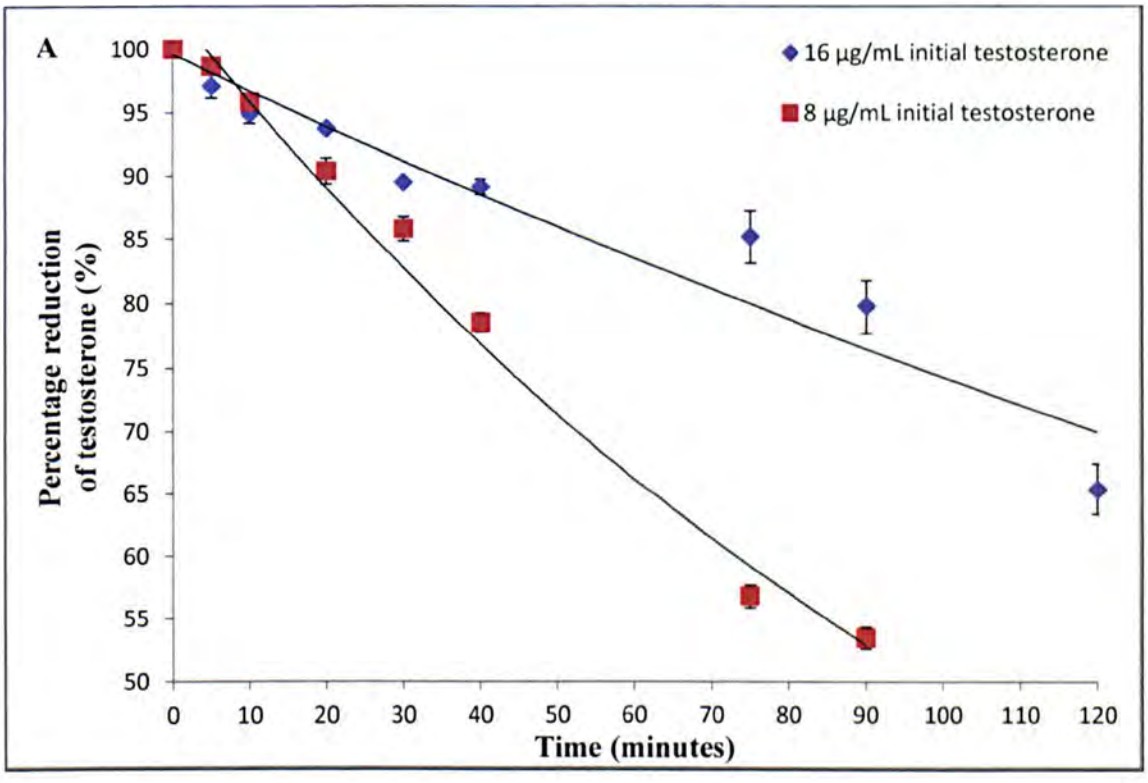
Figure 3.2 Calibration curves, r^2 values and regression curves for testosterone analysis by HPLC over the concentration ranges used in this study and for method validation. A- mobile phase water/methanol, B- mobile phase water/acetonitrile.

Table 3.1 Inter- and intra-day precision and accuracy of testosterone for two HPLC methods used in this study (N=6).

Method	Compound	Concentration (µg/mL)	Level	Precision RSD (%)		Accuracy (%)
				Intra-day N=6+6+6	Inter-day N=18+18+18	
Water: Aceto-nitrile	Testosterone	0.223	Low	6.57	5.63	102.63
		1.804	Medium	0.78	1.19	100.50
		14.420	High	0.58	1.02	99.35
Water: Methanol	Testosterone	0.312	Low	1.71	2.79	97.43
		1.250	Medium	3.15	4.50	96.85
		20	High	1.11	1.39	102.23

3.4.2 Testosterone glucuronidation by UGT2B17 and liver microsomes

The testosterone glucuronidation activity of the UGT2B17 supersomes and pooled human liver microsomes used in this study was assessed. The reaction was terminated over a number of time points and remaining testosterone concentration was monitored (Figure 3.3). Using the UGT2B17 supersomes at 0.2 mg/mL protein concentration, glucuronidation of testosterone continued over 120 minutes. Glucuronidation was greater at the lower substrate concentration used at 8 µg/mL, reducing testosterone by 53% of the initial rate over 90 minutes. The pooled human liver microsomes used at 0.4 mg/mL had greater glucuronidation activity at 15 µg/mL testosterone compared to the UGT2B17 supersomes at 0.2 mg/mL.



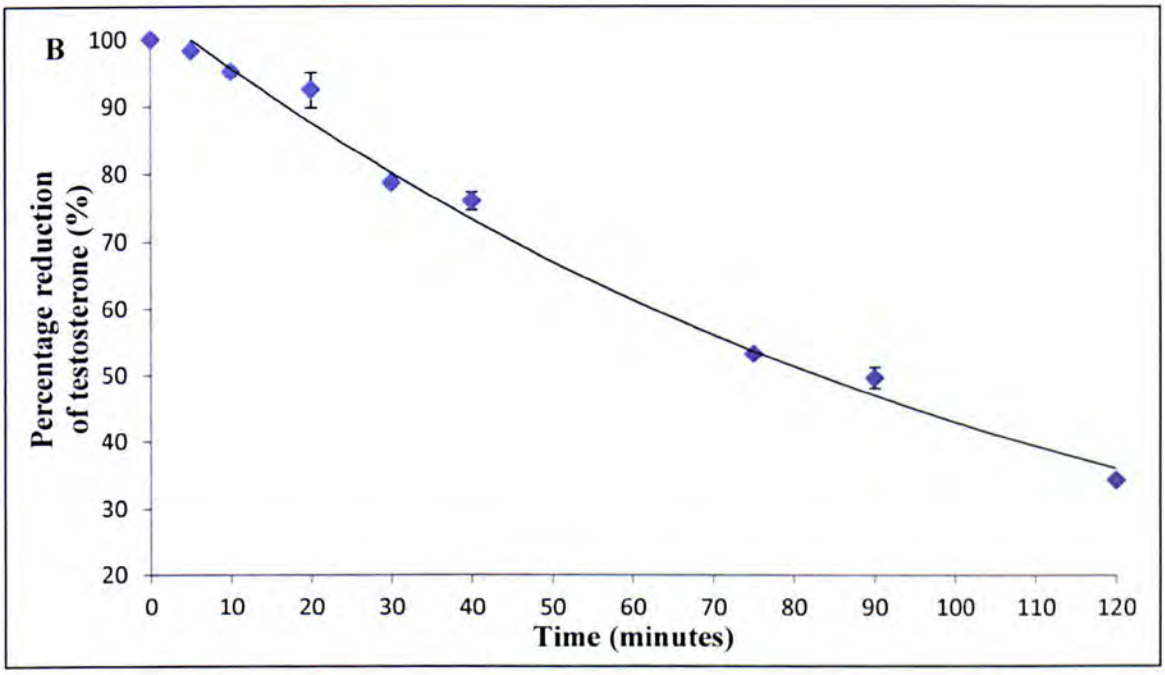


Figure 3.3 Glucuronidation of testosterone by (A) UGT2B17 and (B) pooled human liver microsomes over a time period of 120 minutes. The initial testosterone used in (B) was 16 µg/mL. Each sample represents the mean ± SEM of duplicated samples.

3.4.3 Dietary samples inhibition

3.4.3.1 Inhibition results

To assess whether any of the selected dietary samples inhibit UGT2B17 and reduce testosterone glucuronidation, an initial testosterone concentration of 10 µg/mL and a reaction time of 30 minutes with UGT2B17 concentration of 0.1 mg/mL protein were used. The results are displayed in Table 3.2. A number of foods did not have any significant effects on the glucuronidation of testosterone. However, some of the notable inhibitors of testosterone glucuronidation were the white tea leaf and red wine sample along with pomegranate, and red and black grapes. The rate of testosterone glucuronidation was significantly reduced by 46.8% and 49.3% for red wine and white tea leaves respectively.

Table 3.2 Inhibitory profiles of dietary samples screened for inhibition of testosterone glucuronidation. The initial concentration of testosterone was 10 μg/mL and UGT2B17 concentration was 0.1 mg/mL. The concentration of tea sample added was 2 mg/mL or 10% v/v dietary solution. The reaction time was 30 minutes. Results are displayed as mean ± SEM (* $p<0.05$).

Foods	Testosterone glucuronidation rate (ng/mL/min/mg protein)
Control	512.17 ±29.21
Thai green mango	544.44 ±33.57
Melon tea	534.81 ±50.04
Chocolate	514.04 ±27.11
Beer	500.46 ±19.17
Barley tea	496.89 ±24.00
Coconut	491.96 ±6.06
Dragon fruit	480.59 ±45.11
Korean ginseng tea	476.85 ±23.77
White grape	474.42 ±22.58
White wine	468.30 ±20.14
Ginger tea	462.53 ±33.41
Peppermint tea	458.68 ±11.60
White grapefruit	451.72 ±19.24
Lychees	444.70 ±40.64
Oolong mint tea	407.68 ±6.23
Black grape	404.06 ±7.92
Red grape	393.99 ±19.75
Pomegranate	350.00 ±11.21*
Red wine	272.40 ±20.21*
White tea leaves	259.78 ±26.94*

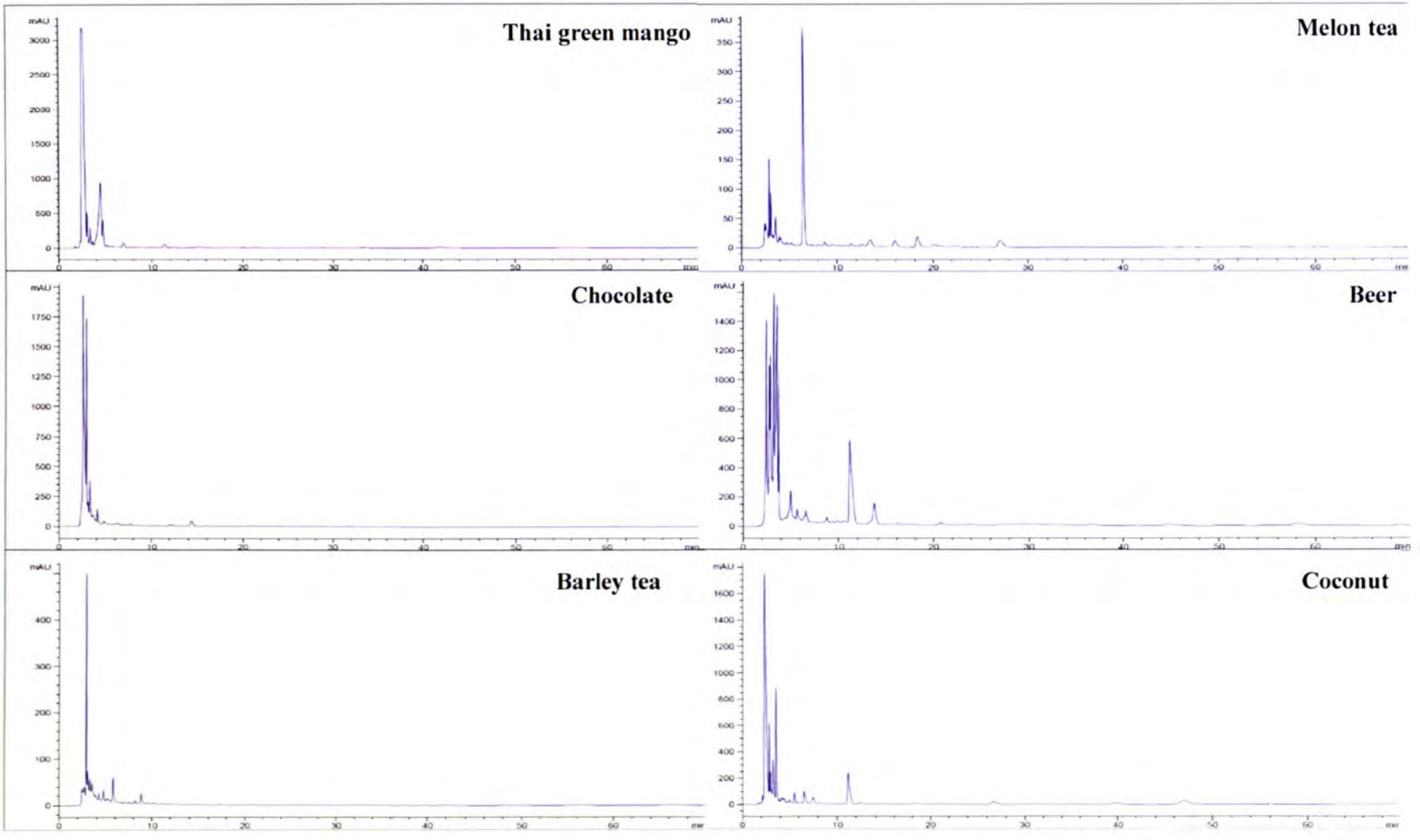
3.4.3.2 Dietary samples catechin analysis

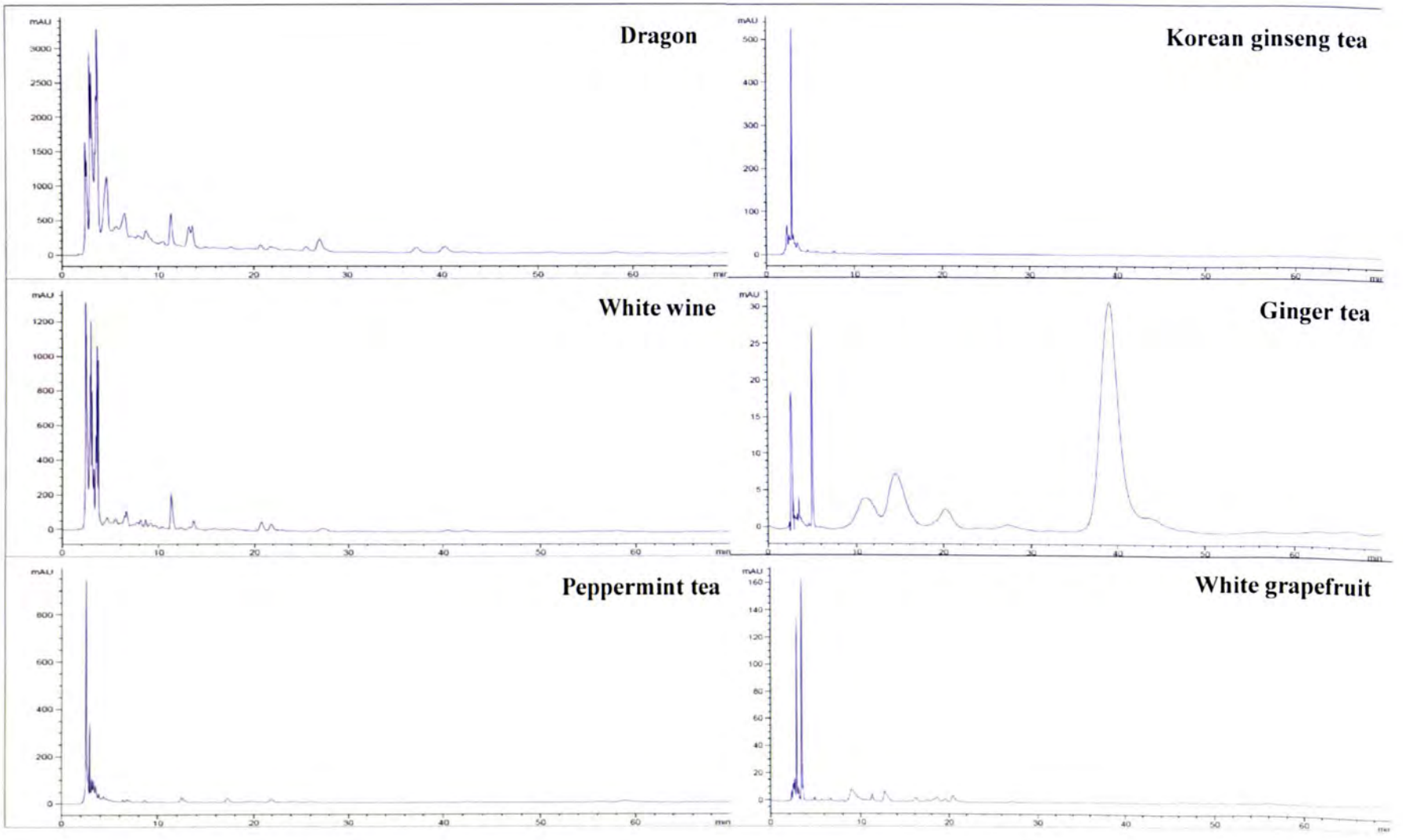
Catechin compounds in the dietary samples were separated and identified using HPLC analysis. Figure 3.4 displays the chromatogram for each dietary sample revealing the key catechins commonly found in teas and dietary samples. The corresponding retention times for each catechin compound are displayed in Table 3.3. The results indicate that the white

tea leaves sample which had the greatest effect of reducing testosterone glucuronidation, had the greatest composition of catechins compared to the other samples screened. Other substances that inhibited testosterone glucuronidation at a lesser rate than the white tea leave sample, showed reduced levels of catechins following screening by HPLC such as the oolong mint tea. However a number of dietary substances that inhibited testosterone glucuronidation significantly such as the red wine and pomegranate sample did not contain the levels of catechins that appeared in the white tea leaves. This suggests that other compounds, perhaps other flavonoid compounds could be responsible for inhibition of UGT2B17 along with catechin compounds.

Table 3.3 Retention times of catechin standards run under the same HPLC conditions as the dietary samples.

Compound	**Retention time (min)**
Gallo catechin	5.00
Epigallocatechin	10.2
Caffeine	18.5
Epigallocatechin gallate	20.2
Epicatechin	27.5
Epicatechin gallate	60.5


Thai green mango
Melon tea
Chocolate
Beer
Barley tea
Coconut


Dragon
Korean ginseng tea
White wine
Ginger tea
Peppermint tea
White grapefruit
mAU
min

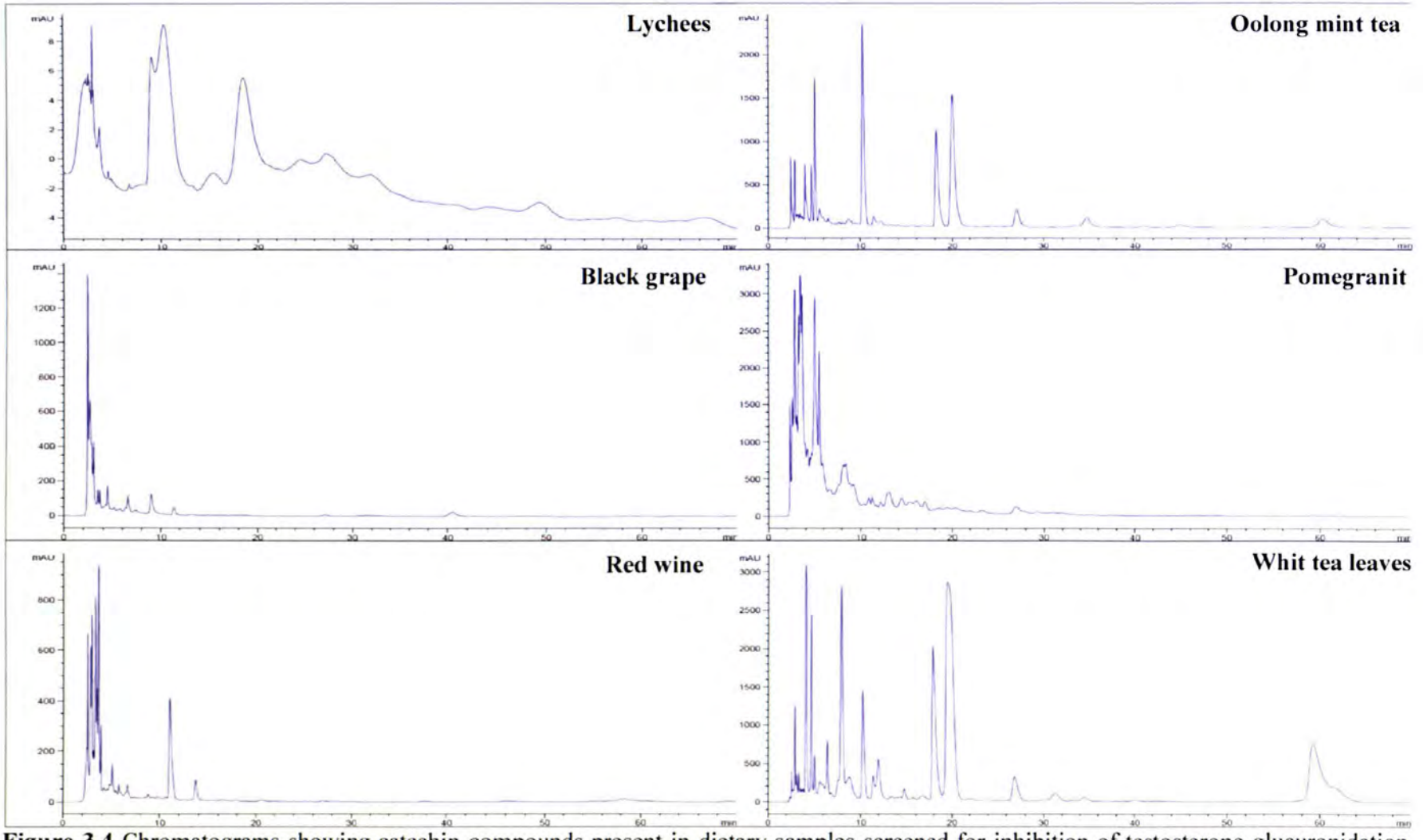


Figure 3.4 Chromatograms showing catechin compounds present in dietary samples screened for inhibition of testosterone glucuronidation.

3.4.4 Screening of tea samples for inhibition of UGT2B17-mediated testosterone glucuronidation

3.4.4.1 Tea sample screening results

Four tea samples; green tea (GT), white tea beard (WTB), white tea leaves (WTL) and white tea powder (WTP), where assessed for inhibitory action on UGT2B17. The results are displayed in Figure 3.5. The initial concentration of testosterone was 28.84 μg/mL with a UGT2B17 protein concentration of 0.2 mg/mL. The reaction was stopped at 30 and 90 minutes. Following termination of the reactions, the samples containing white and green tea extracts had increased remaining testosterone compared to the controls. The GT, WTB and WTP had similar inhibitory effects, reducing testosterone glucuronidation activity up to 18% compared to the control rate. The WTP was most effective having 30% compared to the control sample. The inhibitory action remained over a 90 minute period with the percentage of inhibition reducing slightly from 30 minutes to the second time point at 90 minutes.

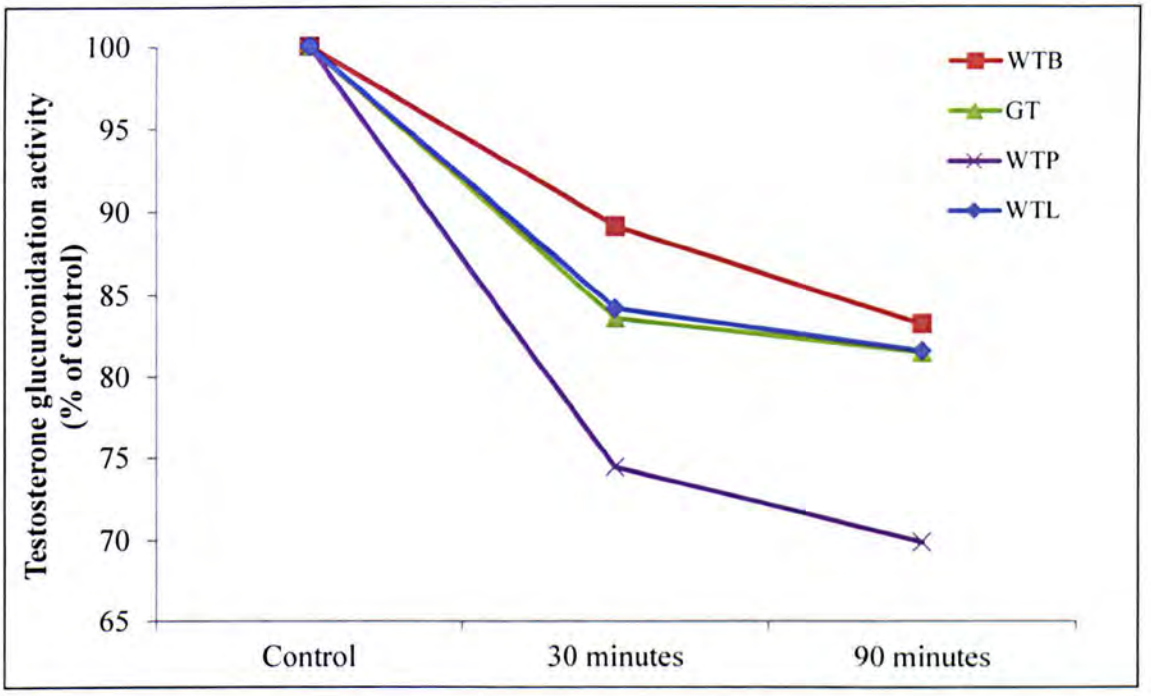


Figure 3.5 Reduction in testosterone glucuronidation activity at 30 and 90 minutes for each tea sample. Each point is the average of duplicated samples. The initial testosterone concentration was 28.84 µg/mL and the UGT2B17 concentration was 0.2 mg/mL. The concentration of tea sample added was 2 mg/mL. The values illustrated in the graph are: 30 min – control 100, GT 83.57 ± 0.96, WTL 84.18 ± 4.50, WT 89.09 ± 4.50, WTP 74.40 ± 3.92; and 90 min – control 100 ± 1.92, GT 81.49 ± 3.71, WTL 81.59 ± 2.69, WT 83.21 ± 3.09, WTP 69.84 ± 4.29.

The four tea samples were further analysed for inhibition of testosterone by UGT2B17, along with commercially available cacao beans and a block of cacao shown in Table 3.4. Previous studies have shown similar phenolic compounds present in cacao samples to that of teas (Sanchez-Rabaneda *et al.*, 2003). At a reduced initial concentration of testosterone (12µg/mL) the tea samples significantly reduced the rate of testosterone glucuronidation over 30 minutes. The WTP was most effective, reducing the rate of glucuronidation by

89.7%. The cacao samples were not as effective as the tea extracts in reducing testosterone glucuronidation rate.

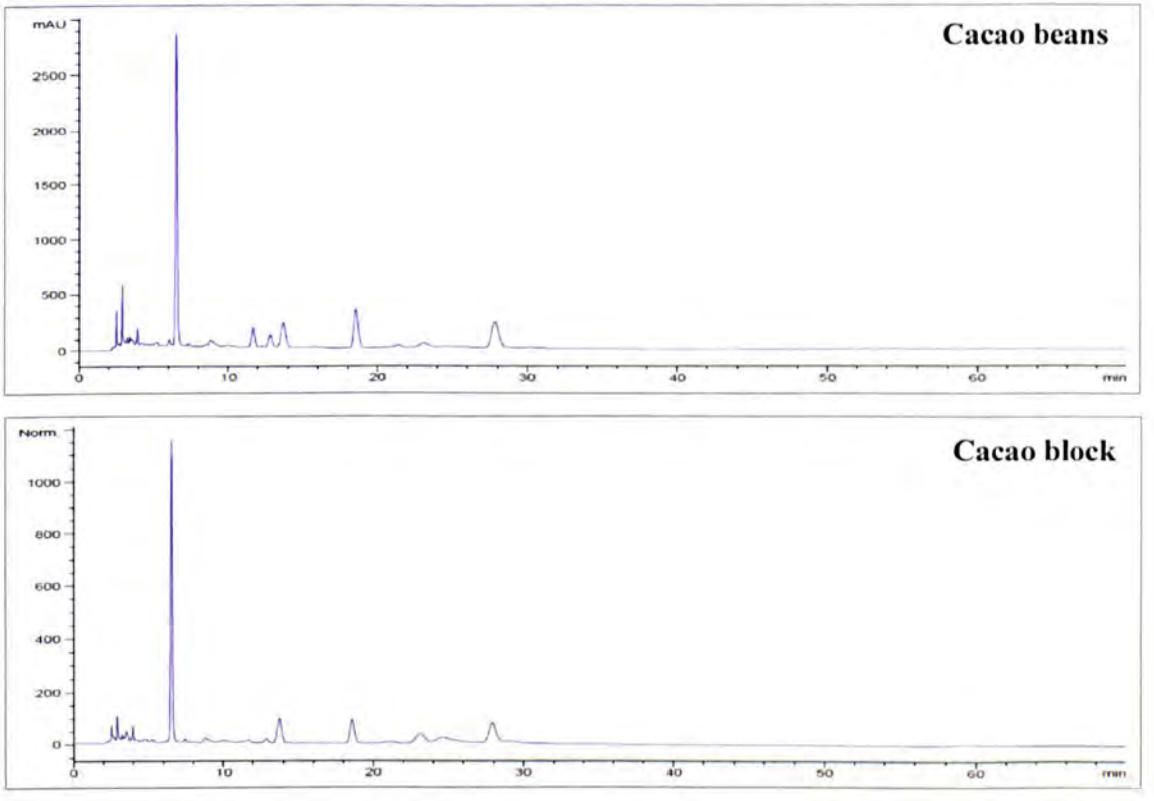
Table 3.4 Inhibition of UGT2B17 testosterone glucuronide profiles by tea and cacao samples. The initial testosterone concentration was 12 μg/mL and the UGT2B17 protein concentration was 0.1 mg/mL. The concentration of tea and cacao present in reaction samples was 1 mg/mL. Results are displayed as mean ± SEM of triplicate samples (* $p<0.05$).

Foods	**Testosterone glucuronidation rate (ng/mL/min/mg protein)**
Testosterone control (12μg/mL)	682.09 ±30.73
Cacao beans	666.22 ±23.55
Cacao block	572.89 ±20.14*
White tea beard	249.83 ±18.87*
White tea leaf	246.22 ±16.61*
Green tea	179.56 ±22.64*
White tea powder	69.57 ±11.04*

3.4.4.2 Analysis of catechin compounds present in tea samples by HPLC

The tea and cacao samples analysed for inhibition of testosterone glucuronidation by UGT2B17 in Table 3.4 were separated by HPLC to identify the catechin compounds present, as a way of providing pure test compounds for determining the active compounds in UGT2B17 inhibition. Figure 3.6 displays the chromatograms of each extract; identification of catechin compounds was based on the retention time of standard

compounds outlined in Table 3.3 along with previous chromatograms based on this method (Wang *et al.*, 2000). The chromatograms revealed that all the green and white teas had a number of common catechins present. These catechins were also present in the cacao samples at much lower amounts than in the teas. The white tea powder which was the most active inhibitor of UGT2B17 had much greater amounts of each of the catechins compared to the other tea samples. These results are further highlighted in Figure 3.7A which shows an overlay chromatogram of the white tea powder and green tea, the next most active substance involved in UGT2B17 inhibition. Figure 3.7B shows the difference in catechin composition between the white tea leaf sample and the cacao block sample, which has much less inhibition activity over the teas. The data from this analysis informed further experimentation with commercial catechin standards to determine if any of these compounds are active in the inhibition of UGT2B17.



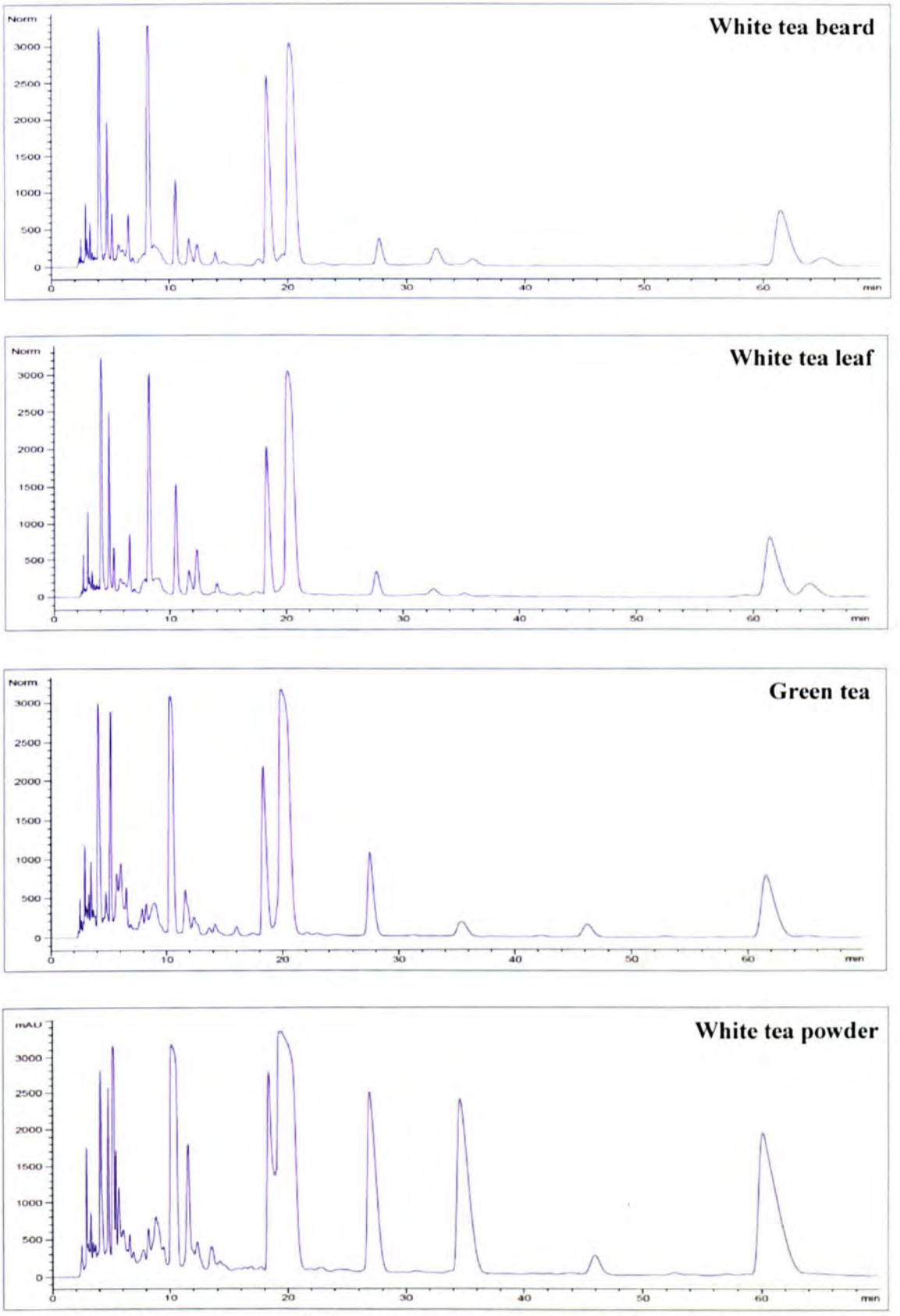


Figure 3.6 Chromatograms displaying the catechin compounds present in each sample analysed for UGT2B17 inhibition. Compound retention times are displayed in Table 3.3. The concentration of sample present was 500 mg/mL

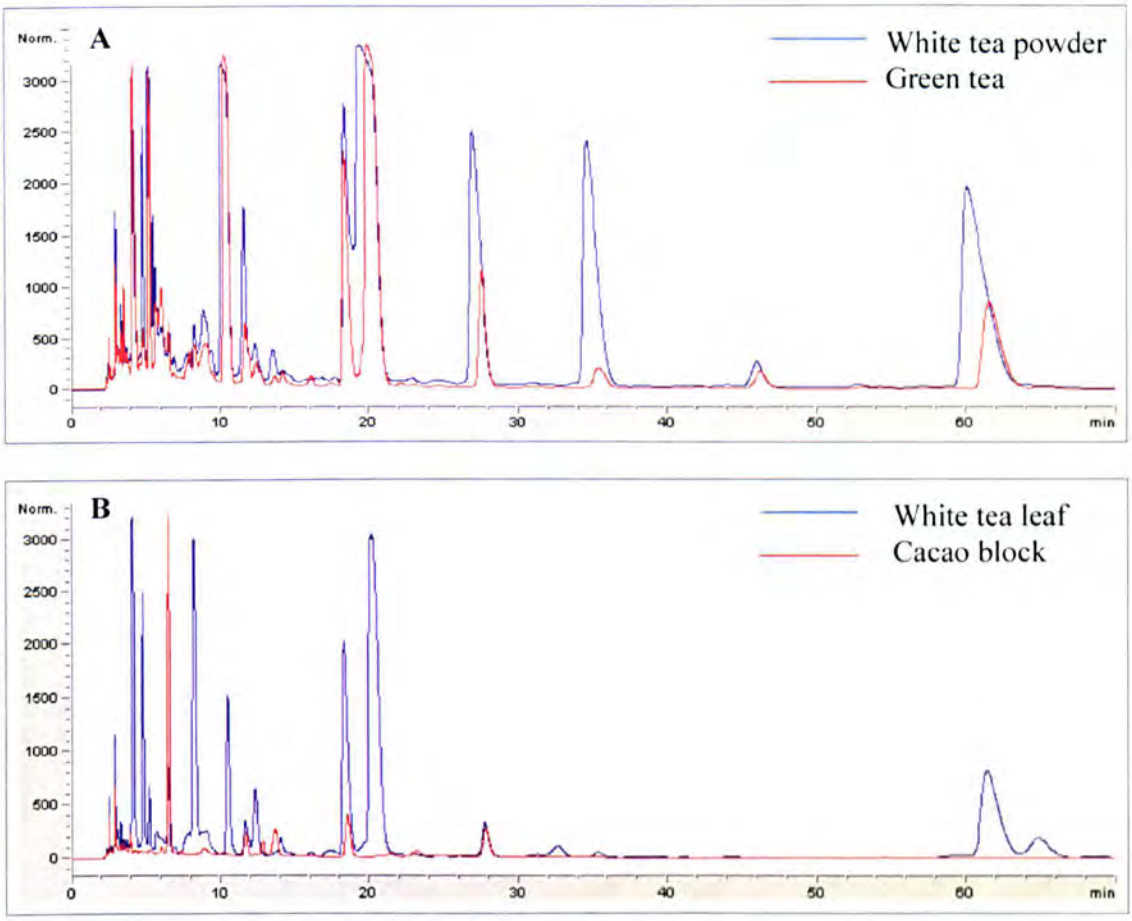


Figure 3.7 Overlaid chromatograms showing A – catechin composition of white tea powder and green tea and B – catechin composition of white tea leaf and cacao block samples. Compound retention times are displayed in Table 3.3.

3.4.5 Catechin inhibition

3.4.5.1 Catechin screening results

Following the inhibition of UGT2B17 testosterone glucuronidation by teas and other dietary samples, commercially available catechins were analysed to determine if any of these compounds are active in the inhibition of UGT2B17. Figure 3.8 displays changes in testosterone glucuronidation rate over 30 minutes following the addition of 250 µg/mL catechin compound. The protein concentration was 0.1 mg/mL and the initial testosterone

was 10 µg/mL. Inhibition was insignificant for the GC, caffeine and EGC compounds. The EC isomers inhibited UGT2B17, the (+)EC isomer was more effective than the (-)EC reducing testosterone glucuronidation rate by 22.4% and 41.8% respectively. ECG, EGCG and CG were the most active compounds, the CG compound reduced the rate by the greatest amount by 84.5%.

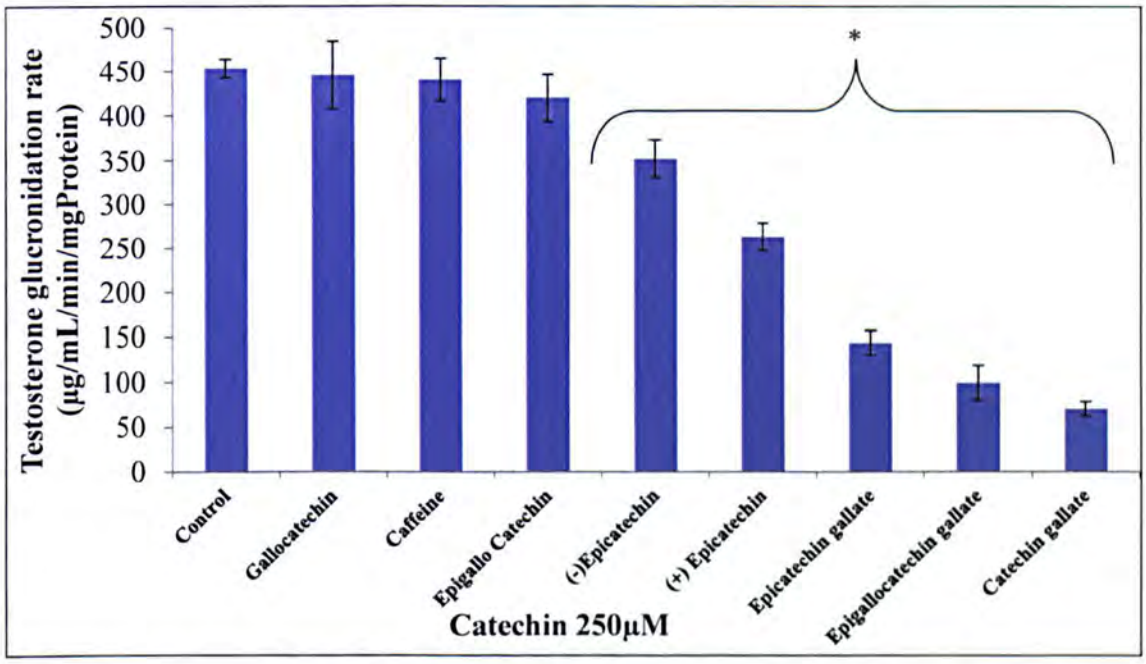


Figure 3.8 Reduction of UGT2B17 mediated testosterone glucuronidation following the addition of 250 µM of individual catechins. Initial testosterone concentrations were 10 µg/mL and UGT2B17 concentration was 0.1 mg/mL. The reaction time was 30 minutes. Results are displayed as mean ± SEM of duplicated samples (* $p<0.05$).

3.4.5.2 Epicatechin and epigallocatechin gallate inhibition

The results from the catechin screening displayed in Figure 3.8 revealed that two compounds of interest, (-)EC and EGCG inhibited UGT2B17 testosterone glucuronidation. As these are two of the primary catechin compounds in tea (Azam *et al.*, 2004), these compounds were further analysed to determine their inhibitory effect of UGT2B17. To

determine the inhibitory effects of (-)EC, a standard concentration of 290.26 µg/mL (-)EC was added to samples with initial testosterone ranging between 2.16 and 43.26 µg/mL, shown in Figure 3.9. When compared to a control with no (-)EC catechin present at each initial testosterone concentration, the results showed that at lower initial testosterone the addition of (-)EC reduced the percentage of testosterone glucuronidation activity compared the control. However as the initial concentration of testosterone increased there was no difference in glucuronidation rates when compared to the control samples. A further study monitored the effect of adding just (-)EC with UGT2B17. This revealed levels of (-)EC remained unaltered when the reaction was terminated after 1 hour and this compound was not glucuronidated by UGT2B17.

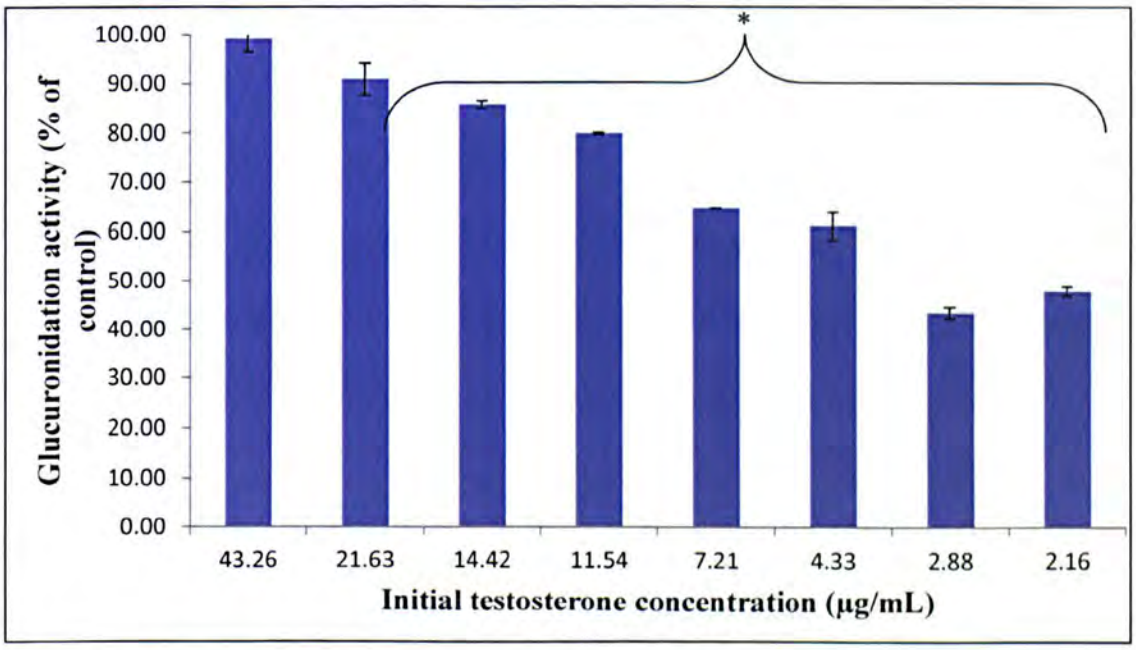


Figure 3.9 UGT2B17 testosterone glucuronidation activity at varying initial testosterone concentrations following the addition of a standard concentration of (-)EC. The values show the percentage of reduced glucuronidation activity compared to a control sample at the same initial testosterone concentrations after 30 minutes. Initial testosterone was 10 µg/mL and the UGT2B17 protein concentration 0.2 mg/mL. Values are displayed as mean ± SEM of duplicate samples (* $P<0.05$).

To assess the competitive activity of EGCG a Lineweaver-Burk plot was constructed. The results are displayed in Figure 3.10 and the Lineweaver-Burk values are displayed in Table 3.5. EGCG showed competitive inhibition against UGT2B17 owing to the consistent Vmax and increasing Km as the concentration of EGCG increased.

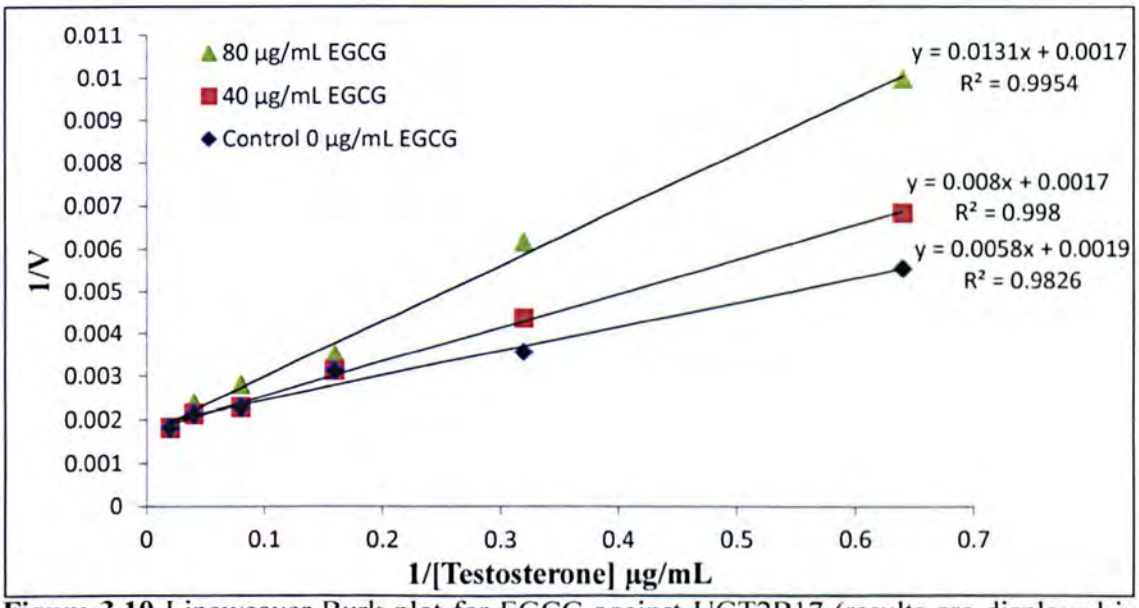


Figure 3.10 Lineweaver-Burk plot for EGCG against UGT2B17 (results are displayed in Table 3.5). Competitive inhibition was displayed with a consistent Vmax and increasing Km when EGCG was added. The reaction time was 45 minutes and UGT2B17 protein concentration was 0.1 mg/mL.

Table 3.5 Kinetic parameters for EGCG inhibition of UGT2B17 taken from the Lineweaver-Burk plot (Figure 3.10).

Enzyme	EGCG concentration	Vmax (ng/mL/ min/mg prot.)	Km (µg/mL)	Inhibition type
UGT2B17	0 µg/mL	526.31	3.03	Competitive
	40 µg/mL	588.23	4.73	
	80 µg/mL	588.23	7.71	

The competitive inhibitory profile of EGCG was confirmed further following the Dixon plot analysis displayed in Figure 3.11. Competitive inhibition was confirmed by the trendlines at each inhibitor concentration intersecting above the X axis. The EGCG IC_{50} was determined to be 64 μM. These results along with the Lineweaver-Burk plot (Figure 3.10) show that increasing concentrations of EGCG reduced the rate of testosterone glucuronidation in comparison to the control with no inhibitor.

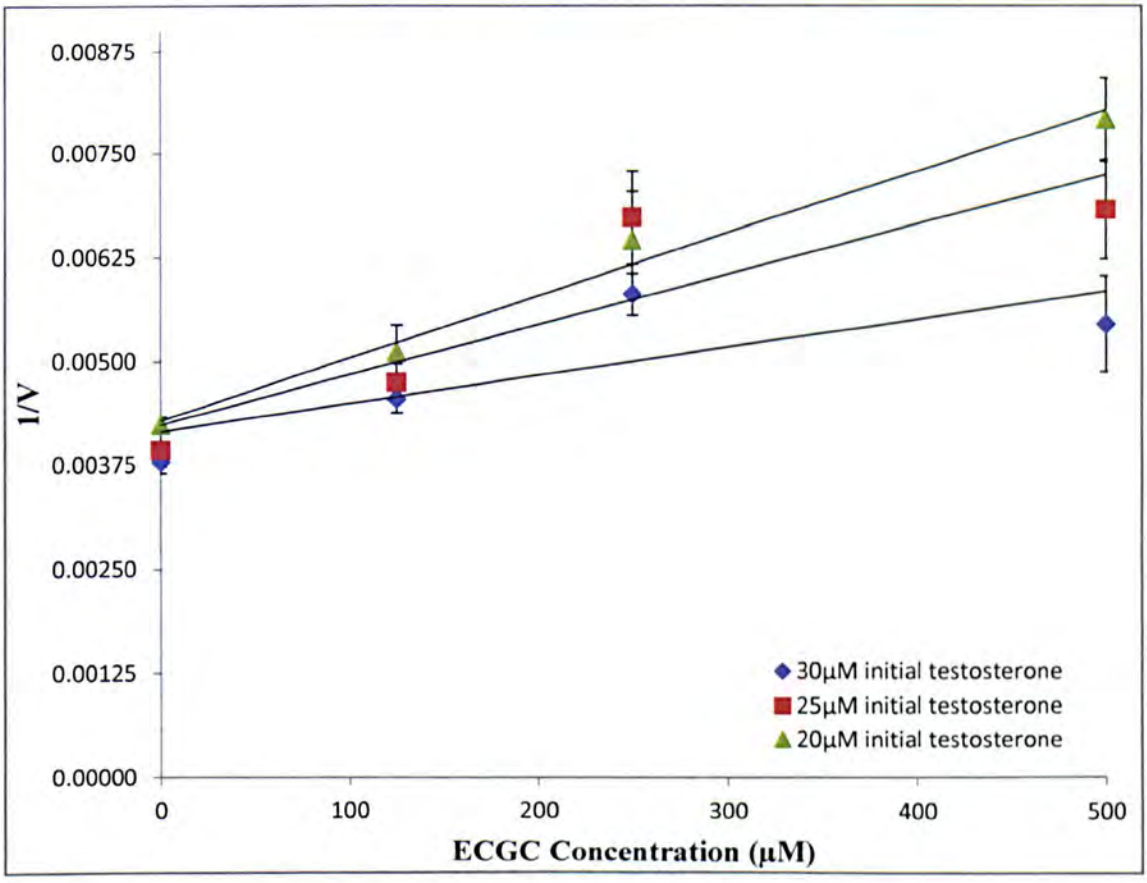


Figure 3.11 Dixon plot analysis of UGT2B17 testosterone glucuronidation by increasing concentrations of EGCG. Initial testosterone ranges were 20, 25 and 30 μM. UGT2B17 concentration was 0.1 mg/mL. The reaction was stopped after 30 minutes. The values are the mean ± SEM of duplicate values.

3.4.6 Red wine inhibition of testosterone glucuronidation

3.4.6.1 Red wine screening

Following on from the screening experiment that indicated red wine inhibited UGT2B17 testosterone glucuronidation (Table 3.2), the inhibitory effects of increasing concentrations of red wine on UGT2B17 were assessed (Figure 3.12). The results show that testosterone glucuronidation activity by UGT2B17 decreased as red wine concentrations in the reaction increased. Reduced testosterone glucuronidation was observed for all samples in which red wine was added compared to the control after both 1 and 2 hours. The activity initially reduced from 10% reduction compared to the control with 2% v/v red wine added, reduced down to over 70% activity with 8% v/v of red wine. At the higher concentrations of red wine, glucuronidation activity was affected more after 2 hours than at 1 hour. At lower concentration of red wine, the inhibition was less in comparison to the control after 2 hours than it was after 1 hour. A further study was carried out that looked into the effect of an evaporated sample of red wine, dried down to remove ethanol content and reconstituted with the same volume of water. The sample was added to reactions at 4% and 8% v/v. These samples reduced the glucuronidation of testosterone activity to 59.18% ±3.154 (p = 0.035) and 23.48% ±4.405 (p = 0.026) respectively compared to the control rate after two hours.

Based on the percentage of ethanol present in the red wine sample, it was estimated that the concentration of ethanol present in the reaction samples used in the study (Figure 3.12), ranged between 0.26%-1.04% v/v based on the additions of 2%-8% v/v respectively of red wine. Figure 3.13 shows the effects of UGT2B17 testosterone glucuronidation following the addition of pure ethanol when added to reactions at 1, 2 and 3% v/v. At an intial

testosterone concentration of 14.42 µg/mL, testosterone glucuronidation did not appear to be significantly altered by the addition of 1% v/v ethanol, however glucuronidation activity of UGT2B17 was reduced following the additions of 2 and 3% v/v of ethanol. Owing to the lower levels of ethanol that would have been added with the red wine volumes used mentioned above, it is likely that any inhibitory effect of UGT2B17 occurred from the components in red wine and any effect of testosterone glucuronidation from the ethanol present in the sample would be minimal.

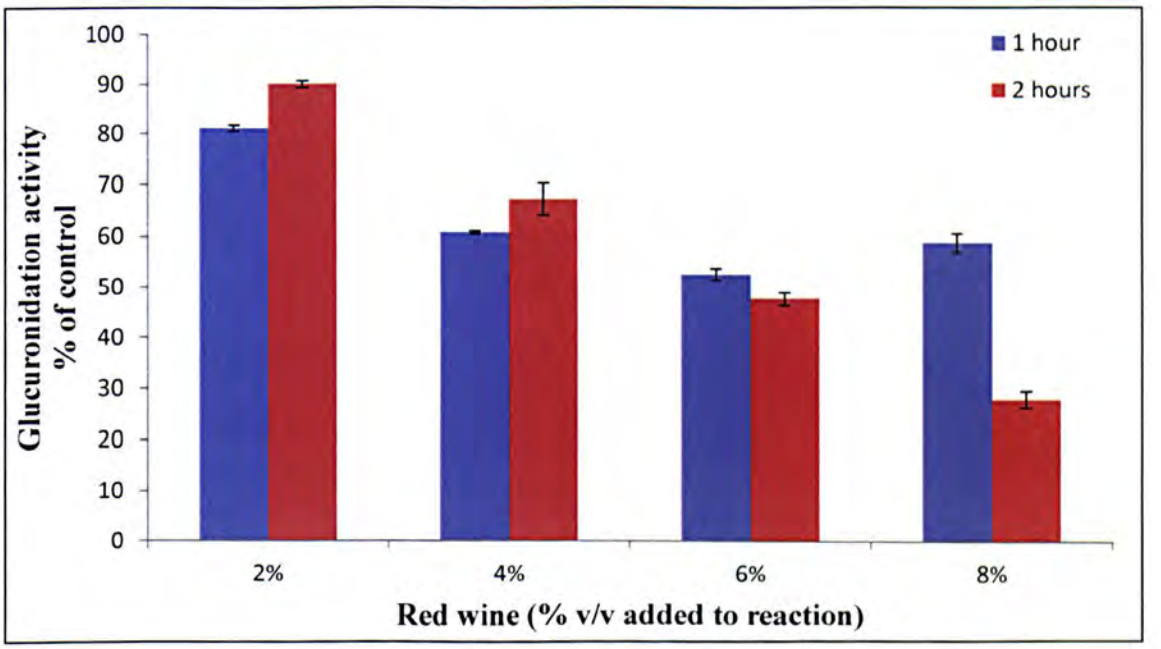


Figure 3.12 Reduction of UGT2B17 testosterone glucurindation activity by the addition of red wine at increasing volumes with an initial testosterone concentration of 28.84 µg/mL and UGT2B17 protein concentration of 0.2 mg/mL. The results are the mean ± SEM of duplicate samples. $p<0.05$ for all samples.

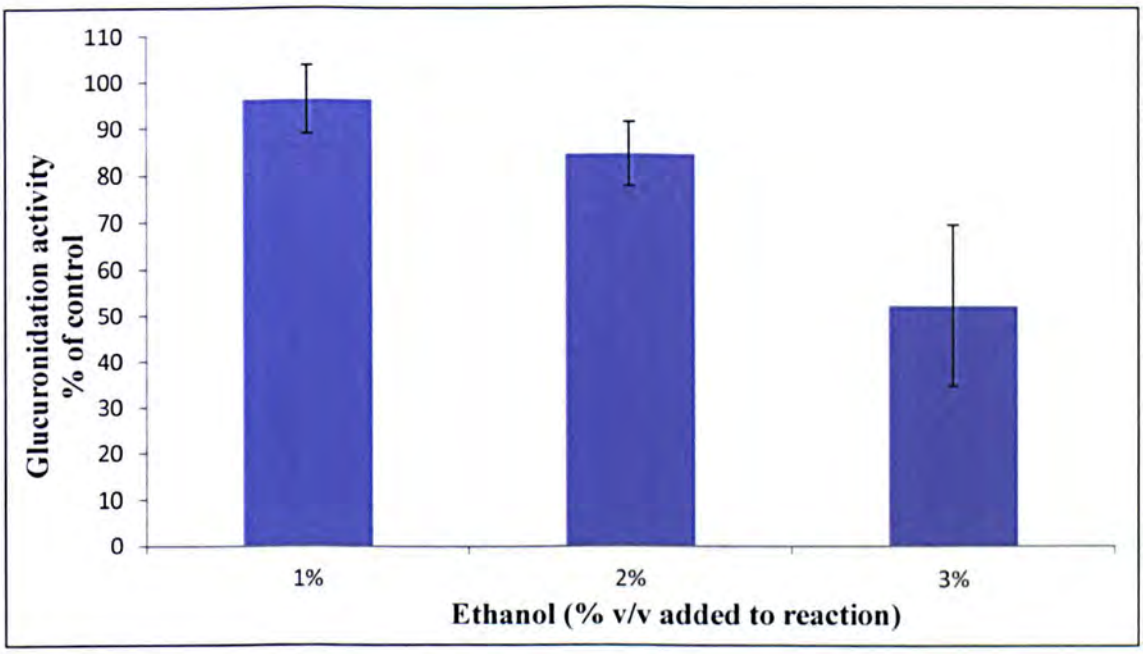


Figure 3.13 Effects of increases in ethanol concentration on testosterone glucuronidation activity of UGT2B17. Initial testosterone concentration was 14.42 μg/mL and initial UGT2B17 protein concentration 0.2 mg/mL. The reaction time was 30 minutes, results are displayed as mean ± SEM. 1% ($p = 0.669$), 2% ($p = 0.265$), 3% ($p = 0.220$).

3.4.6.2 Red wine analysis by HPLC

To identify if any of the key phenolic compounds commonly present in red wine were also present in the sample used in this study, the red wine sample was analysed and phenolic compounds identified by HPLC (Figure 3.14). A number of phenolic compounds were found to be present based on comparing the retention time of the chromatogram with reference phenolic standards ran under the same conditions. The retention time of each phenolic compound is outlined in Table 3.6.

Table 3.6 Retention times of phenolic compound reference standards based on the HPLC method used for the analysis of red wine.

Compound	Retention time (min)
Gallic acid	4.8
Chlorogenic acid	8.2
Caffeic acid	11.6
p-Coumaric acid	21.5
Quercetin	29.5

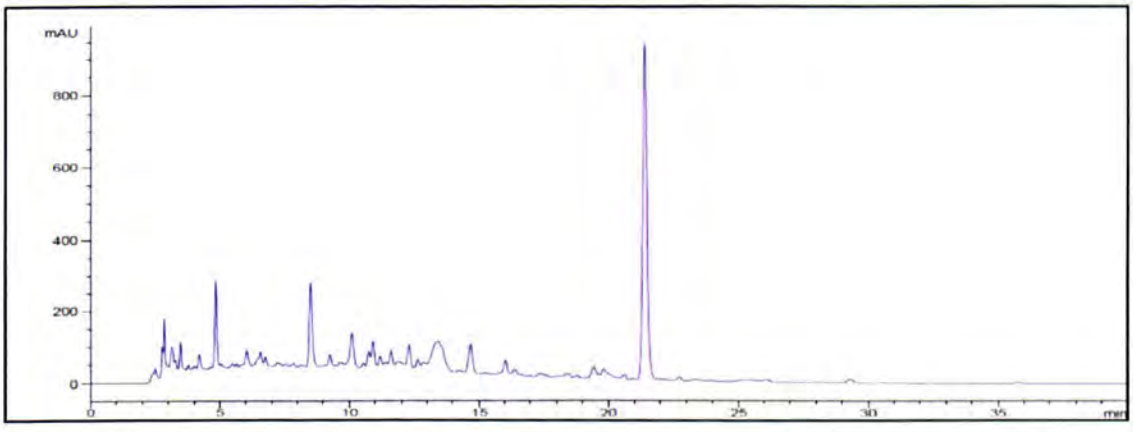


Figure 3.14 Chromatogram displaying phenolic compounds in the red wine sample used in this study. Retention times are displayed in Table 3.6.

3.4.6.3 Screening of phenolic compounds for UGT2B17 inhibition.

Following the confirmation of phenolic compounds in the red wine sample (Figure 3.14), these phenolic compounds were screened for any inhibition of UGT2B17 testosterone glucuronidation. Initial screening of five common phenolic compounds on testosterone glucuronidation is outlined in Table 3.7.

Table 3.7 Reduction in UGT2B17 testosterone glucuronidation over 60 minutes by the addition of 250 μM of phenolic compound with 100 μM initial testosterone. UGT2B17 protein concentration was 0.2 mg/mL. Values are the mean ± SEM of duplicate samples (* $p<0.05$).

Compound	**Glucuronidation**
250 μM	**% of control**
Gallic acid	91.01±7.740
Chlorogenic acid	78.65±4.020
Caffeic acid	98.47±6.814
p-Coumaric acid	100.84±5.385
Quercetin	28.01±1.980*

Given that quercetin was the most effective inhibiting phenolic compound, further studies were performed at reduced concentrations of quercetin and lower initial concentrations of testosterone shown in Table 3.8. Lower concentrations of quercetin at 1.5 and 2.0 μM still appeared to reduce testosterone glucuronidation activity despite the fact there was still a high concentration of testosterone at 20 and 30 μM.

Table 3.8 Effect of low concentrations of quercetin on testosterone glucuronidation over 60 minutes with a UGT2B17 protein concentration of 0.1 mg/mL. Values are the mean ± SEM of duplicate samples.

Initial Testosterone (μM)	Quercetin (μM)	Glucuronidation % of control
20	50	65.62±10.110
20	20	79.93±8.040
20	2	82.42±4.990
30	2	97.69±6.530
20	1.5	91.37±5.520

The phenolic compound gallic acid showed a minor reduction in testosterone glucuronidation under the conditions shown in Table 3.7. Using a lower initial concentration of testosterone (50 μM) and increasing concentrations of gallic acid ranging from 0-250 μM, testosterone glucuronidation activity reduced gradually as gallic acid increased, shown in Figure 3.15. However, this level of UGT2B17 inhibition was minor in comparison to quercetin.

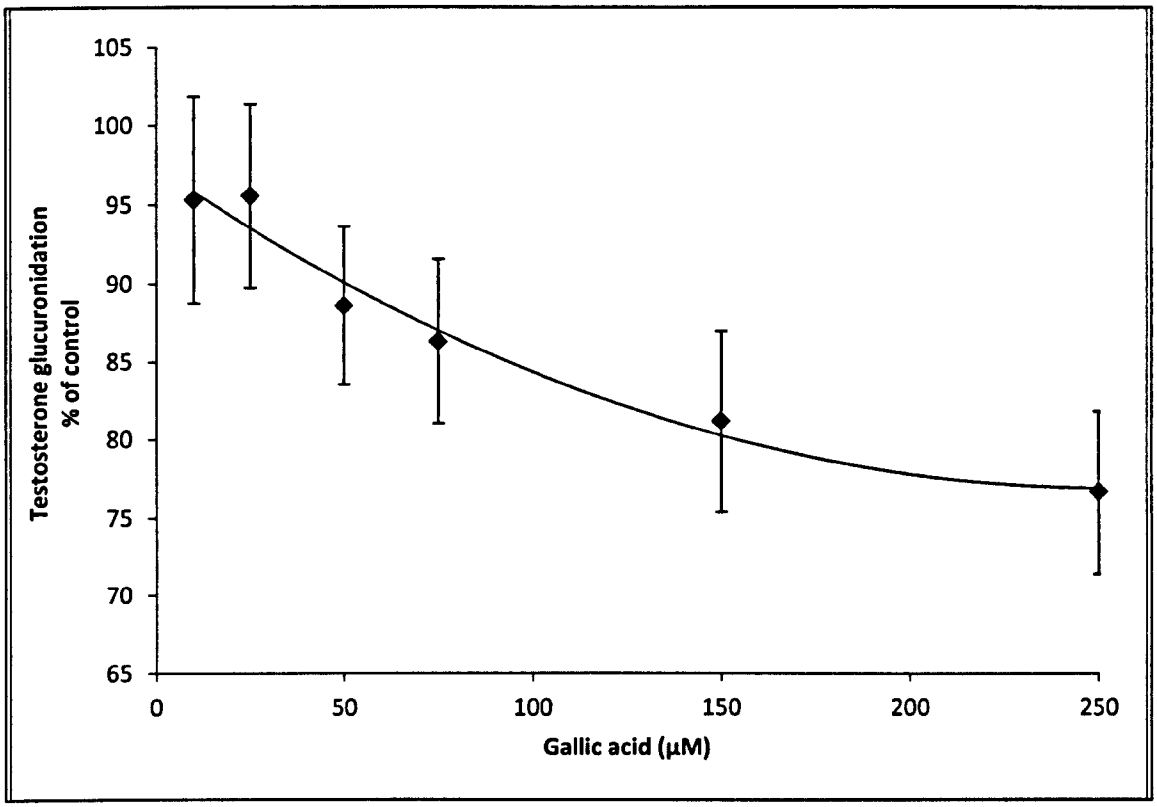


Figure 3.15 Effects of increasing gallic acid concentration on the glucuronidation of a 50 μM initial testosterone after 60 minutes. UGT2B17 protein concentration was 0.1 mg/mL. Values are the mean ± SEM of duplicate samples.

3.4.7 Inhibition of other phenolic compounds on testosterone glucuronidation

The phenolic compounds 1-naphthol and 4-ethylphenol have been shown to be glucuronidated when screened with UGT2B17 (Turgeon *et al.*, 2003). These compounds are of interest for their activity, if any, on glucuroniation of testosterone when combined with UGT2B17. A dose response curve was set up for 1-naphthol with added concentrations ranging from 25-300 μM and a set initial testosterone concentration of 100 μM displayed in Figure 3.16. The EC_{50} value of 1-naphthol was determined at 138.2 μM.

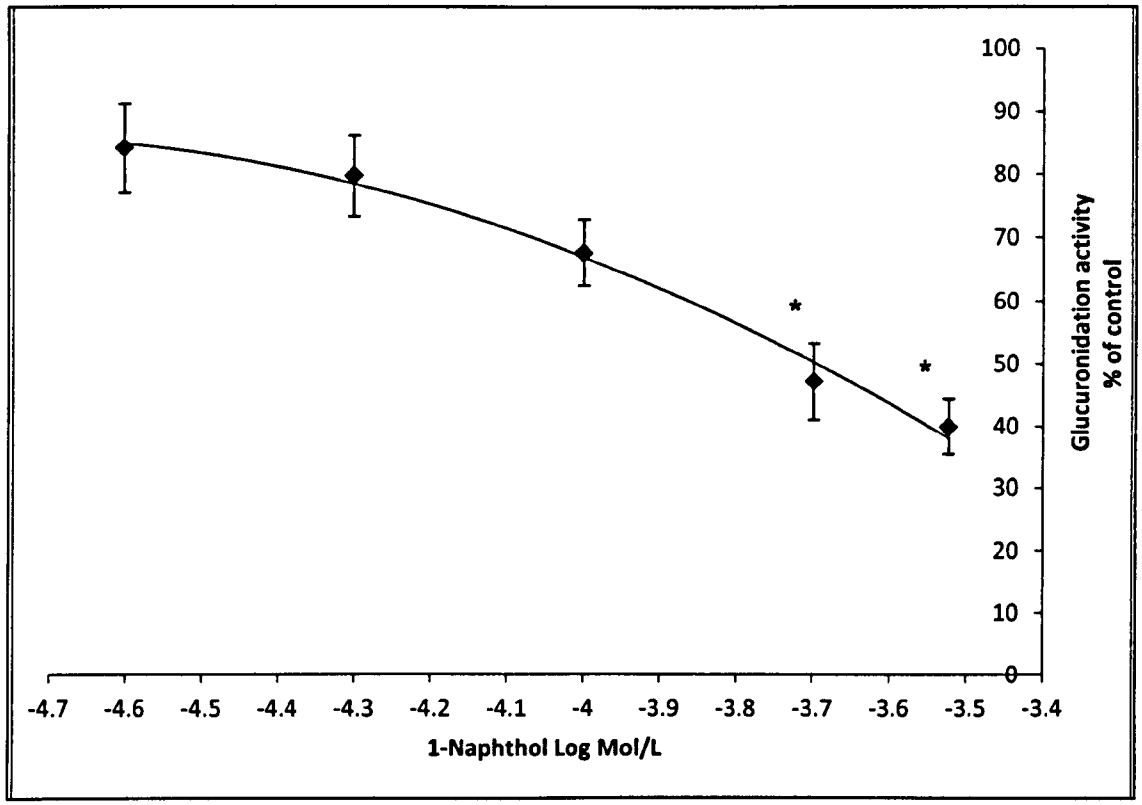


Figure 3.16 Dose response curve showing the reduction in testosterone glucuronidation following the addition of increasing concentrations of 1-naphthol. The reaction was carried out over two hours using 100 μM initial testosterone and 0.1 mg/mL UGT2B17 protein concentration. The concentrations of 1-Napthol used were 25, 50, 100, 200* and 300* μM. Values are the mean ± SEM of duplicate samples (* $p<0.05$).

A Lineweaver-Burk plot was constructed to determine any inhibitory effects of 4-ethylphenol on UGT2B17 testosterone glucuronidation. Figure 3.17 and Table 3.9 display the Lineweaver-Burk plot and changes in Vmax and Km following the addition of 40 μg/mL 4-ethylphenol. The addition of 4-ethylphenol had no effect on Vmax however the Km value increased. This indicates the phenolic compound has competitive inhibition on UGT2B17.

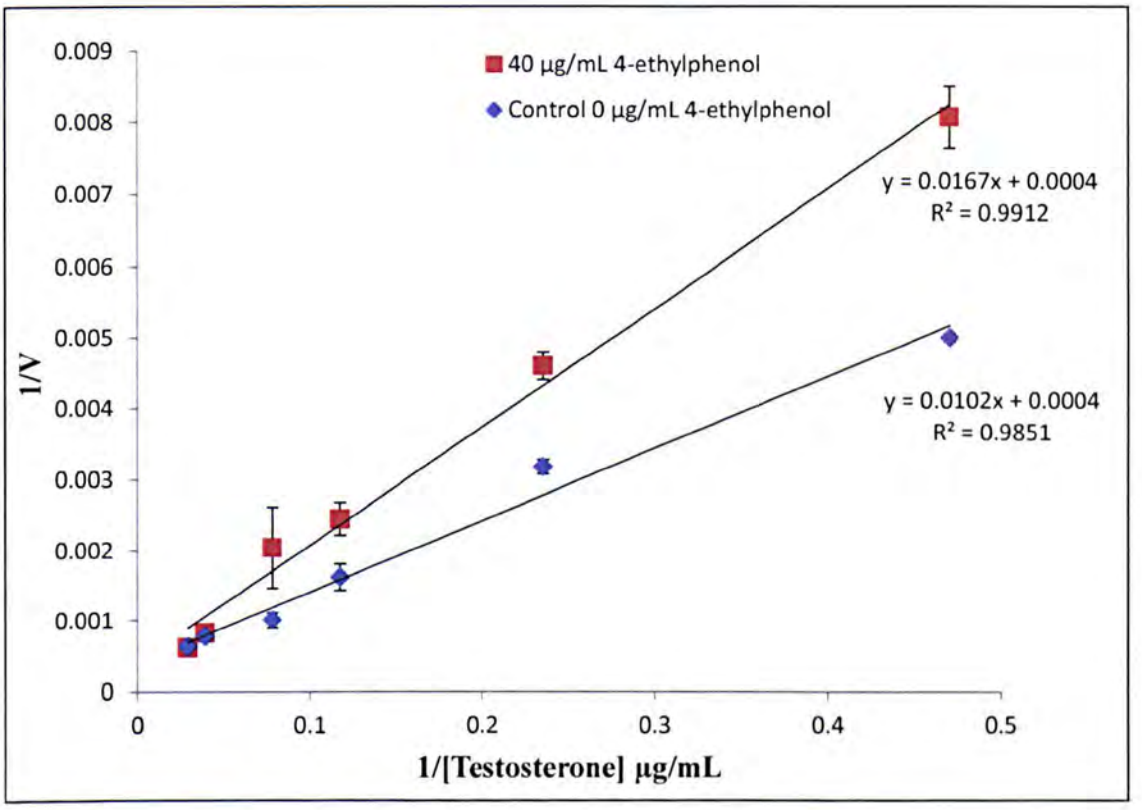


Figure 3.17 Lineweaver-Burk plot generated for 4-ethylphenol against UGT2B17 testosterone glucuronidation activity. The reaction was for 30 minutes with 0.2 mg/mL protein concentration. Results are the mean ± SEM of duplicate samples.

Table 3.9 Kinetic parameters for the Lineweaver-Burk plot displayed in Figure 3.17. Competitive inhibition was displayed by 4-ethylphenol owing to the identical Vmax values and increased Km following the addition of the inhibitor compound.

Enzyme	4-ethylphenol concentration	Vmax (ng/mL/ min/mg prot.)	Km (µg/mL)	Inhibition type
UGT2B17	0 µg/mL	2500	25.5	
	40 µg/mL	2500	33.4	Competitive

3.5 Discussion

The results from this chapter revealed that a number of dietary substances and their active compounds inhibit the glucuronidation of testosterone by UGT2B17, along with identifying the active compounds in these substances that are active in inhibition. On the other hand, there were also numerous dietary components that did not alter testosterone activity or only had a minor effect compared to some of the most active foods. This could be due to variations in the composition and amounts of phenolic and catechin compounds in the samples. HPLC analysis of these foods revealed variations in catechin compounds between samples; this was linked to the activity of UGT2B17 inhibition of testosterone glucuronidation.

The analysis of the tea samples revealed the green and white teas used in this study inhibited UGT2B17 at similar rates, apart from the white tea powder sample which exhibited the greatest inhibition. HPLC analysis of tea samples revealed catechin compounds present in these teas, it was clear that the white tea powdered sample had greater quantities of measured catechins present. This is likely to be due to the enhanced solubility of this powdered sample in water compared to the other samples which were granular.

Each catechin was screened for inhibition of UGT2B17 and five compounds significantly inhibited testosterone glucuronidation; (-)EC, (+)EC, ECG, EGCG and CG. The catechin compounds that had no effect on testosterone glucuronidation were GC, caffeine and EGC. Further analysis with (-)EC and EGCG at varying initial testosterone concentrations showed that inhibition was greater with lower initial testosterone concentrations. As the concentration of testosterone was increased, the level of inhibition was reduced to having

little of or no effect. This indicated that these compounds were acting competitively with the substrate owing to the testosterone overcoming the inhibitor as the concentration is increased. The kinetic data from EGCG also supported this competitive inhibition type as Vmax remained constant whilst Km increased as the inhibitor concentration increased. It has yet to be shown if EGCG is a substrate for UGT2B17. However, EGCG is glucuronidated by other UGT enzymes, including UGT1A1 (Lu *et al.*, 2003). Given that these compounds are competitive it suggests these inhibitors are competing with the substrate testosterone for the active site of UGT2B17 (Nelson and Cox, 2002). It was shown that (-)EC was not glucuronidated by UGT2B17 following a glucuronidation assay with (-)EC as the substrate. Given there are differences between the structure of (-)EC and testosterone around the hydroxyl moieties, this further supports the suggestion that (-)EC is not glucuronidated by UGT2B17. However owing to the competitive inhibition displayed by (-)EC and its polyphenolic structure, along with phenols being substrates for UGT2B17 (Turgeon *et al.*, 2003), this indicates it is binding to the active site of the enzyme without being glucuronidated. Average plasma testosterone levels are 35.9 ±6.5 nMol/L n=16 (Behre *et al.*, 1997) and (-)EC concentrations after 1 and 2 hours can be raised between 21.2-133 nMol/L following consumption of seven cups of green tea per day and 20 mg/mL green tea consumption (Behre *et al.*, 1997; Hirano-Ohmori *et al.*, 2005). This indicates that competitive inhibition under these concentrations *in vivo* could be possible. In competitive sport, a common method of testosterone doping regimes is for testosterone to be taken at low dosages at frequent intervals, in cycles (Sottas *et al.*, 2010; Evans *et al.*,2004). A greater inhibitory effect could be benefitted from this regime as if an inhibitor is co-administered with testosterone, the competitive inhibition would be more effective at lower substrate dosages.

Following on from the analysis of tea and catechin samples, the study was extended to red wine and the phenolic compounds present in the red wine sample. Red wine was chosen as a suitable screening sample as it has lower concentrations of catechins compared to green and white teas, whilst having a variety of other phenolic compounds (USADA; Waterhouse, 2002). This study showed that some of the phenolic compounds found in red wine inhibited UGT2B17 testosterone glucuronidation. The red wine sample inhibited UGT2B17 over one and two hour periods. This inhibition was similar when the sample was evaporated to dryness and reconstituted with an equal volume of water to remove ethanol. This further indicates inhibition was caused by the phenolic compounds in the wine sample. The phenolic compounds that inhibited glucuronidation at different efficacies were 4-ethylphenol, gallic acid, caffeic acid and quercetin, whereas p-coumeric acid and chlorogenic acid did not have any effect on the enzyme. The most active inhibiting phenolic compound was quercetin, this compound remained active at reducing testosterone glucuronidation at concentrations of 1.5 and 2 μM with an initial testosterone concentration of 20 μM. Serum concentrations of quercetin have been shown to increase up to 1.5 μM following supplementation (Conquer *et al.*, 1998). It is likely that at a lower concentration of testosterone that would be present in serum, inhibition from quercetin would be greater. Plasma concentrations of the other phenolic compounds gallic acid and caffeic acid increase following consumption of red wine (Simonetti *et al.*, 2001; Caccetta *et al.*, 2000). The increases in concentrations of the phenolic compounds through supplementation and red wine consumption could lead to enhanced reductions in testosterone glucuronidation by inhibiting UGT2B17 with these phenolic compounds. The phenolic compound 4-ethylphenol produces distinct aromas in red wine and is found in a number of other dietary substances as it is a by-product from the spoilage of yeast (Barata *et al.*, 2006). This compound is a substrate of UGT2B17 (Turgeon *et al.*, 2003) and

inhibition was greater at a lower initial concentration of testosterone. It is likely that 4-ethylphenol is acting competitively and being glucuronidated by UGT2B17 along with testosterone.

The phenolic compound 1-naphthol reduced UGT2B17 activity up to 39.9% activity compared to the control sample following the addition of 300 μM 1-napthol with an initial concentration of 100 μM testosterone over 2 hours. The EC_{50} for this compound under these conditions was 138.2 μM. This compound is a substrate of UGT2B17 and is glucuronidated by the enzyme (Turgeon *et al.*, 2003). 1-Naphthol is a key metabolite of fertilisers carbaryl and naphthalene (Meeker *et al.*, 2007), the likely exposure to this compound from the extensive use of fertilisers has shown detectable urinary levels of 1-naphthol in individuals (Meeker *et al.*, 2007). Exposure to 1-naphthol has been shown to reduce excreted testosterone concentrations (Meeker *et al.*, 2007; http://www.jhsph.edu /research/centers-and-institutes/center-for-excellence-in-environmental-health-tracking/Second_Report.pdf). An explanation for this could be due to this compound inhibiting UGT2B17 through being a substrate of the enzyme and reducing testosterone glucuronidation and excretion.

These results highlight the interactions of dietary components and compounds on the interactions with UGT2B17 mediated testosterone glucuronidation. The results showed that catechin and phenolic compounds that are substrates and non substrates of UGT2B17 are capable of inhibiting this enzyme. Apart from UGT2B17 and at a lower rate UGT2B15, other UGT enzymes that glucuronidate testosterone do so at significantly reduced rates (Sten *et al.*, 2009a; Sten *et al.*, 2009b). In a previous study, other minor testosterone glucuronidating UGTs were insensitive to the inhibitor ibuprofen, suggesting

that following inhibition of UGT2B17, other UGT enzymes do not contribute further to the glucuronidation of testosterone in human liver microsomes *in vitro* (Sten *et al.*, 2009b).

Given the role of UGT2B17 in testosterone regulation and excretion, these results indicate common dietary substances could impact on excreted and circulating levels of testosterone. Inhibition of testosterone glucuronidation could have implications for testosterone doping. Performance could be enhanced through elevating levels of free testosterone given that less testosterone will be glucuronidated and excreted. Excreted levels of testosterone glucuronide could be reduced through inhibiting UGT2B17. This could lead to altered T/E levels and an inaccurate representation of testosterone use. Determining any effects that these inhibitors have on the most active epitestosterone glucuronidation enzyme UGT2B7 (Sten *et al.*, 2009a) will be required, to determine if these inhibitors are active in reducing epitestosterone along with testosterone glucuronidation. This will indicate the extent T/E levels could be impacted on by these inhibitors.

3.6 Conclusion

The inhibitory effects of commonly consumed dietary substances and compounds on UGT2B17 have been shown here for the first time. Whilst some of the inhibitors are substrates of UGT2B17, the other compounds are not substrates which could indicate other similar compounds that are not linked to UGT2B17 having similar inhibition effects. These results could have implications on testosterone doping and detection through reducing excreted testosterone and increasing circulating free testosterone, leading to the steroid being active for longer.

Chapter 4. Inhibition of epitestosterone glucuronidation by dietary compounds and pharmaceuticals and the interaction of epitestosterone on testosterone glucuronidation

4.1 Introduction

Epitestosterone is a naturally occurring steroid that is the epimer of testosterone. Whilst the role of testosterone is known as an anabolic sex steroid, epitestosterone does not possess anabolic activity and its hormonal and physiological roles if any, are unknown (Aguilera *et al.*, 2002; Starka, 2003; Sten *et al.*, 2009a). Epitestosterone is used as a marker to monitor testosterone abuse in athletes by measuring the T/E ratio in urine. This marker is chosen given that epitestosterone excretion levels are similar to testosterone and whilst testosterone excretion will increase following its administration, epitestosterone levels will not be altered, therefore increasing the T/E ratio (Saudan *et al.*, 2006). However, the efficiency of the T/E ratio is counteracted by administration of epitestosterone alongside testosterone given that urinary concentration of epitestosterone increases following intake (Dehennin, 1994; Aguilera *et al.*, 2002). This has led epitestosterone to be classed as a urine manipulation agent and suspicious samples are considered if epitestosterone concentrations are raised above 200 ng/mL (Aguilera *et al.*, 2002). However, due to variations between individuals' epitestosterone levels, unless concentrations have exceeded 1000 ng/mL, it has proven difficult to determine if samples between 200 and 1000 ng/mL are adverse (Aguilera *et al.*, 2002; Brooks *et al.*, 1979). Epitestosterone levels have also been known to be reduced naturally that has lead to reports of false positive T/E ratios (Raynaud *et al.*, 1992; Oftebro, 1992).

The results presented in the previous chapter found that a number of dietary components, particularly catechin compounds in teas, inhibit UGT2B17 testosterone glucuronidation. Determination of the inhibitory effects of epitestosterone glucuronidation by UGT2B7 will provide an insight on the glucuronidation of both steroids by these compounds.

The most active enzyme in the glucuronidation of epitestosterone is UGT2B7, other UGT isoforms also glucuronidate epitestosterone at lower rates, notably UGT2A1 and UGT2A3. Epitestosterone is not glucuronidated by UGT2B17, however the addition of epitestosterone has reduced the rate of testosterone glucuronidation by interacting with the enzyme (Sten *et al.*, 2009a). This inhibitory action was found to occur at μM concentrations, however it has not yet been determined if this occurs at lower ng/mL levels that would be representative of those found in human serum. The expression and activity of UGT2B17 varies between males and females (Gallagher *et al.*, 2010). These differences could lead to altered testosterone glucuronidation activity and sensitivities to testosterone glucuronidation.

UGT2B7 is involved in the glucuronidation of a number of compounds and pharmaceuticals including two anti-retroviral drugs efavirenz and zidovudine. When combinations of these drugs have been exposed to UGT2B7, drug-drug interactions occurred, leading to competitive inhibition between both compounds (Belanger *et al.*, 2009). The implication of these anti-retroviral drugs on UGT2B7 epitestosterone glucuronidation is unknown and combinations of these pharmaceuticals with epitestosterone could alter the glucuronidation rate.

UGT2B17 and UGT2B7 are similar in structure, with the 2B subfamily being 76% identical amino acid sequence (Sten *et al.*, 2009a). Given the similarity in structure and sequence between these enzymes, it is postulated that some of the inhibitors of UGT2B17 could also inhibit epitesoterone glucuronidation by UGT2B7. Given that efavirenz and zidovudine are glucuronidated by UGT2B7 it would be expected that when combined with epitestosterone, the glucuronidation of epitestosterone will be reduced as these compounds will compete for the active site. It is also postulated that epitestosterone will interfere with UGT2B17 and reduce testosterone glucuronidation at ng/mL levels.

4.2 Aims

The aims of this chapter are to investigate inhibition of UGT2B7 epitestosterone by dietary and pharmaceutical components, in addition another aim is to determine the inhibitory action of epitestosterone on UGT2B17 testosterone glucuronidation. To meet this aim –

- Tea and catechin inhibitors of UGT2B17, displayed in chapter 3, were analysed to determine the inhibitory effect these teas and catechins have on the glucuronidation of epitestosterone by UGT2B7.
- Inhibitory effects of UGT2B7 substrates efavirenz and zidovudine on epitestosterone glucuronidation was determined.
- The effect epitestosterone has on testosterone glucuronidation with UGT2B17 and pooled male and female human liver microsomes was determined.

4.3 Methods

The full methods are described in chapter 2. Briefly:

- Solutions of tea extracts were prepared and filtered (1.6g in 80 mL water), added to epitestosterone glucuronidation assays (5% v/v). Catechins were added to the reactions to give a catechin concentration of 250 μM (2.3.1, 2.3.2, 2.4.1).
- HPLC and LC-MS/MS analysis was used for determining concentrations of testosterone and epitestosterone. HPLC analysis was used for determining efavirenz concentrations (2.4.2, 2.4.3.2, 2.4.4). Samples were extracted by LLE extraction prior to LC-MS/MS analysis (2.4.1).
- Stock solutions of efavirenz and ziduvodine were dissolved in methanol and water respectively, prepared at 2.5 mg/mL stock solutions and stored at 4°C. Serial dilutions of these drugs were prepared and added to epitestosterone glucuronidation assays 2% v/v.
- Stock solutions of testosterone and epitestosterone were prepared as 5 mg/mL concentrations dissolved in acetonitrile (testosterone) or methanol (epitestosterone). These solutions were diluted to working standards and samples for the standard curve and QC samples. Stock and working standard solutions were stored in silanised vials at -20°C.

The HPLC and LC-MS/MS method validation for testosterone, epitestosterone and efavirenz analysis was performed by spiking the compounds dissolved in the relevant solvent (1% v/v) into a 1 mL matrix solution consisting of 25 μg/mL alamethicin , 8 mM magnesium chloride, 50 mM of a pH 7.5 Tris-HCl buffer and deionised water. The validation of testosterone and epitestosterone analysis was performed together.

4.4 Results

4.4.1 Method validation

4.4.1.1 HPLC analysis of epitestosterone

The validation of epitestosterone was performed alongside the validation of testosterone from the previous chapter (section 3.4.1). A chromatogram showing the separation of testosterone and epitestosterone using a mobile phase of water:acetonitrile (61:39) is displayed in Figure 4.1. The calibration curve, r^2, and regression equation for epitestosterone at reference concentrations (0.39-50 µg/mL) used in this study is shown in Figure 4.2. The r^2 value for the calibration curve was above 0.999 for epitestosterone showing good linearity. Inter and intra-day precision for epitestosterone is shown in Table 4.1. The accuracy and precision levels for epitestosterone was within the limits set by FDA guidelines. Testosterone validation for this method was performed in the previous chapter (section 3.4.1), the validation results for testosterone were within FDA guideline limits.

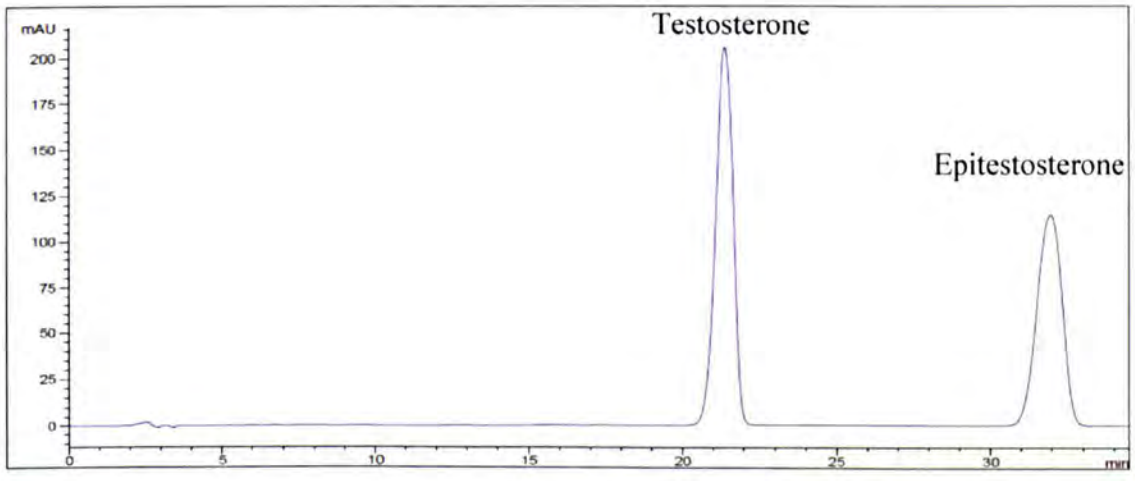


Figure 4.1 Chromatogram showing the separation of testosterone and epitestosterone for the HPLC method used in this study to separate these compounds.

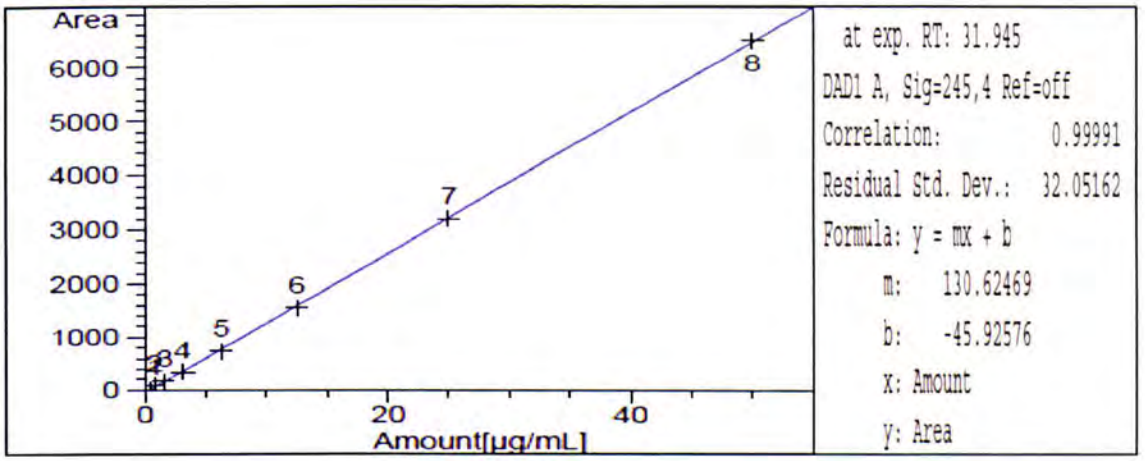


Figure 4.2 Calibration curve, r^2 value and regression curve for epitestosterone analysis using HPLC for the concentration ranges used in this study and method validation.

Table 4.1 Inter- and intra-day precision and accuracy results for the HPLC analysis of epitestosterone using QC under assay conditions (N=6).

Method	Compound	Concentration (µg/mL)	Level	Precision RSD (%)		Accuracy (%)
				Intra-day N=6+6+6	Inter-day N=18+18+18	
Water: Aceto-nitrile	Epitestosterone	0.781	Low	2.27	2.57	99.66
		3.125	Medium	1.01	4.42	106.04
		25.00	High	2.36	3.13	104.20

4.4.1.2 Thermo Scientific LC-MS/MS (method B) analysis of testosterone and epitestosterone

Chromatogram and mass spectra of separated 1.563 ng/mL testosterone and epitestosterone are shown in Figure 4.3. The calibration curves of testosterone and epitestosterone over the reference concentrations (testosterone: 0.781-100 ng/mL, epitestosterone: 1.563-100 ng/mL) used in this study are shown in Figure 4.4. Inter- and intra-day accuracy and precision data for testosterone and epitestosterone are shown in Table 4.2 and the

extraction recoveries of each analyte are shown in Table 4.3. Accuracy and precision data for both analytes were within the limits set by FDA guidelines.

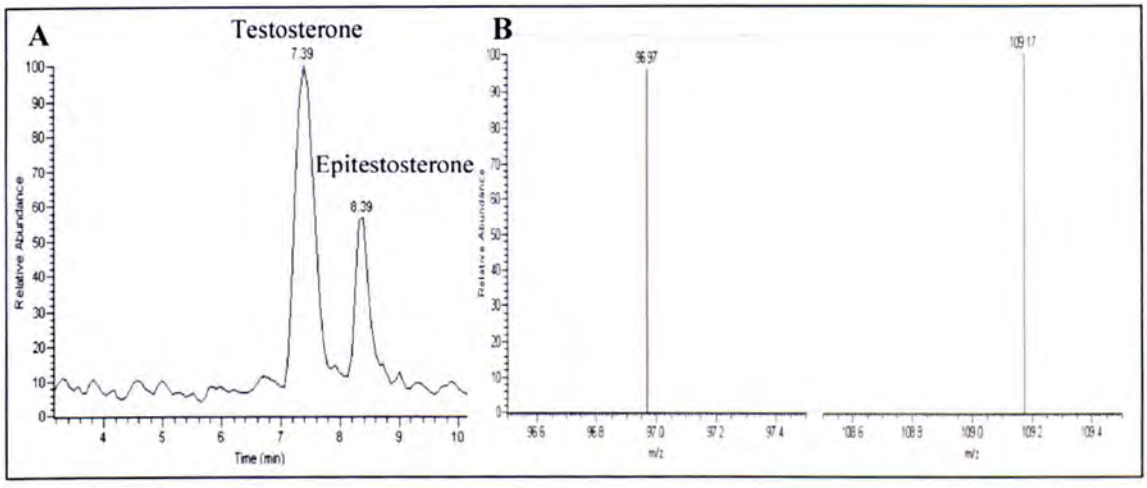


Figure 4.3 Chromatogram and mass spectrum for analysis of testosterone and epitestosterone at 1.563 ng/mL (LLOQ level for epitestosterone). A – Chromatogram of testosterone and epitestosterone, B – Mass spectrum of product ions at 97 and 109.

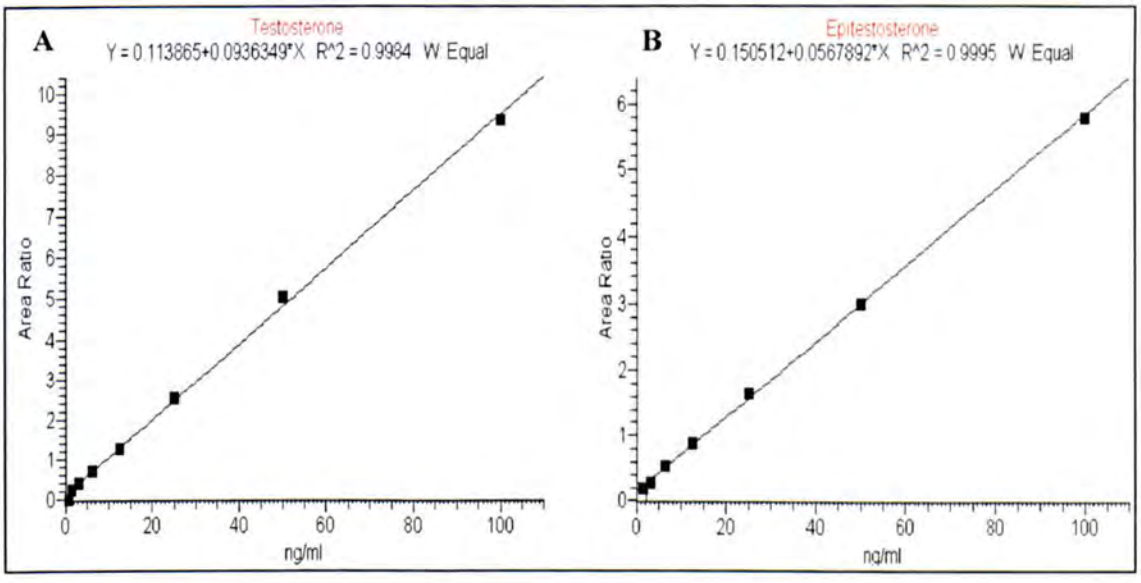


Figure 4.4 Calibration curves of A – testosterone and B – epitestosteron, r^2 values and regression equations for testosterone and epitestosterone during the first validation run. Calibration curves are the concentration against the ratio of analyte area over internal standard area.

Table 4.2 Assay validation results for testosterone and epitestosterone.

Compound	Concentration (ng/mL)	Level	Precision RSD (%)		Accuracy (%)
			Intra-day N=6+6+6	Inter-day N=18+18+18	
Testosterone	6.25	Low	1.73	2.05	96.81
	12.5	Medium	1.31	1.49	97.22
	50	High	6.89	7.05	98.02
Epitestosterone	6.25	Low	1.81	2.20	95.61
	12.5	Medium	2.05	2.89	96.87
	50	High	4.69	4.97	96.41

Table 4.3 Extraction recoveries results for testosterone and epitestosterone (N=6).

Compounds	Concentration (ng/mL)	Extraction recovery (%)
Testosterone	1.563 (LLOQ)	91.68
	12.5	93.82
	25	93.11
Epitestosterone	3.125 (LLOQ)	90.84
	12.5	94.34
	25	92.77

4.4.1.3 Analysis of efavirenz by HPLC

Chromatogram of a spiked sample with efavirenz is shown in Figure 4.5. A calibration curve with reference standards ranging between 0.781 – 100 µg/mL efavirenz was prepared, the calibration curve showed good linearity with an r^2 value above 0.999. The calibration curve, r^2 and regression values of efavirenz are displayed in Figure 4.6. Table 4.4 displays the inter- and intra-day precision and accuracy of efavirenz, the accuracy and precision were found to be within the limits set by FDA guidelines.

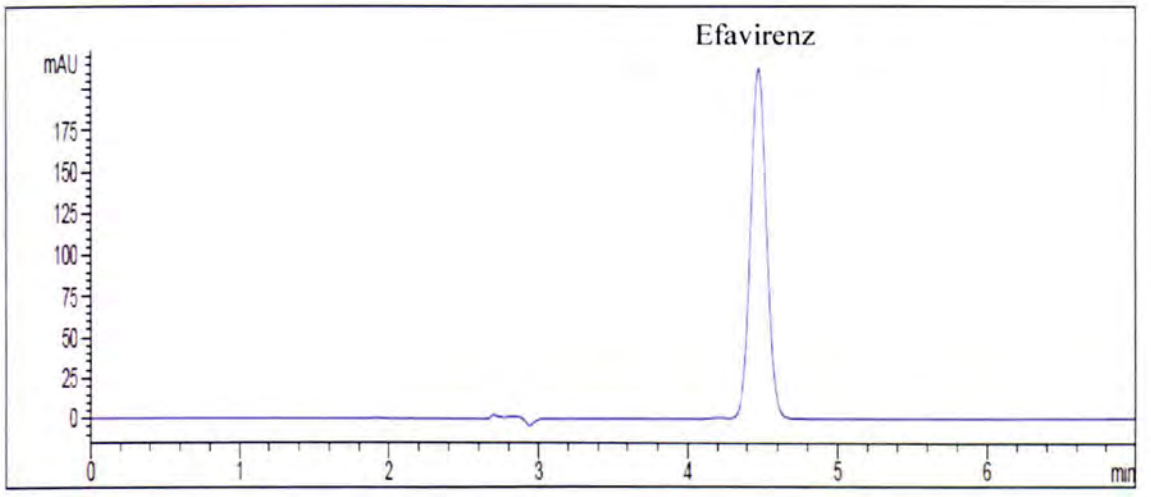


Figure 4.5 HPLC chromatogram of efavirenz dissolved in methanol.

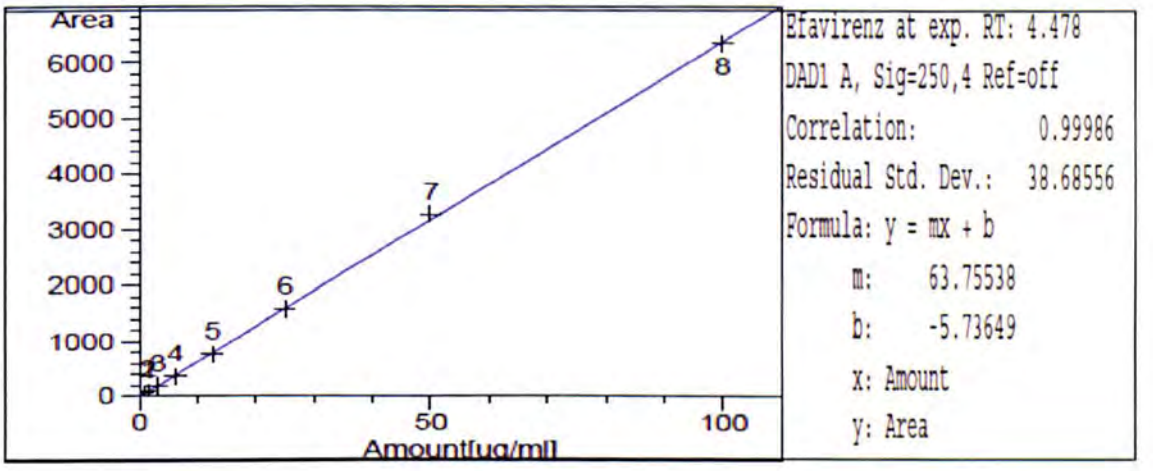


Figure 4.6 Calibration curve of efavirenz displaying the linear range of concentrations used in this study, along with the r^2 and regression equations.

Table 4.4 Precision and accuracy validation results for the HPLC analysis of efavirenz.

Compound	Concentration (ng/mL)	Level	Precision RSD (%)		Accuracy (%)
			Intra-day N=6+6+6	Inter-day N=18+18+18	
Efavirenz	1.563	Low	3.25	2.71	94.52
	6.250	Medium	1.45	1.59	98.11
	25	High	2.85	1.70	99.20

4.4.2 Epitestosterone glucuronidation by UGT2B7

The glucuronidation of epitestosterone by UGT2B7 used in this study was monitored over set time points. Epitestosterone glucuronidation assays were performed using an initial epitestosterone concentration of 12 μg/mL as the only substrate and terminating the reaction at set time points. Figure 4.7 displays the percentage reduction of epitestosterone over a 90 minute period at set time points. A reduction in epitestosterone was observed over at each time point with the addition of 0.2 mg/mL UGT2B7 protein concentration. After 90 minutes, 54% of epitestosterone remained from the initial concentration added.

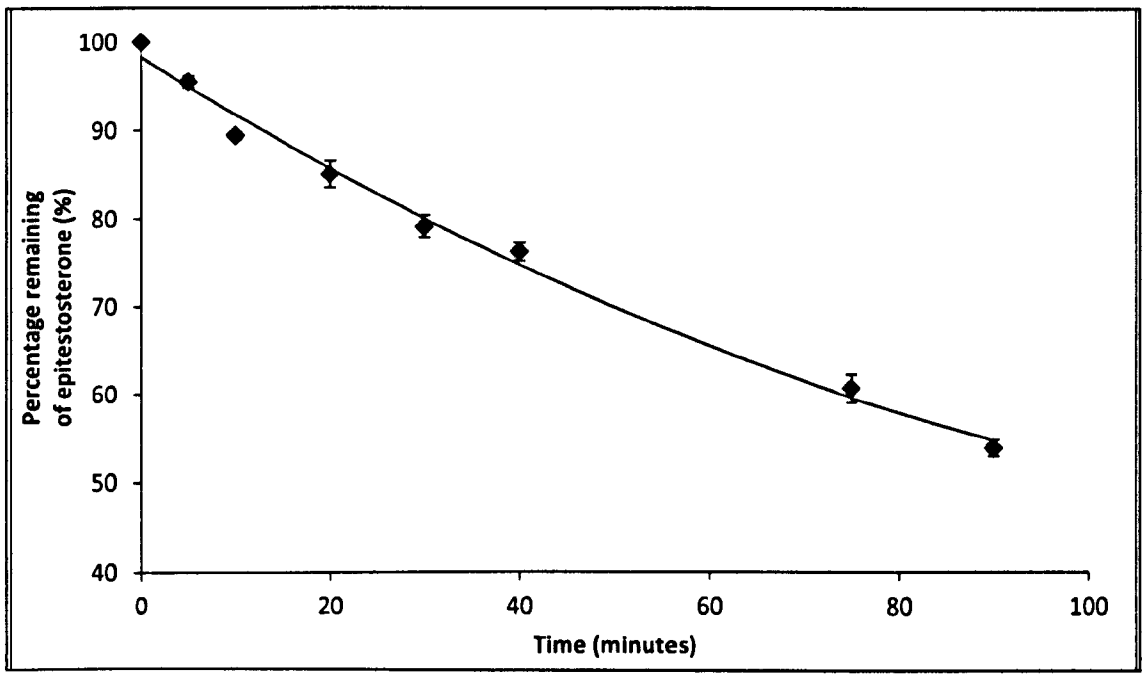


Figure 4.7 Glucuronidation of 12 μg/mL epitestosterone by UGT2B7 over 90 minutes. Each point represents the mean ± SEM of duplicate values.

4.4.3 Effects of tea samples and catechins on epitestosterone glucuronidation

Four tea samples and two reference catechin standards (-)EC and EGCG at a concentration of 250 μM were screened to determine if they have inhibitory effects on epitestosterone by

UGT2B7. The changes in epitestosterone glucuronidation rate after 60 minutes are displayed in Figure 4.8. The rate of epitestosterone glucuronidation was reduced by all four of the tea samples and EGCG by similar amounts compared to the control, however the addition of (-)EC did not reduce glucuronidation rate and was similar to the control.

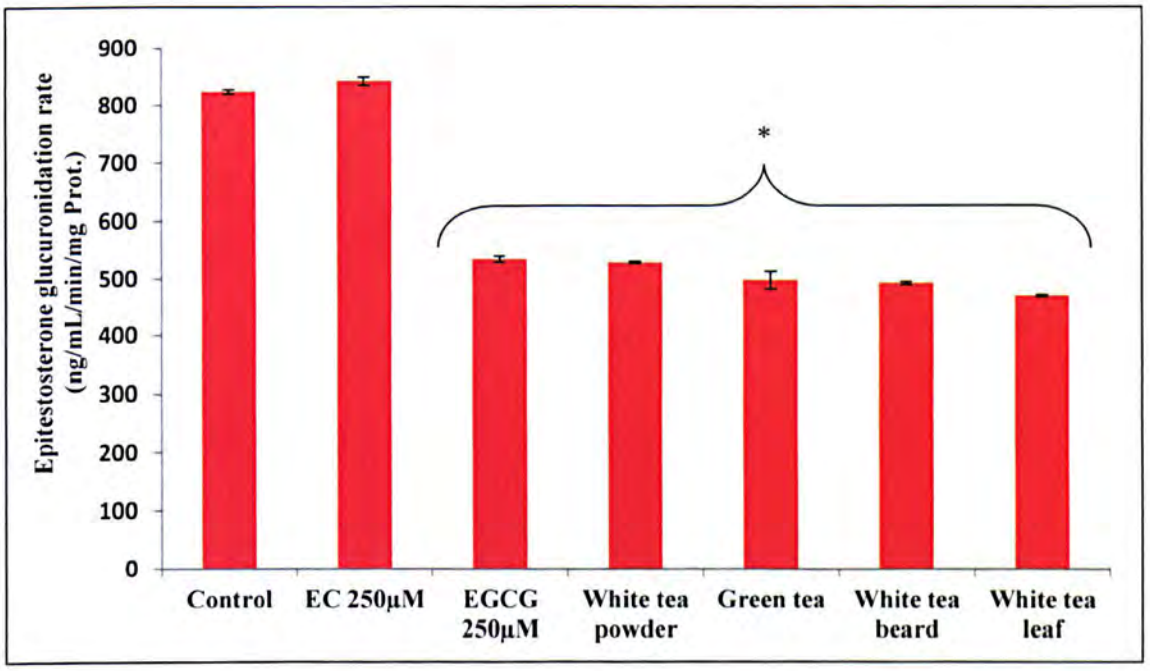


Figure 4.8 Changes to UGT2B7 epitestosterone glucuronidation rate following the additions of tea samples or catechin standards. The initial concentration of epitestosterone was 15.8 µg/mL and the UGT2B7 protein concentration was 0.2 mg/mL. The concentration of tea sample in reactions was 1 mg/mL. The reaction time was 60 minutes. Values are the mean ± SEM of duplicate samples (* $p<0.05$).

As the tea samples significantly inhibit UGT2B7 mediated epitestosterone glucuronidation, catechin compounds were screened to determine if these components inhibit UGT2B7. Figure 4.9 shows the changes in epitestosterone glucuronidation rate following the addition of 250 µM standards of catechin compounds after 60 minutes. The catechin compounds were (-)EC, (+)EC, ECG and EGCG. These compounds were found to be present in the

four tea samples (Figure 3.6) and in the previous chapter it was shown that all of these compounds significantly inhibit UGT2B17 testosterone glucuronidation, within the range of 22.42-84.48% reduction in glucuronidation rate (Figure 3.8). (+)EC, ECG and EGCG significantly reduced epitestosterone glucuronidation. The order of inhibition activity of these compounds was similar to that found for the UGT2B17 enzyme, with EGCG being most active and (-)EC being the least active of these four compounds for both UGT2B17 and UGT2B7.

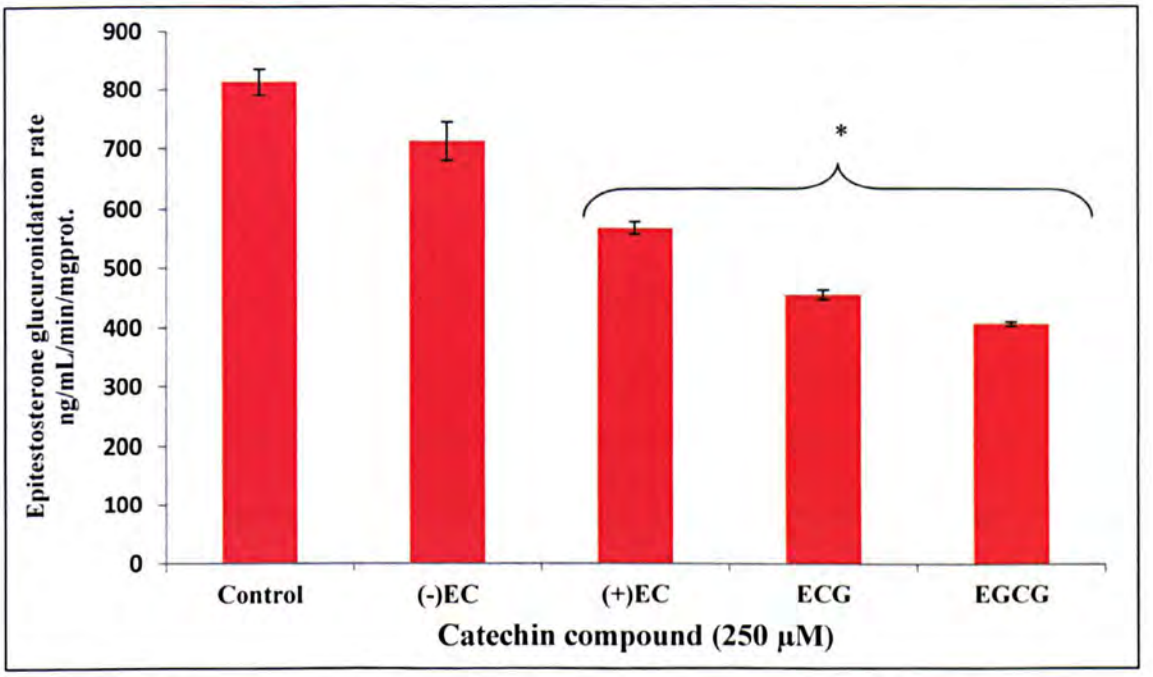


Figure 4.9 UGT2B7 epitestosterone glucuronidation rate following the addition of 250 µM individual catechin standard. The initial epitestosterone concentration was 15.8 µg/mL and the UGT2B7 protein concentration was 0.1 mg/mL. The reaction time was 60 minutes. Values are the mean ± SEM of triplicate samples (* $p<0.05$).

4.4 Interaction of antiretroviral drugs on UGT2B7 epitestosterone glucuronidation

The following sections display the results of the inhibitory effects of two antiretroviral drugs efavirenz and ziduvodine on UGT2B7 epitestosterone glucuronidation. The results also demonstrate any effects of adding epitestosterone on the rate of efavirenz glucuronidation by UGT2B7.

4.4.4.1 Efavirenz

The inhibitory activity of efavirenz on UGT2B7 epitestosterone glucuronidation was assessed by the use of a Lineweaver-Burk plot (displayed in Figure 4.10 with the values from this shown in Table 4.5). The initial epitestosterone concentrations ranged from 1.25-20 μg/mL with increasing concentrations of efavirenz added to each set of initial concentrations. The results indicated efavirenz acts competitively to inhibit UGT2B7 epitestosterone glucuronidation. As the inhibitor concentration was gradually increased Vmax remained consistent whereas Km increased, Vmax only increased as a high concentration of efavirenz was added.

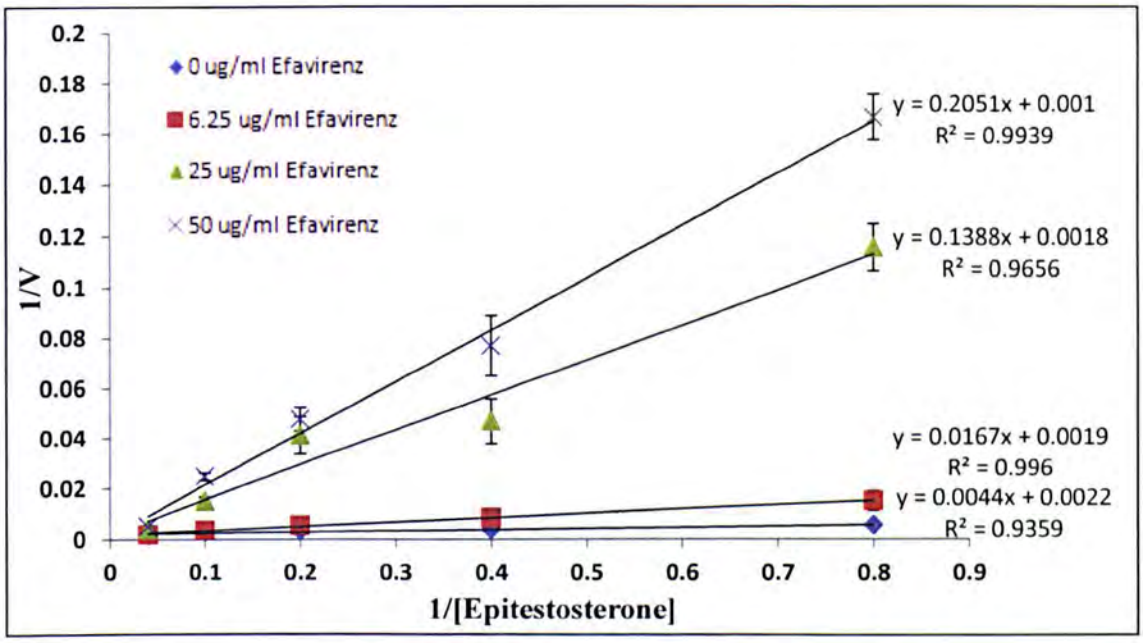


Figure 4.10 Lineweaver-Burk plot for efavirenz against UGT2B7 epitestosterone glucuronidation (results are displayed in Table 4.5). Competitive inhibition was displayed with a consistent Vmax that was only raised by adding a high concentration of efavirenz, an increase in Km was observed as the efavirenz concentration added was raised. The reaction time was 30 minutes and the UGT2B7 protein concentration was 0.1 mg/mL. Values are the mean ± SEM of duplicate samples.

Table 4.5 Kinetic parameters for efavirenz inhibition of UGT2B7 epitestosterone glucuronidation, taken from the Lineweaver-Burk plot from Figure 4.10.

Enzyme	Efavirenz concentration	Vmax (ng/mL/ min/mg prot.)	Km (µg/mL)	Inhibition type
UGT2B7	0 µg/mL	454.5	2.00	Competitive
	6.25 µg/mL	526.3	8.79	
	25 µg/mL	555.6	77.11	
	50 µg/mL	1000.0	205.10	

As efavirenz was shown to competitively inhibit the glucuronidation of epitestosterone by UGT2B7, this study was extended to determine the effects of increasing concentrations of epitestosterone on efavirenz glucuronidation by UGT2B7. A Lineweaver-Burk plot of increasing epitestosterone on the glucuronidation of efavirenz is shown in Figure 4.11; the kinetic data are displayed in Table 4.6. The addition of 10 and 50 µg/mL epitestosterone to initial concentrations of efavirenz ranging between 3.6-60 µg/mL did not alter the glucuronidation of efavirenz significantly. The kinetic data from the Lineweaver-Burk plot (Table 4.6) show that Vmax remained constant whilst Km only increased slightly following the addition of epitestosterone.

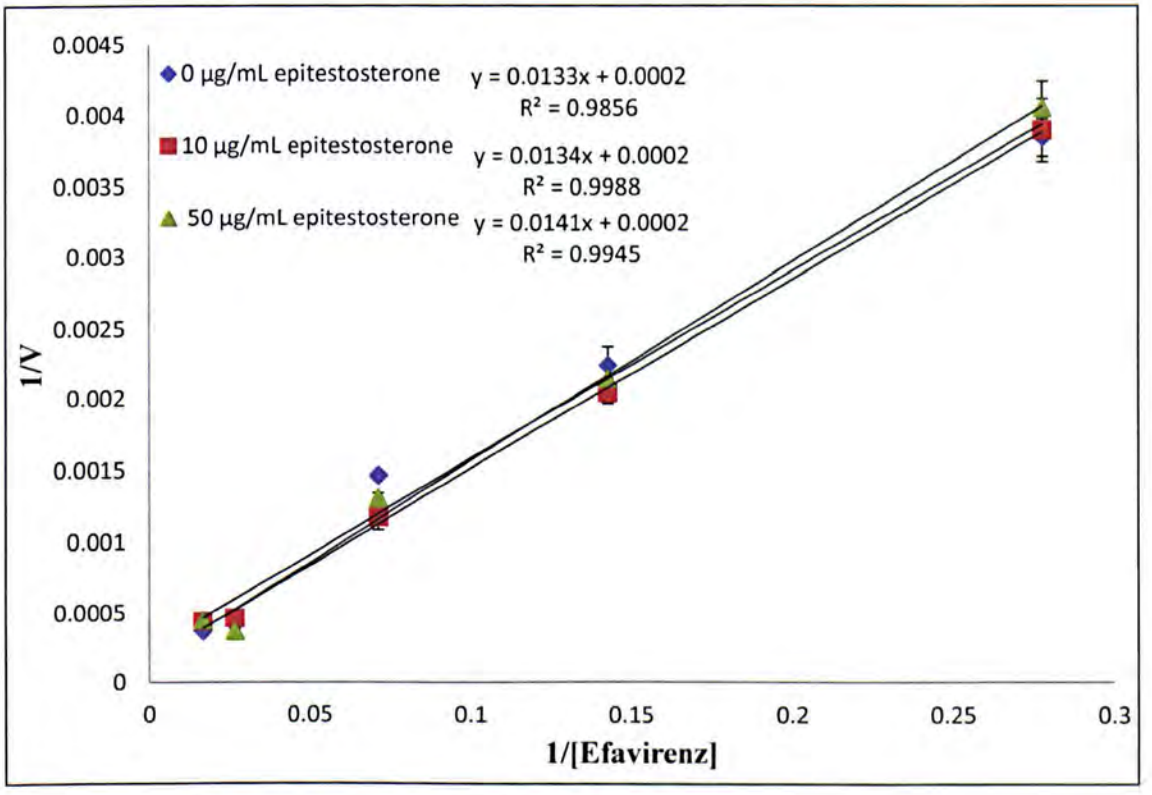


Figure 4.11 Lineweaver-Burk plot for epitestosterone against UGT2B7 efavirenz glucuronidation (results are displayed in Table 4.6). Vmax remained constant following the addition of epitestosterone and only minimal effects were observed on the Km values. The reaction time was 30 minutes and the UGT2B7 protein concentration was 0.1 mg/mL. Values are the mean ± SEM of duplicate samples.

Table 4.6 Kinetic parameters for the Lineweaver-Burk plot (Figure 4.11) on the effect of epitestosterone on efavirenz glucuronidation.

Enzyme	Epitestosterone concentration	Vmax (ng/mL/ min/mg prot.)	Km (μg/mL)	Inhibition type
UGT2B7	0 μg/mL	5000	66.5	None
	10 μg/mL	5000	67.0	
	50 μg/mL	5000	69.5	

4.4.4.2 Ziduvidine

The inhibitory action of another antiretroviral drug, ziduvodine on UGT2B7 epitestosterone was investigated. The Lineweaver-Burk plot in Figure 4.12 shows the effect of increasing concentrations of ziduvodine on epitestosterone glucuronidation. Initial concentrations of epitestosterone ranged from 1.25-20 μg/mL. Ziduvodine had contrasting effects compared to efavirenz as only minimal inhibitory activity towards UGT2B7 epitestosterone glucuronidation was displayed. The kinetic data from the Lineweaver-Burk plot is displayed in Table 4.7. This data shows that increasing ziduvodine increased Vmax slightly whilst only having a minor increase in Km, however this increase would not be considered to make this drug a viable inhibitor at these concentrations.

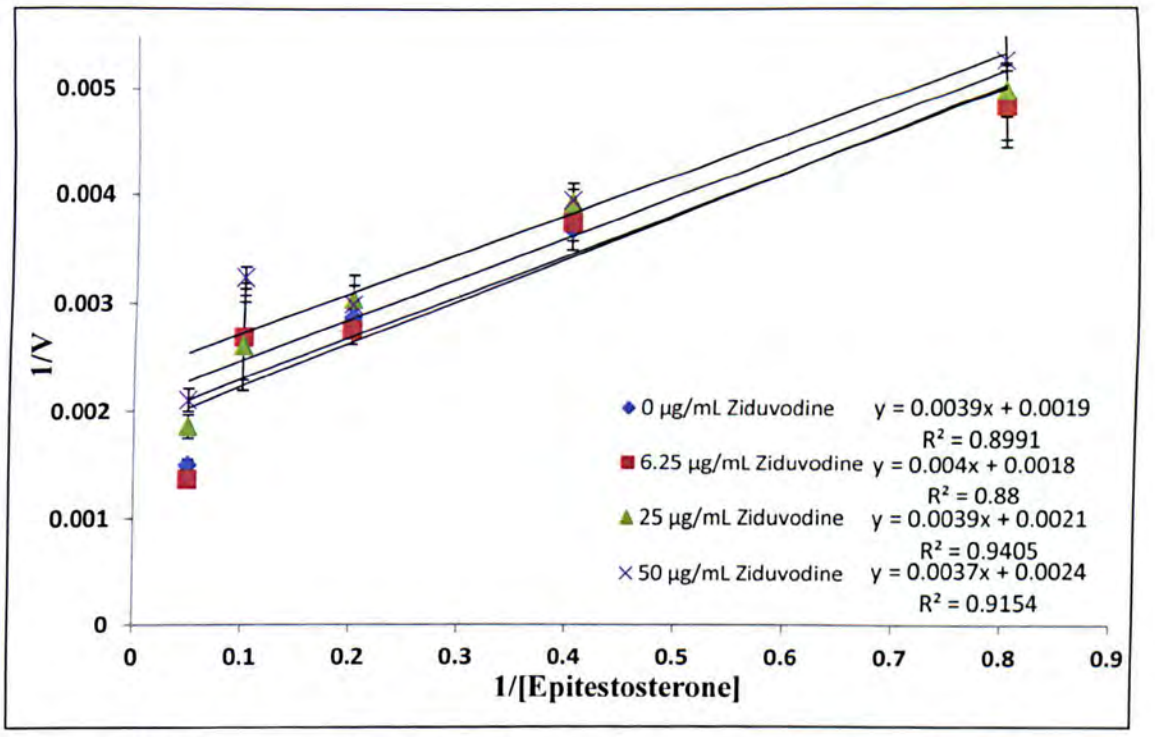


Figure 4.12 Lineweaver-Burk plot for ziduvodine against UGT2B7 epitestosterone glucuronidation (results are displayed in Table 4.7). There was no viable inhibition displayed owing to the minor increases in Vmax and Km with increasing concentrations of ziduvodine added. The reaction time was 30 minutes with a UGT2B7 protein concentration of 0.1 mg/mL. Values are the mean ± SEM of duplicate samples.

Table 4.7 Kinetic parameters for the effect of ziduvodine on UGT2B7 epitestosterone glucuronidation, taken from the Lineweaver-Burk plot from Figure 4.12.

Enzyme	Efavirenz concentration	Vmax (ng/mL/ min/mg prot.)	Km (µg/mL)	Inhibition type
UGT2B7	0 µg/mL	416.7	1.63	None
	6.25 µg/mL	476.2	1.90	
	25 µg/mL	555.6	2.33	
	50 µg/mL	526.3	2.11	

4.4.5 Interaction of epitestosterone on testosterone glucuronidation

The role of epitestosterone on UGT2B17 and gender pooled microsome testosterone glucuronidation was investigated. This study followed on from a previous study (Sten *et al.*, 2009a) to analyse these effects at both μg/mL and physiological ng/mL concentrations of these steroids. It has previously been found that increases in epitestosterone between 10-125 μM, reduces UGT2B17 glucuronidation of testosterone, this was determined at initial concentrations of testosterone ranging between 10-50 μM (Sten *et al.*, 2009a).

4.4.5.1 UGT2B17

The effect of increasing concentrations of epitestosterone on the rate of testosterone glucuronidation by UGT2B17 supersomes is displayed in Figure 4.13 at μg/mL concentrations and Table 4.8 for ng/mL concentrations. Figure 4.13 shows the effect of increasing concentrations of epitestosterone ranging between 0-30 μg/mL on the rate of 6.8 μg/mL initial testosterone. The addition of epitestosterone at 7.5 μg/mL and above significantly reduced the rate of testosterone glucuronidation, these epitestosterone levels were above the initial concentration of testosterone.

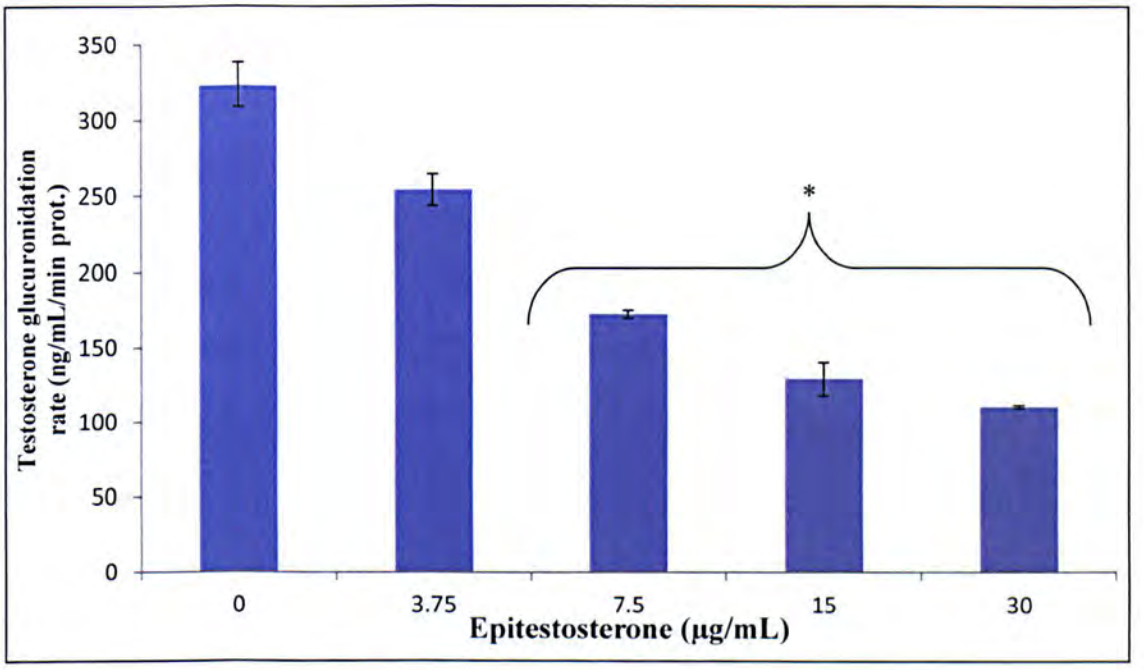


Figure 4.13 Changes in UGT2B17 testosterone glucuronidation rate by epitestosterone. The reaction time was 30 minutes using 0.1 mg/mL UGT2B17 protein concentration. Values are the mean ± SEM of duplicate samples (* $p<0.05$).

Table 4.8 Effect of epitestosterone on UGT2B17 testosterone glucuronidation at levels similar to physiological concentrations. The reaction time was 30 minutes using 0.05 mg/mL UGT2B17 protein concentration. Values are the mean ± SEM of duplicate samples.

Epitestosterone (ng/mL)	Testosterone (ng/mL)	Testosterone/ Epitestosterone	Testosterone glucuronidation Rate (ng/mL/min/mg prot.)
0	50		8.27 ±0.129
3.125	50	16	8.30 ±0.099 (p=0.876)
6.25	50	8	8.28 ±0.057 (p=0.962)
12.5	50	4	8.16 ±0.149 (p=0.619)
25	50	5	7.97 ±0.240 (p=0.634)
50	50	1	7.85 ±0.064 (p=0.145)
100	50	0.5	7.64 ±0.085 (p=0.091)

4.4.5.2 Effects of increasing epitestosterone on glucuronidation by male and female pooled microsomes

The effects of epitestosterone on testosterone glucuronidation by pooled male and female microsomes was analysed and the differences between the two sets of gender microsomes on the rate of testosterone glucuronidation. Increasing epitestosterone levels ranging between 0-60 μg/mL were added to 13.16 μg/mL of initial testosterone (Figure 4.14). A reduction in the rate of testosterone glucuronidation was observed as epitestosterone was added with a significant reduction for males at 30 μg/mL and 60 μg/mL epitestosterone using male microsomes. Testosterone glucuronidation rate was significantly reduced at 60 μg/mL epitestosterone using both male and female pooled microsomes. This study was then applied at lower concentration of steroids using LC-MS/MS analysis, using increasing epitestosterone concentrations ranging between 0-200 ng/mL with an initial testosterone glucuronidation rate of 50 ng/mL (Figure 4.15). Under these conditions the rate of testosterone glucuronidation was not altered by the addition of epitestosterone. However there were significant differences between the testosterone glucuronidation rates between the male and female pooled microsomes in the samples regardless of any epitestosterone added. The male pooled microsomes glucuronidated testosterone at a greater rate than female pooled microsoes under these concentrations.

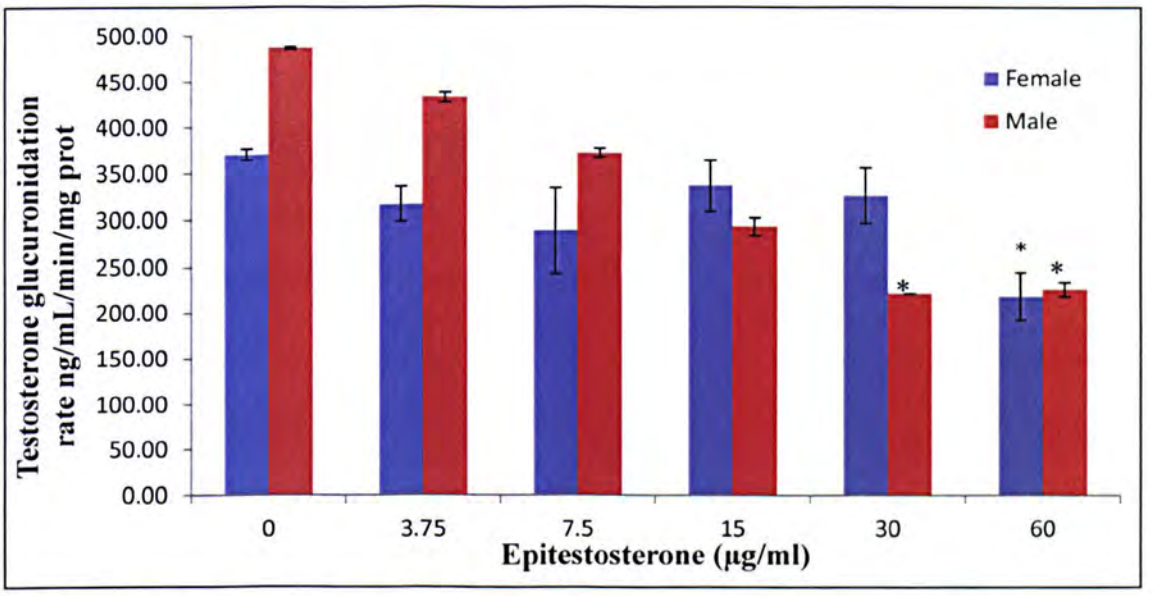


Figure 4.14 Alteration in testosterone glucuronidation from the addition of epitestosterone between gender-pooled microsomes. The reaction time was 30 minutes using 0.4 mg/mL microsomal protein. Values are the mean ± SEM of duplicate samples (* $p<0.05$).

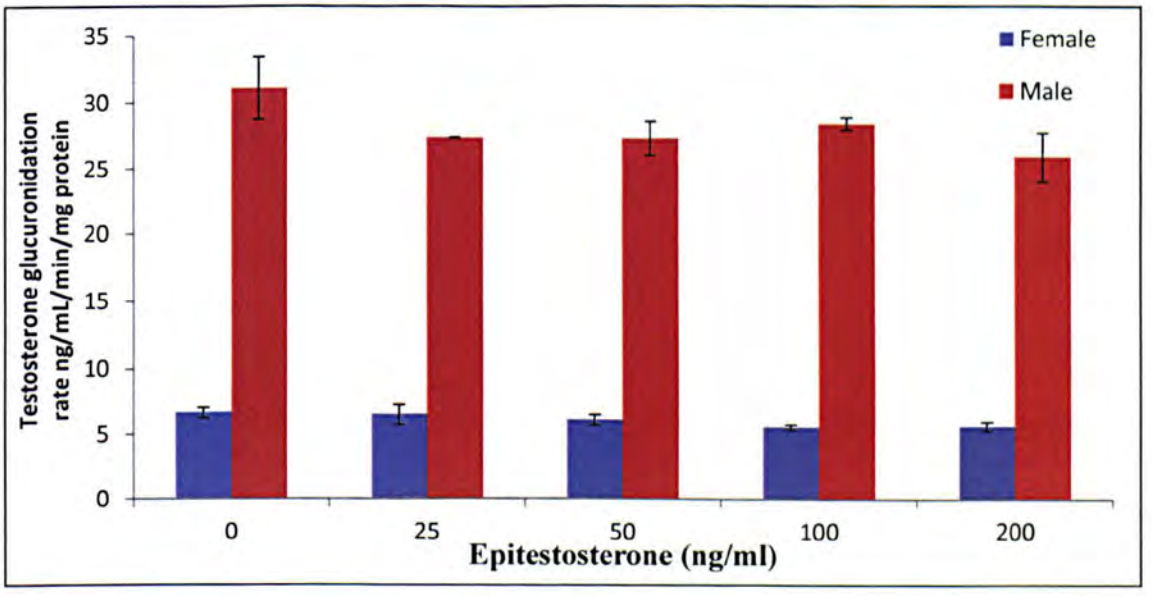


Figure 4.15 Increasing epitestosterone on microsomal testosterone glucuronidation at ng/mL concentrations. The rate of microsomal testosterone glucuronidation was not altered by the addition of epitestosterone. The reaction time was 30 minutes with a microsomal protein concentration of 0.2 mg/mL. Values are the mean ± SEM of duplicate samples.

4.5 Discussion

These results showed that dietary teas and catechin compounds inhibit UGT2B7 glucuronidation of epitestosterone, extending on the results of the previous chapter showing the inhibition of the same teas and catechins inhibiting UGT2B17 testosterone glucuronidation. The four tea samples and 250 μM EGCG inhibited epitestosterone glucuronidation, reducing the rate by similar amounts that ranged between 35.3-42.8% for these samples. However the (-)EC catechin did not have any significant effect on UGT2B7 epitestosterone glucuronidation. (-)EC was found to significantly reduce the rate of testosterone glucuronidation by UGT2B17 (Figure 3.8), whereas under these condition it did not have any significant effect on epitestosterone glucuronidation by UGT2B7. The four tea samples reduced epitestosterone glucuronidation by similar efficacies. Whereas a solution of the white tea powdered sample was a more effective inhibitor of UGT2B17 testosterone glucuronidation than the other teas. This could be due to all four tea samples expressing maximum inhibitory effect of UGT2B7 with the amount of tea sample added to the reactions.

The effects of the four catechins that inhibited UGT2B17, (-)EC, (+)EC, ECG and EGCG, were analysed to determine if these compounds inhibit UGT2B7 epitestosterone glucuronidation. As the tea samples had inhibited UGT2B7 it would be likely that these catechin compounds could be active in UGT2B7 inhibition, similar to UGT2B17. The results showed that (+)EC, ECG and EGCG inhibit UGT2B7 and significantly reduce the rate of epitestosterone glucuronidation. The inhibitory activity of each catechin resembled the results of how each of these compounds inhibit UGT2B17 testosterone glucuronidation. ECG and EGCG were more active in reducing epitestosterone than (-)EC

and (+)EC. As with the UGT2B17 enzyme the (+)EC isomer was more active inhibiting UGT2B7 than the (-)EC isomer.

Two anti-retroviral drugs ziduvodine and efavirenz, which are substrates of UGT2B7 (Belanger *et al.*, 2009), were analysed for their inhibition activity against UGT2B7 epitestosterone glucuronidation. Efavirenz displayed competitive inhibition of epitestosterone glucuronidation. At lower initial concentrations of epitestosterone the inhibition was more effective, which was reduced as the epitestosterone concentration was increased. This was shown in the increased Km with increasing efavirenz concentration whereas Vmax remained constant apart from increasing at the highest concentration of efavirenz added. Following on from these results a study investigating the effects of epitestosterone on efavirenz glucuronidation revealed epitestosterone did not alter the UGT2B7 glucuronidation rate of efavirenz. This could be due to UGT2B7 glucuronidating efavirenz at a greater rate than epitestosterone and the additional concentration of epitestosterone did not have a significant effect on efavirenz glucuronidation with the concentration of enzyme present in the reaction. Analysis of ziduvodine at the same concentrations of efavirenz revealed that ziduvodine was not a viable inhibitor of UGT2B7 testosterone glucuronidation. It has previously been shown the efavirenz is glucuronidated at a much greater rate than ziduvodine (Belanger *et al.*, 2009), this means it is likely that a much greater concentration of ziduvodine would be required in order to have any potential reduction in epitestosterone glucuronidation by inhibition of UGT2B7. Both anti-retroviral drugs are used in the treatment of HIV. Plasma concentrations of efavirenz have been shown to vary depending on enzyme polymorphisms with measured plasma concentrations ranging between 1.09-6.07 μg/mL (Rodriguez-Novoa *et al.*, 2005). These plasma samples were analysed from subjects receiving 600 mg efavirenz daily for 3 months. Plasma

concentrations of efavirenz are much greater than plasma epitestosterone concentrations. These high plasma efavirenz concentrations may competitively inhibit UGT2B7 epitestosterone glucuronidation *in vivo*. Increases in the plasma concentrations of epitestosterone from administration are likely to be less than the plasma concentrations of efavirenz if used at therapeutic doses. It would therefore be likely that the effect of inhibiting efavirenz glucuronidation by epitestosterone administration, if any, would be minimal. Another aspect that was analysed was the interaction of epitestosterone on efavirenz glucuronidation. As efavirenz reduced the rate epitestosterone glucuronidation, it was expected that increases in epitestosterone substrate would reduce UGT2B7 efavirenz glucuronidation. This could lead to implications on efavirenz glucuronidation rates and bioavailability from varying epitestosterone levels. However it was found that epitestosterone had little effect on efavirenz glucuronidation. This indicates that efavirenz is a stronger substrate of UGT2B7 than epitestosterone.

The interaction of epitestosterone on the glucuronidation of testosterone by UGT2B17 and microsomes has been investigated. This study extended a previous study that observed increasing epitestosterone concentration reduced the rate of testosterone glucuronidation by interacting with UGT2B17, however in this study epitestosterone was not glucuronidated by UGT2B17 (Sten *et al.*, 2009a). Using gender pooled microsomes, a reduction in testosterone glucuronidation rate was observed as epitestosterone was increased for both male and female pooled microsomes at μg/mL concentrations of testosterone and epitestosterone. However in a similar study with reduced ng/mL concentrations of testosterone and epitestosterone, the rate of testosterone glucuronidation remained unaltered in both male and female pooled microsomes with the addition of epitestosterone. In this study, a significant difference was observed between the rates of

testosterone glucuronidation between the two gender microsomes. The male pooled microsomes had a significantly greater rate of testosterone glucuronidation compared to the female pooled microsomes. This could be due to enhanced expression of UGT enzymes in the male microsomes. A previous study highlights differences in RNA expression of UGT2B17 enzymes between males and females (Gallagher *et al.*, 2010). At ng/mL levels of testosterone and epitestosterone using UGT2B17 supersomes, a reduction of testostersone glucuronidation rate was observed following the addition of epitestosterone. However, following the addition of epitestosterone, the reduced rate was less than when μg/mL concentrations of these steroids were used. The observed reduction in glucuronidation rate at ng/mL levels was not significant at the highest concentration of epitestosterone which was double the initial 50 ng/mL concentration of testosterone.

The results from this chapter highlight the role of dietary compounds and pharmaceuticals on epitestosterone glucuronidation through the inhibition of UGT2B7. This includes tea and catechin compounds commonly found in teas reducing the rate of epitestosterone glucuronidation by UGT2B7. This study was an extension of the results obtained from the previous chapter that showed that sample tea and catechin compounds inhibit the glucuronidation of testosterone by UGT2B17. These results highlight the role commonly consumed dietary samples and pharmaceuticals can have on the regulation of testosterone and epitestosterone. There was also variation between some of the tea and catechin compounds interactions between the two enzymes. For example (-)EC had no effect on UGT2B7 epitestosterone, whilst in a study on the previous chapter this compound significantly reduced UGT2B17 testosterone glucuronidation rate. This could have an impact if applied *in vivo* towards the regulation and excretion of both of these steroids. These results also showed that efavirenz is a competitive inhibitor of epitestosterone

glucuronidation. Efavirenz has been shown to be a substrate of UGT2B7 (Belanger *et al.*, 2009), the substrate specify of UGT2B17 is not known, it is therefore likely that this drug will inhibit only epitestosterone glucuronidation. If applied *in vivo* the use of efavirenz could lead to the reduction of epitestosterone excretion whilst testosterone glucuronidation and excretion remained unaltered, this could have implications in testosterone doping and detection. An increased T/E ratio could result, owing to decreased urinary epitestosterone levels. At the same concentrations ziduvodine did not have any inhibitory effect on epitestosterone. This means the drug chosen in HIV treatment at the same dosages could be key in the regulation of epitestosterone glucuronidation.

At ng/mL concentration levels it was found that increasing concentrations of epitestosterone had a minor effect of reducing testosterone glucuronidation by interacting with UGT2B17, this was a lesser effect than at μg/mL levels. If this minor effect was applied *in vivo* it could have an impact on T/E levels as epitestosterone excretion would likely be increased from administration and testosterone excretion could be reduced owing to the role of epitestosterone reducing testosterone glucuronidation.

4.6 Conclusion

Dietary teas and catechin compounds inhibit UGT2B7 mediated epitestosterone along with UGT2B17 mediated testosterone glucuronidation. It has also been shown that the anti-retroviral drug Efavirenz competitively inhibits UGT2B17 epitestosterone glucuronidation, whilst at the same concentrations ziduvodine is not a viable inhibitor. Increasing epitestosterone concentration at ng/mL levels reduced UGT2B17 glucuronidation rate of a 50 ng/mL initial testosterone. However, under similar conditions with gender pooled microsomes no changes in testosterone were observed by additional epitestosterone. This

study highlighted the enhanced testosterone glucuronidation activity of male gender pooled microsomes over female pooled microsomes. To conclude, these results combine with the previous chapter to highlight a number of inhibitory activities for dietary compounds that show the regulation of either testosterone or epitestosterone, or both steroids could be altered. This could alter excreted levels of these steroids that could have implications on circulating steroid and T/E levels by either increasing or decreasing ratios, which effects doping controls.

Chapter 5. Effects of diclofenac and stanozolol administration on urinary testosterone and epitestosterone excretion in rats and implications on T/E ratios

5.1 Introduction

The previous chapters have highlighted the *in vitro* inhibition by commonly consumed dietary components and pharmaceuticals on testosterone and epitestosterone glucuronidation, through inhibiting the most active enzymes involved in their glucuronidation, UGT2B17 and UGT2B7. Two NSAIDs diclofenac and ibuprofen have been reported to inhibit the glucuronidation of testosterone by UGT2B17 *in vitro* (Sten *et al.*, 2009b). A subsequent human *in vivo* study revealed that these drugs did not influence T/E ratios (Lundmark *et al.*, 2013). However this study was limited in terms of the dosages of NSAIDS that could be used *in vivo* and the study only used a single dose of testosterone on day 0 of the study. This would not reflect a real life scenario in which it is common to administer low dosages of anabolic steroids at regular intervals and often in cycles (Sottas *et al.*, 2010; Evans *et al.*, 2004; Graham *et al.*, 2008). UGT2B17 has been found to contribute to the metabolism of NSAIDs (Turgeon *et al.*, 2003). NSAIDs including diclofenac and ibuprofen are used in the treatment of pain and inflammation by athletes (Warden, 2009).

Stanozolol is a synthetic derivative of testosterone and is glucuronidated to several conjugated compounds. Following stanozolol administration, 3'-hydroxystanozolol was one of the main conjugated compounds excreted in urine as 3'-hydroxystanozolol glucuronide, along with stanozolol glucuronide. This was confirmed by the presence of de

conjugated stanozolol and 3'hydroxystanozolol following deconjugation by β-glucuronidase (Deshmukh *et al.*, 2012b; Pozo *et al.*, 2009). Stanozolol suppresses endocrine negative feedback mechanisms which reduce LH and FSH secretion (Evans, 2004).

The use of exogenous anabolic steroids such as stanozolol has been found to suppress negative feedback mechanisms reducing the stimulation of LH and FSH hormone (Evans 2004). This endocrine suppression can lead to a number of endocrine disorders in males which have been found to worsen with increased anabolic use for longer periods (Evans 2004; Jarow Lipshultz 1990; MacIndoe et al. 1997). Whilst glucuronide metabolites of stanozolol and 3'hyroxystanozolol have been observed in urine following administration of stanozolol, little is known about the UGT enzymes involved in the glucuronidation of these compounds. There is also no knowledge of the role of stanozolol in altering testostosterone glucuronidation along with testosterone and epitestosterone metabolites.

The study reported in this chapter is designed to extend previous observations involving the NSAID diclofenac and its effect on testosterone and epitestosterone metabolites. This study analysed the role of stanozolol on testosterone and epitestosterone metabolites. An *in vivo* study using rat models was used to investigate the effects of administering 0-25 mg/kg/day diclofenac and 5 mg/kg/day stanozolol, on rat urinary concentrations of testosterone and epitestosterone. The results from this study provide comparable results between previous *in vitro* and *in vivo* studies analysing the effects of diclofenac on testosterone glucuronidation. To carry out the analysis, an LC-MS/MS based method was optimised and validated to determine free and deconjugated testosterone and

epitestosterone in rat serum and urine. An *in vitro* study investigated the role of stanozolol and 3'hydroxystanozolol on testosterone glucuronidation by UGT2B17.

5.2 Aims

The aims of this chapter are to determine the effects of stanozolol and diclofenac on urinary testosterone and epitestosterone excretion in rats. To meet this aim –

- Optimisations of the deconjugation and extraction procedure for the LC-MS/MS analysis of testosterone and epitestosterone in rat blood and urine matrices was performed. Validation of the LC-MS/MS method for testosterone and epitestosterone was also performed.
- Analysis of free and deconjugated rat urine samples throughout the study was performed, determining any changes to testosterone, epitestosterone and T/E ratio levels.
- Glucuronidation assays with the microsomes of rats to determine the testosterone glucuronidation activity of each microsome sample *in vitro* was performed.
- The effect of stanozolol and 3'hydroxystanozolol on UGT2B17 mediated glucuronidation of testosterone glucuronidation *in vitro* was determined.

5.3 Methods

5.3.1 Standard solutions

Reference standards of testosterone (1 mg/mL dissolved in acetonitrile) and epitestosterone (1 mg/mL dissolved in methanol) were used as stock solutions. Working solutions of these analytes were prepared by dilution of the stock solution to 1000 ng/mL and 250 ng/mL. A

stock solution of 1 mg/mL stanozolol D3 dissolved in 1,2 dimoxyethane was mixed and diluted with methanol to obtain a working solution of 40 ng/mL. Stanozolol was prepared at 1 mg/mL dissolved in acetonitrile and 3'hydroxystanozolol was prepared at 1 mg/mL dissolved in methanol.

5.3.2 Method validation

Method validation was performed as described in section 2.4.5. Briefly-

- Inter- and intra-day validation was performed for determining accuracy and precision of testosterone.
- Method extraction recovery along with method selectivity and deconjugation activity was assessed as part of the method validation.

5.3.3 Rat preparation

This section expands on the method description described in chapter 2 (section 2.5.1). Twenty four male brown Norway rats were used in this study, split into four groups of six. The rats were kept in an animal house in Semmelweis University, Budapest, Hungary. Animals were kept at room temperature with alternating 12 hour light dark cycles and food and water were available *ad-libitum*.

Rats were administered with varying concentrations of diclofenac ranging between 0-25 mg/kg/day, subcutaneously in saline. The 24 individual rats were split into four groups of six that determined the amount of diclofenac that would be administered throughout the

study. All rat groups received the same dosage of stanozolol at 5 mg/kg/day six days a week, administered intraperitoneally as saline solutions. The duration of diclofenac treatment was six weeks; stanozolol treatment lasted for 3 weeks. Both diclofenac and stanozolol were administered at the same time each treatment day. The dosage group concentrations of administered diclofenac are shown in Table 5.1 and the drug treatment timeline is displayed in Figure 5.1.

Table 5.1 Diclofenac concentrations administered to rat groups throughout the study.

Group	Diclofenac
1 (rats 1-6)	0 mg/kg/day
2 (rats 7-12)	1 mg/kg/day
3 (rats 13-18)	5 mg/kg/day
4 (rats 19-24)	25 mg/kg/day

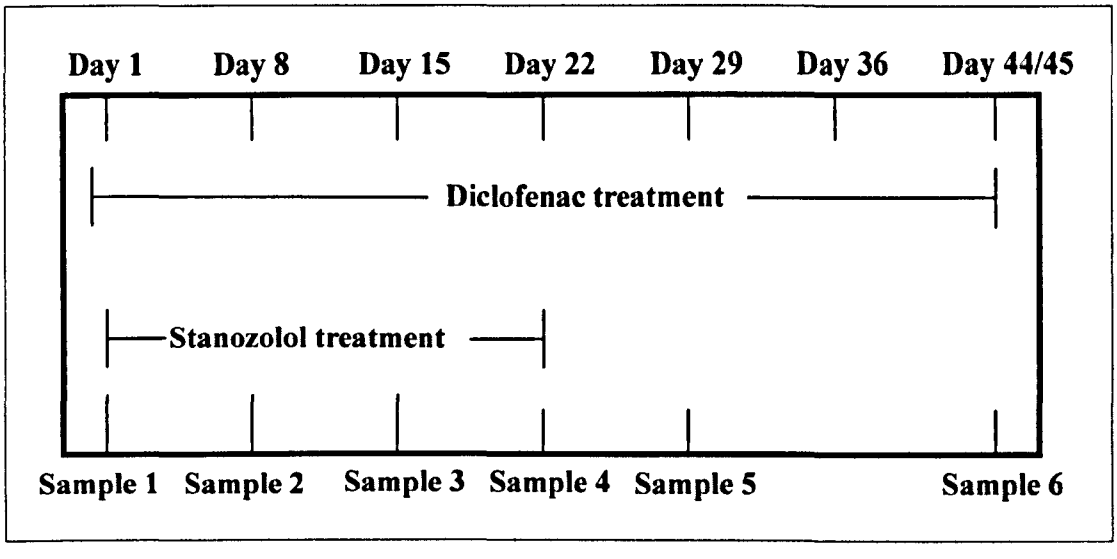


Figure 5.1 Drug treatment and sample collection timeline.

5.3.4 Rat urine sample collection

The administration of stanozolol and diclofenac and the collection of samples were performed in accordance with European Commission council directives on laboratory animals (86/609/EEC) (http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri= CELEX:31986L0609:EN:NOT), conducted under the license of Semmelweis University, Budapest, Hungary. Sample analysis was performed by the author at Kingston University. These samples were also used for analysis of stanozolol and 3'hydroxystanozolol by another auther, Dr. Nawed Deshmukh, presented in their PhD thesis. During the study, five of the rats from group four died following ten days of treatment, the study continued from this point without any further samples from the rats in this group. Two urine samples were collected every seven days during the study with the exception of no samples being collected on day 36. Prior to the collection of urine samples, rats were anaesthetised using ketamine and xylazine. One of the urine samples were collected before drug treatment, the other urine sample was collected one hour after injections of stanozolol and diclofenac.

5.3.5 Sample preparation and extraction of testosterone and epitestosterone for LC-MS/MS analysis

The methods are described fully in chapter 2. Briefly:

- Blood and urine samples were prepared for extraction by deconjugating testosterone and epitestosterone glucuronides (2.6.2).
- LLE was used for extraction of testosterone, epitestosterone and internal standard stanozolol D3 (2.6.2).

- LC-MS/MS analysis was used to determine concentrations of testosterone, epitestosterone, using the internal standard stanozolol D3 (2.4.3.1).

5.3.6 Analysis of rat microsomal testosterone glucuronidation activity

- Microsome samples were obtained from the individual rats following the termination of the rat study at day 45 of the study. A protein Bradford assay was performed to determine the protein concentration present in each microsome sample.
- Testosterone glucuronidation assays were performed with each microsome. The assay is described in detail in chapter 2 (2.4.1).

5.3.7 Determining the effects of stanozolol and 3'hydroxystanozolol on *in vitro* UGT2B17 testosterone glucuronidation

The methods are described in chapter 2. Briefly:

- Testosterone glucuronidation assays were performed (2.4.1) with the addition of stanozolol and 3'hydroxystanozolol (1% v/v) concentrations ranging between 0-10 µg/mL.
- HPLC analysis using a mobile phase of water and acetonitrile was used to determine remaining testosterone concentrations following the termination of reactions (2.4.2).

5.4 Results

5.4.1 Method validation

Method selectivity was confirmed by extracting blank charcoal stripped urine and plasma and analysing these samples by LC-MS/MS under the same method. This confirmed no peaks could be detected that would interfere with the retention times of the analytes and internal standard. Chromatograms of testosterone and epitestosterone extracted from rat plasma and urine matrices at 0.250 ng/mL are shown in Figure 5.2. The calibration curves, r^2 and regression equations for testosterone used within the reference ranges of the study (0.25-1000 ng/mL) are displayed in Figure 5.3. Owing to the concentration ranges used, calibration curves for each analyte were prepared at a low and high concentration ranges. The calibration curves showed good linearity with r^2 values above 0.992 for testosterone and 0.999 for epitestosterone.

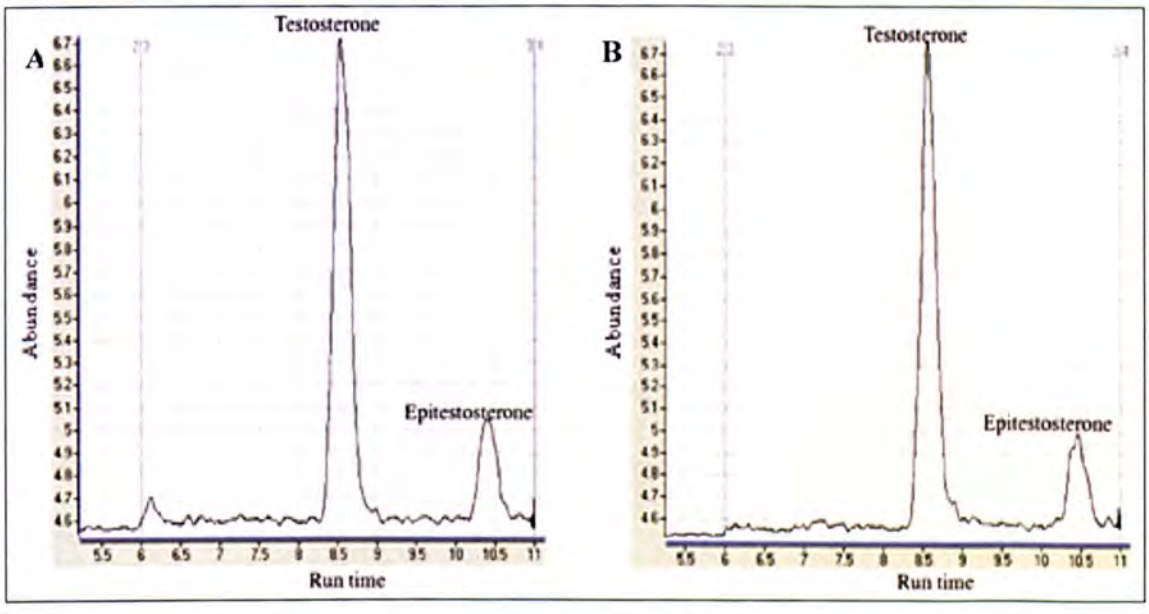


Figure 5.2 Chromatograms of testosterone and epitestosterone extracted from A - plasma and B - urine at 0.250 ng/mL, LLOQ concentration level for epitestostosterone.

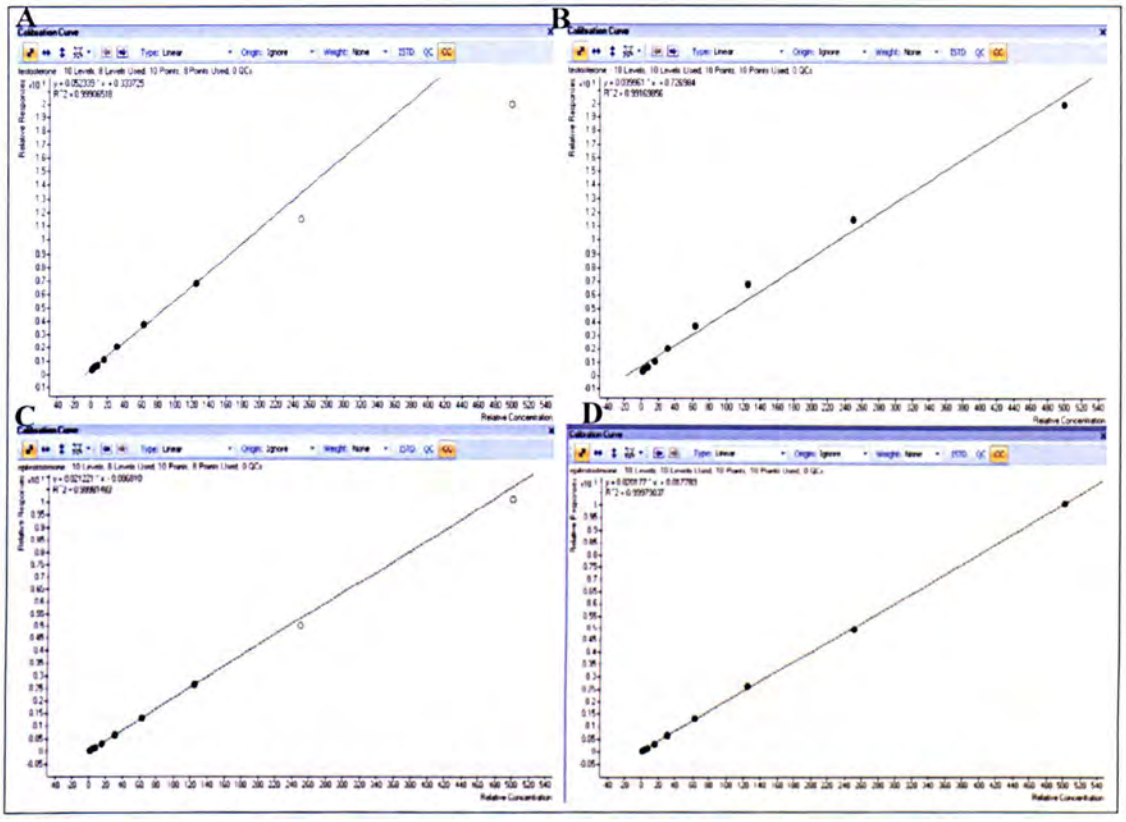


Figure 5.3 Calibration curves in urine matrices, R^2 values and regression curves for A - testosterone low concentration, B - testosterone high concentration, C - epitestosterone low concentration and D - epitestosterone high concentration. The low concentration range was 0.25-250 ng/mL, the high concentration range was 0.25-1000 ng/mL.

5.4.1.1 Accuracy

The accuracy and precision for inter- and intra-day validation of testosterone and epitestosterone QC samples extracted from rat blood and urine matrices are displayed in Table 5.2. The validation results were within the FDA guideline limits (http://www.fda.gov/downloads/Drugs/.../Guidances/ucm070107.pdf).

Table 5.2 Assay validation results in urine and plasma matrices.

Urine

Compound	Concentration (ng/mL)	Level	Precision RSD (%)		Accuracy (%)
			Intra-day N=6	Inter-day N=18	
	0.25	LLOQ	10.01	10.11	107.03
Testosterone	1.9	Low	2.66	5.51	96.61
	15.6	Medium	4.20	10.29	104.02
	250	High	1.13	3.39	97.11
Epitestosterone	0.25	LLOQ	1.25	9.56	100.50
	1.9	Low	1.05	4.36	100.39
	15.6	Medium	8.17	8.94	96.45
	250	High	1.67	3.70	102.60

Plasma

Compound	Concentration (ng/mL)	Level	Precision RSD (%)		Accuracy (%)
			Intra-day	Inter-day	
	0.25	LLOQ	8.14	8.21	109.14
Testosterone	1.9	Low	3.94	4.26	98.26
	15.6	Medium	5.47	5.92	102.66
	250	High	1.86	2.29	96.55
Epitestosterone	0.25	LLOQ	2.58	6.87	98.20
	1.9	Low	1.44	2.96	103.01
	15.6	Medium	7.05	6.50	98.29
	250	High	1.82	3.19	100.86

5.4.1.2 Recovery

The extraction recoveries of testosterone and epitestosterone from rat plasma and urine matrices are displayed in Table 5.3. The extraction recovery percentages were high for testosterone and epitestosterone in both matrices at the concentration ranges analysed. Extraction recoveries ranged from 81.63-94.49% for testosterone and 84.72-92.77% for epitestosterone. These results indicate that the LLE extraction method would be effective for both matrices for the analysis of testosterone and epitestosterone, owing to the consistent and reproducible recoveries at these concentrations.

Table 5.3 Extraction recovery results (%) for epitestosterone and testosterone extracted in rat plasma and urine.

Matrix	Compound	Concentration (ng/mL)	Extraction recovery % N=6
Plasma	Testosterone	0.25	94.49
		1	81.63
		4	85.60
		16	85.98
	Epitestosterone	0.25	92.77
		1	89.67
		4	84.72
		16	89.48
Urine	Testosterone	0.25	91.23
		1	87.63
		4	88.29
		16	89.16
	Epitestosterone	0.25	92.57
		1	91.44
		4	89.60
		16	90.16

5.4.1.3 Matrix effect

To determine the effects of the matrices on method selectivity, blank charcoal stripped rat plasma and urine were extracted and then spiked with known concentrations of steroids. These concentrations were compared with the same known concentration standards of testosterone and epitestosterone in acetonitrile. Table 5.4 displays the matrix effects of rat plasma and urine on testosterone and epitestosterone compared with standard concentrations. At each concentration analysed for testosterone and epitestosterone, there were no observed differences in analyte concentrations in both matrices that had been spiked following extraction, when compared with standard concentrations of testosterone and epitestosterone spiked in acetonitrile at the same concentrations.

Table 5.4 Summary of matrix effect results, N=6.

Compound	Concentration (ng/mL)	Extracted plasma (ng/mL)	Extracted urine (ng/mL)	Standard in acetonitrile (ng/mL)
Testosterone	0.25	0.248	0.241	0.250
	1	0.977	0.952	0.957
	2	2.019	2.005	2.053
	8	8.089	8.129	8.455
Epitestosterone	1	1.002	1.094	0.992
	2	2.051	1.961	1.977
	8	8.038	8.122	8.110

5.4.1.4 Deconjugation assay

The results of the testosterone glucuronide and epitestosterone glucuronide deconjugation assays in urine and plasma are displayed in Table 5.5. Under these assay conditions and concentrations used in this study, β-glucuronidase was effective at deconjugating testosterone and epitestosterone glucuronides in plasma and urine matrices.

Table 5.5 Deconjugation validation results of testosterone and epitestosterone glucuronides using β-glucuronides at standard concentrations, N=6.

Matrix	Compound	Concentration (ng/mL)	Extracted spiked steroid in matrix (ng/mL)	De-conjugated steroid concentration (ng/mL)
Plasma	Testosterone	1	0.98	0.91
		2	2.08	2.03
		15	16.96	14.03
	Epitestosterone	1	1.09	1.15
		2	2.11	2.14
		15	16.18	14.85
Urine	Testosterone	1	1.02	1.09
		2	2.04	2.07
		15	15.81	15.29
	Epitestosterone	1	1.08	0.96
		2	1.97	1.94
		15	15.87	15.06

5.4.1.5 Application of method with rat blood and urine samples

The validated method was applied to determine the total conjugated and unconjugated concentrations of testosterone and epitestosterone in six rat urine and blood samples. The urine samples were control samples supplied from the study performed in this chapter. These samples were collected before the administration of the drugs diclofenac and stanozolol. The blood samples from six brown Norway rats were obtained from Sera Laboratories International (West Sussex, UK).

The concentrations of testosterone in urine ranged between 63.43-615.22 ng/mL and 318.52-386.39 ng/mL in blood. Epitestosterone concentrations in urine ranged between 16.28-160.46 ng/mL and ranged between 1.40-2.11 ng/mL in blood. The individual rat urinary and blood testosterone and epitestosterone concentrations are displayed in Table 5.6. It was observed from these results that the T/E ratios are much greater in blood than urine owing to lower circulating epitestosterone compared to excreted levels.

Table 5.6 Concentrations of testosterone and epitestosterone in the blood and urine of six individual male brown Norway rats. Each value represents the mean ± SEM of triplicate injections. The average value represents the mean ± SEM average value of the six individual rat samples.

Matrix	Animal	Testosterone (ng/mL)	Epitestosterone (ng/mL)	Ratio (T/E)
Urine	1	280.30 ±3.798	75.65 ±0.165	3.71
	2	615.22 ±4.595	160.46 ±1.515	3.83
	3	142.64 ±1.973	48.32 ±0.156	2.95
	4	71.63 ±0.258	16.25 ±0.176	4.40
	5	36.80 ±0.304	86.14 ±0.03	0.42
	6	63.46 ±0.035	125.37 ±0.815	0.51
	Average	**201.68 ±90.16**	**85.37 ±21.20**	**2.64 ±0.71**
Serum	1	386.39 ±6.268	1.70 ±0.286	227.89
	2	378.84 ±17.143	1.64 ±0.129	230.69
	3	318.52 ±1.517	1.40 ±0.025	227.12
	4	381.15 ±15.214	2.11 ±0.065	180.26
	5	378.80 ±8.662	1.55 ±0.028	244.54
	6	336.70 ±1.835	2.08 ±0.063	161.54
	Average	**363.40 ±11.615**	**1.75 ±0.118**	**212.01 ±13.47**

5.4.2 Alterations in rat testosterone levels

The average concentrations of urinary testosterone between groups during and post stanozolol treatment are displayed in Table 5.7.

Table 5.7 Average concentrations of urinary conjugated and deconjugated testosterone between rat groups during and post stanozolol treatment. Urine samples were collected and analysed before and after stanozolol injections. Results are displayed as the average ± SEM of each individual rat urine measured in the group. Each group of rats were administered with 5 mg/kg stanozolol. Diclofenac administration was G1 – 0 mg/kg, G2 – 1 mg/kg and G3 – 5 mg/kg. BLD – below limit of detection of this analytical method which was under 0.125 ng/mL testosterone.

Average urinary testosterone concentrations (ng/mL) in rat groups		
Groups	**During stanozolol treatment (sample days 7, 14 and 21 pre stanozolol injection)**	**Post stanozolol treatment (sample days 28 and 42 pre stanozolol injection)**
G1 (rats 1-6)	165.44 ±43.084	0.35 ±0.101
G2 (rats 7-13)	349.90 ±90.343	1.52 ±1.015
G1 (rats 13-18)	258.77 ±84.054	BLD
	During stanozolol treatment (sample days 7, 14 and 21 post stanozolol injection)	**Post stanozolol treatment (sample days 28 and 42 post stanozolol injection)**
G1 (rats 1-6)	77.93 ±35.128	0.28 ±0.304
G2 (rats 7-13)	168.59 ±82.400	0.76 ±0.760
G1 (rats 13-18)	63.62 ±27.076	BLD

The average urinary concentrations of testosterone for the three rat groups over the course of the study are displayed in Figure 5.4 for urinary samples collected before stanozolol injection and Figure 5.5 for urinary samples taken post stanozolol injection. Individual urinary testosterone concentrations for each rat are displayed in the appendix. During the stanozolol treatment, the average urinary concentrations of testosterone in the three groups did not appear to vary between groups and there were no significant differences observed in excreted testosterone between groups in samples taken before or after stanozolol injection (before stanozolol injection [$F(2, 49) = 1.446$, $p = 0.245$, partial eta-squared = 0.056]) (post stanozolol injection [$F(2, 49) = 0.687$, $p = 0.508$, partial eta-squared = 0.028]). The magnitude of group effect size was determined using partial eta-squared, this revealed the

group effect size was low in the urine samples before and post stanozolol injection as the partial eta-squared values were below 0.06. However, in all three groups there were significant reductions in the average concentrations of testosterone between the samples analysed during stanozolol treatment and post stanozolol treatment. This reduction in testosterone was observed in both urine samples collected before and after stanozolol injection from day 7 to 42 (urine samples before stanozolol injection between day 7 and 42 G1 p = 0.010, G2 p = 0.017, G3 p = 0.005) (urine samples post stanozolol injection between day 7 and 42 G1 p = 0.069 [eta-square = 0.303], G2 p = 0.062 [eta-square = 0.334], G3 p = 0.209 [eta-square = 0.169]). A large partial eta-squared value was obtained for the groups that were not considered significant, which have contributed to the large variation. Urinary testosterone excretion reduced throughout the study, initially high concentrations of testosterone excretion were observed, however at the end of the study a number of samples in all three groups had undetectable levels of testosterone below the 0.125 ng/mL limit of detection. The concentrations of urinary testosterone remained low following the termination of stanozolol administration after day 21 until day 42.

It was only possible to gain samples from group 4 (rats 19-24) on sample days 0 and 7 as the rats died 10 days into the study. The average urinary testosterone concentrations for this group were 199.47 ±40.688 ng/mL before the initial stanozolol injection and 244.30 ±52.833 ng/mL post stanozolol injection on day 0 of the study. These concentrations were similar to the levels of the other groups at this point in the study. The average group 4 urinary testosterone at day 7 before stanozolol injection was 263.02 ±141.91 ng/mL, which rose to 534.63 ±141.91 ng/mL post stanozolol injection.

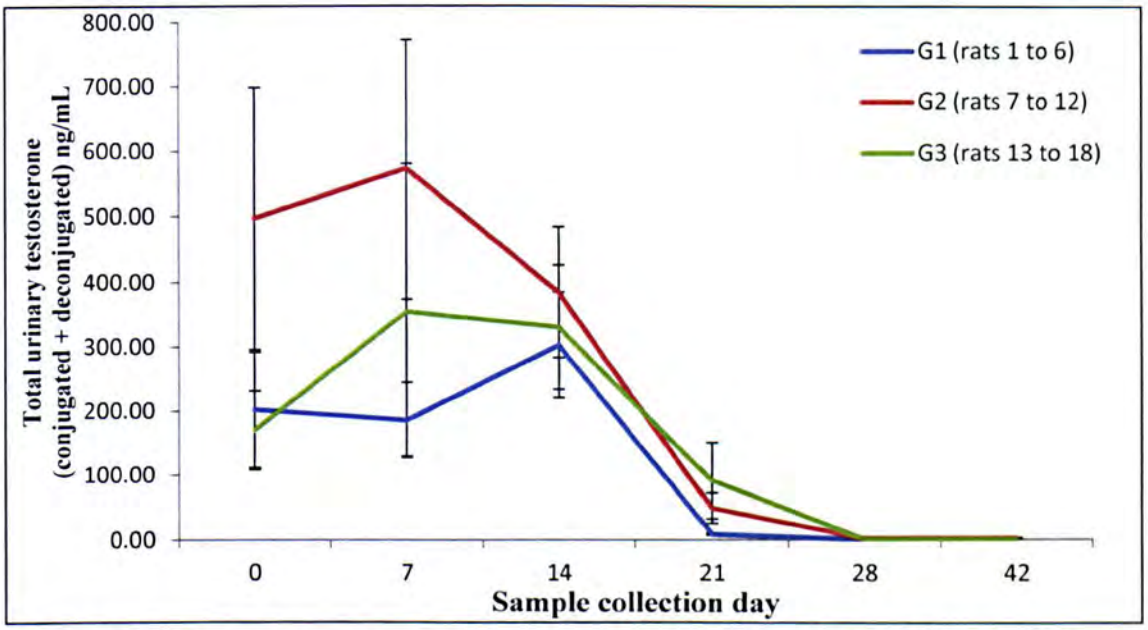


Figure 5.4 Urinary testosterone concentrations between groups 1, 2 and 3 over the course of the study from samples taken before stanozolol injection. All groups were administered with 5 mg/kg stanozolol. G1 – 0 mg/kg diclofenac, G2 – 1mg/kg diclofenac, G3- 5 mg/kg diclofenac. Data are presented as the mean ± SEM of each group.

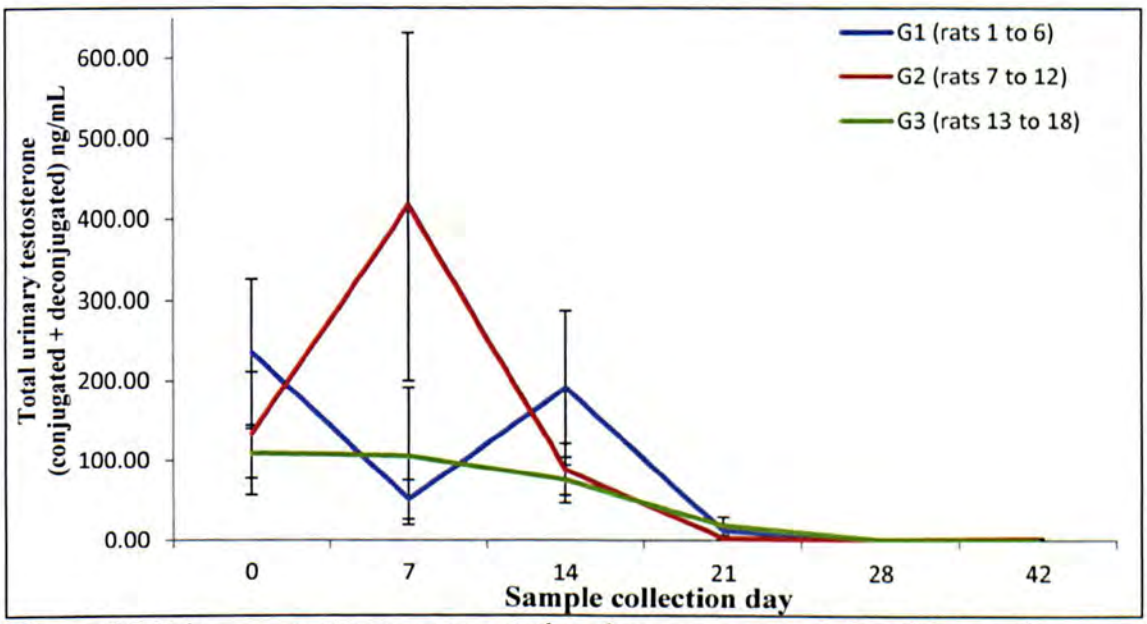


Figure 5.5 Urinary testosterone concentrations between groups 1, 2 and 3 over the course of the study from samples taken after stanozolol injection. All groups were administered with 5 mg/kg stanozolol. G1 – 0 mg/kg diclofenac, G2 – 1mg/kg diclofenac, G3- 5 mg/kg diclofenac. Data are presented as the mean ± SEM of each group.

5.4.3 Alterations in rat epitestosterone levels

The average excreted urinary total epitestosterone (conjugated and unconjugated) concentrations excreted between groups, during and post stanozolol treatment is displayed in Table 5.8.

Table 5.8 Average urinary epitestosterone between rat groups during and post treatment of stanozolol. Results are displayed as the average ± SEM of each individual rat urine measured in the group. Each group of rats were administered with 5 mg/kg stanozolol. Diclofenac administration was G1 – 0 mg/kg, G2 – 1 mg/kg, G3 – 5 mg/kg.

Groups	During stanozolol treatment (sample days 7, 14 and 21 pre stanozolol injection)	Post stanozolol treatment (sample days 7, 14 and 21 pre stanozolol injection)
G1 (rats 1-6)	17.46 ±4.116	284.09 ±56.346
G2 (rats 7-13)	54.88 ±41.421	403.33 ±72.335
G1 (rats 13-18)	10.40 ±4.069	265.27 ±93.384
	Post stanozolol treatment (sample days 7, 14 and 21 post stanozolol injection)	**Post stanozolol treatment (sample days 7, 14 and 21 post stanozolol injection)**
G1 (rats 1-6)	19.49 ±4.727	292.60 ±51.230
G2 (rats 7-13)	14.05 ±3.627	407.65 ±128.912
G1 (rats 13-18)	8.89 ±1.895	288.83 ±83.378

The average urinary epitestosterone concentrations between groups at each sample collection day during the study is displayed in Figure 5.6 for samples taken before stanozolol injection and Figure 5.7 for samples taken post stanozolol injection. The urinary concentrations of epitestosterone for each individual rat are displayed in the appendix. During the period of stanozolol treatment, there were no significant differences in excreted epitestosterone levels between the groups for both the samples taken before and after stanozolol injections (before stanozolol injection [$F(2, 49) = 1.094$, $p = 0.343$, partial eta-squared = 0.043]) (post stanozolol injection [$F(2, 46) = 1.720$, $p = 0.190$, partial eta-squared =

0.070]). Partial eta-squared values showed the group effect sizes were small for samples before stanozolol injection and medium post stanozolol injection. Group 2 had a higher average of excreted epitestosterone on several sample days compared to groups 1 and 2 during the study; however this was due to a high concentration observed in one individual rat within this group. For groups 1, 2 and 3 there was a significant change in urinary epitestosterone levels over the course of the study (urine samples before stanozolol injection between day 7 and 42 G1 $p = 0.001$, G2 $p = 0.065$ [partial eta-squared = 0.329], G3 $p = 0.008$) (urine samples post stanozolol injection between day 7 and 42 G1 $p = 0.001$, G2 $p = 0.020$, G3 $p = 0.014$). Although no significant increase was observed in group 2 before stanozolol injection, the partial eta-squared value indicate a large sample effect size. Initial low levels of epitestosterone were observed during the beginning of the study which remained low or reduced at each sample collection until day 21. Levels of epitestosterone increased after day 21, with concentrations at the highest levels on the final sample collection on day 48 in all three groups. The increase in epitestosterone in urine continued following the termination of stanozolol treatment. The average group 4 (rats 19-24) urinary epitestosterone concentrations on day 0 were 8.29 ±3.942 ng/mL for samples taken before stanozolol injection and 4.10 ±1.002 for sample collected after stanozolol injection. On day 7 for the group 4 (rats 19-24), urinary epitestosterone was 79.42 ±36.65 ng/mL for samples taken before stanozolol injection and 150.60 ±86.523 ng/mL for samples collected after stanozolol injection. This indicates a rapid rise of urinary epitestosterone in this group from day 0 to day 7.

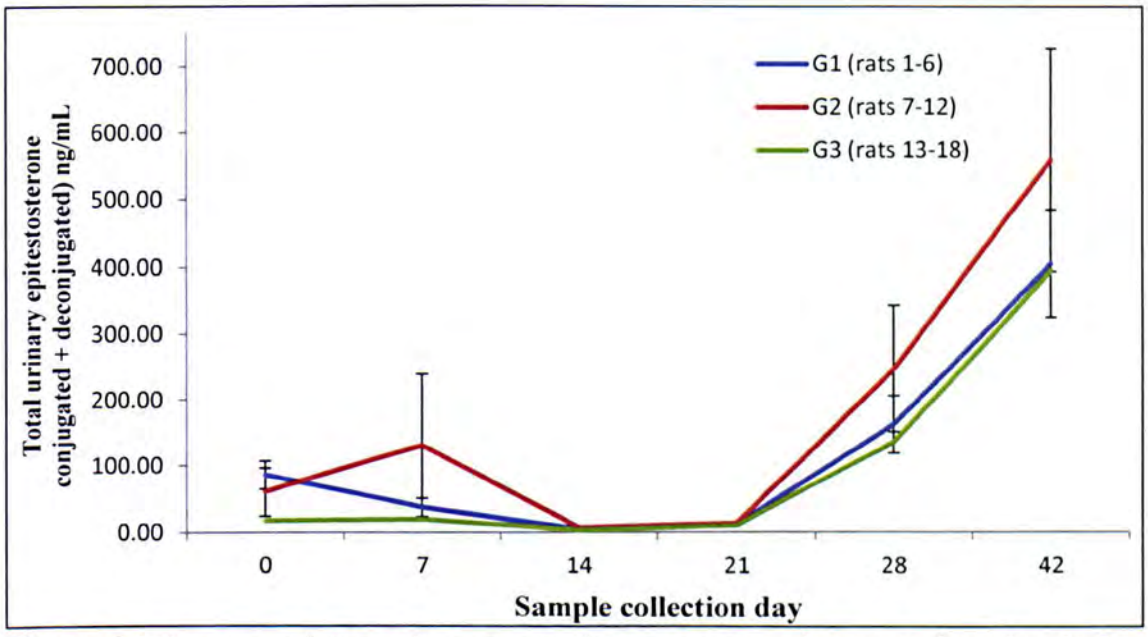


Figure 5.6 Average urinary epitestosterone between groups from samples taken before stanozolol injection over the course of the study. All groups were administered with 5 mg/kg stanozolol. Diclofenac treatment was G1 – 0 mg/kg, G2- 1 mg/kg, G3 – 5 mg/kg. Data are displayed as the mean ± SEM of each group.

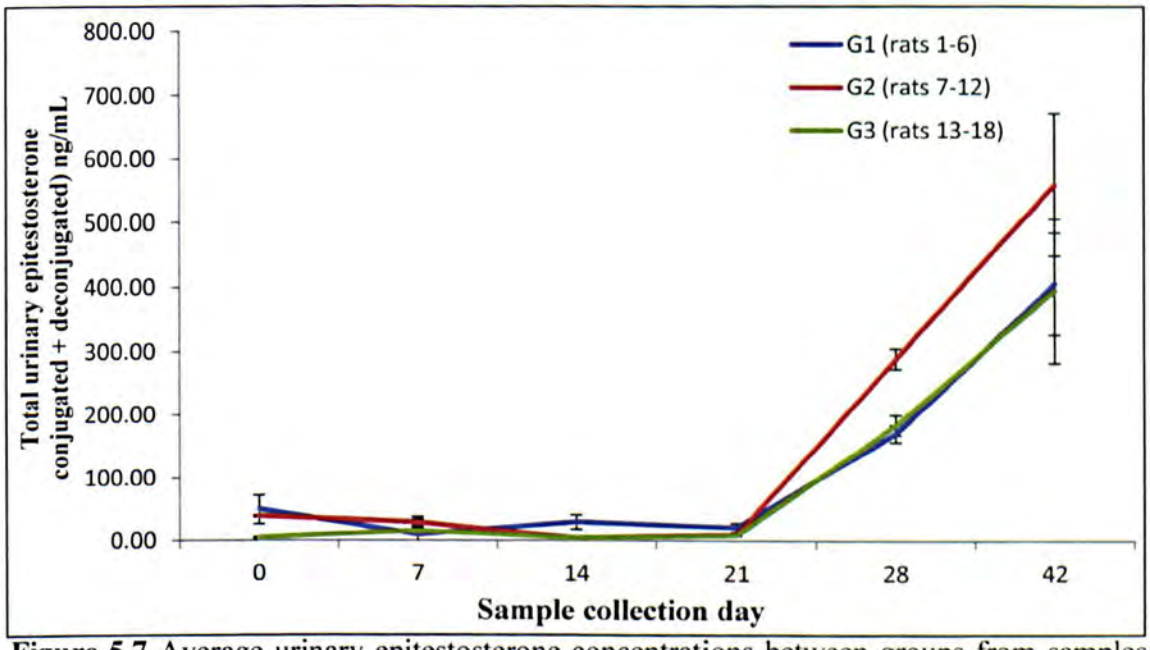


Figure 5.7 Average urinary epitestosterone concentrations between groups from samples collected post stanozolol injections through the duration of the study. All groups were administered with 5 mg/kg stanozolol. Diclofenac administration was G1 – 0 mg/kg, G2 – 1 mg/kg, G3 – 5 mg/kg. Data are presented as the mean ± SEM of each group.

5.4.4 Alterations to T/E levels

The average urinary T/E ratios between groups during and post stanozolol treatment are displayed in Table 5.9.

Table 5.9 Average urinary T/E ratios between rat groups during and post stanozolol treatment. Urine samples were collected and analysed every 7 days for periods during and post treatment of stanozolol. Results are displayed as the mean ± SEM of individual rat T/E ratios within each group. Each rat group was administered with 5 mg/kg stanozolol. Diclofenac admistration was G1 – 0 mg/kg, G2 – 1 mg/kg and G3 – 5 mg/kg. * The T/E ratios were lower than the number of decimal places expressed in this table.

Groups	During stanozolol treatment (sample days 7, 14 and 21 pre stanozolol injection)	Post stanozolol treatment (sample days 28 and 42 pre stanozolol injection)
G1 (rats 1-6)	40.86 ±15.612	0.001 ±0.0006
G2 (rats 7-13)	50.23 ±12.558	0.007 ±0.0053
G1 (rats 13-18)	62.50 ±14.731	0.000 ±0.0000*
	During stanozolol treatment (sample days 7, 14 and 21 post stanozolol injection)	**Post stanozolol treatment (sample days 28 and 42 post stanozolol injection)**
G1 (rats 1-6)	4.07 ±1.015	0.001 ±0.0006
G2 (rats 7-13)	25.78 ±6.656	0.005 ±0.0048
G1 (rats 13-18)	31.60 ±8.159	0.000 ±0.0000*

The average urinary T/E ratios for each rat group on sample collection days over the course of the study are displayed in Figure 5.8 for samples taken before stanozolol injections and Figure 5.9 for samples collected post stanozolol injections. Individual rat T/E ratios throughout the study are displayed in the appendix. During the treatment of stanozolol from sample days 7 – 21, there was a slight increase in T/E ratios between the groups. The T/E ratios increased as the groups with higher concentrations of diclofenac increased, however the differences in T/E ratios between groups was not found to be

significant (before stanozolol injection [$F(2, 49) = 0.413$, $p = 0664$, partial eta-squared = 0.017]) (post stanozolol injection [$F(2, 46) = 1.753$, $p = 0.185$, partial eta-squared = 0.071]). The partial eta-squared effect size was medium for urine samples before and after stanozolol injection. The T/E ratios in all three groups were significantly lower post stanozolol treatment than during stanozolol treatment (urine samples before stanozolol injection between day 7 and 42 G1 $p = 0.053$ [eta-squared = 0.325], G2 $p = 0.044$, G3 $p = 0.158$ [eta-squared = 0.189]) (urine samples post stanozolol injection between day 7 and 42 G1 $p = 0.004$, G2 $p = 0.014$, G3 $p = 0.282$ [eta-squared = 0.127]). The samples that did not have a significant difference in the T/E ratio between day 7 and 42 had large group effect sizes based on the partial eta-square values, apart from group 3 samples collected post stanozolol injection which had a medium effect size. This means the lack of statistical significance is owing to the sample size used and the variations between the samples in groups. In all three groups the T/E ratios reduced to less than 0.010, these low T/E ratios were due to the increase in urinary epitestosterone along with testosterone excretion reducing to very low levels.

At the beginning of the study between samples collected at day 0 and day 7, the T/E ratio of each group did not alter significantly, the ratios in each group either slightly increased or decreased. There was a significant increase in the T/E ratios between day 7 and 14 for samples collected before stanozolol administration, this increase was observed in groups 1 and 3, the group effect size was high in group two passed on the partial eta-squared value (G1 $p = 0.017$, G2 = 0.059 [partial eta-squared = 0.342], G3 = 0.008). The peak T/E ratio levels were on day 14 of sample collection for most groups, the samples taken after this showed a significant reduction in T/E ratios between day 14 and 21 for samples collected before stanozolol administration (G1 $p = 0.008$, G2 $p = 0.019$, G3 $p = 0.001$). The samples

collected after stanozolol administrations were also significantly reduced for groups 1 and 2 between days 14 and 21 (G1 $p = 0.039$, G2 $p = 0.039$, G3 $p = 0.081$ [partial eta-squared = 0.273]). Group 3 T/E ratios also reduced between days 14 and 21, but not significant, although a large partial eta-squared group effect size was observed. The T/E ratios continued to reduce to very low values from day 21 to 48 in all groups from samples taken before and after stanozolol administration. The average urinary T/E ratios in group 4 on day 0 sample collection was 38.05 ±7.714 before stanozolol injection and 61.30 ±4.620 post stanozolol injection. The final set of T/E ratios obtained from group 4 on day 7 was 3.28 ±0.61before staozolol administration and 22.209 ±17.895 post stanozolol injection.

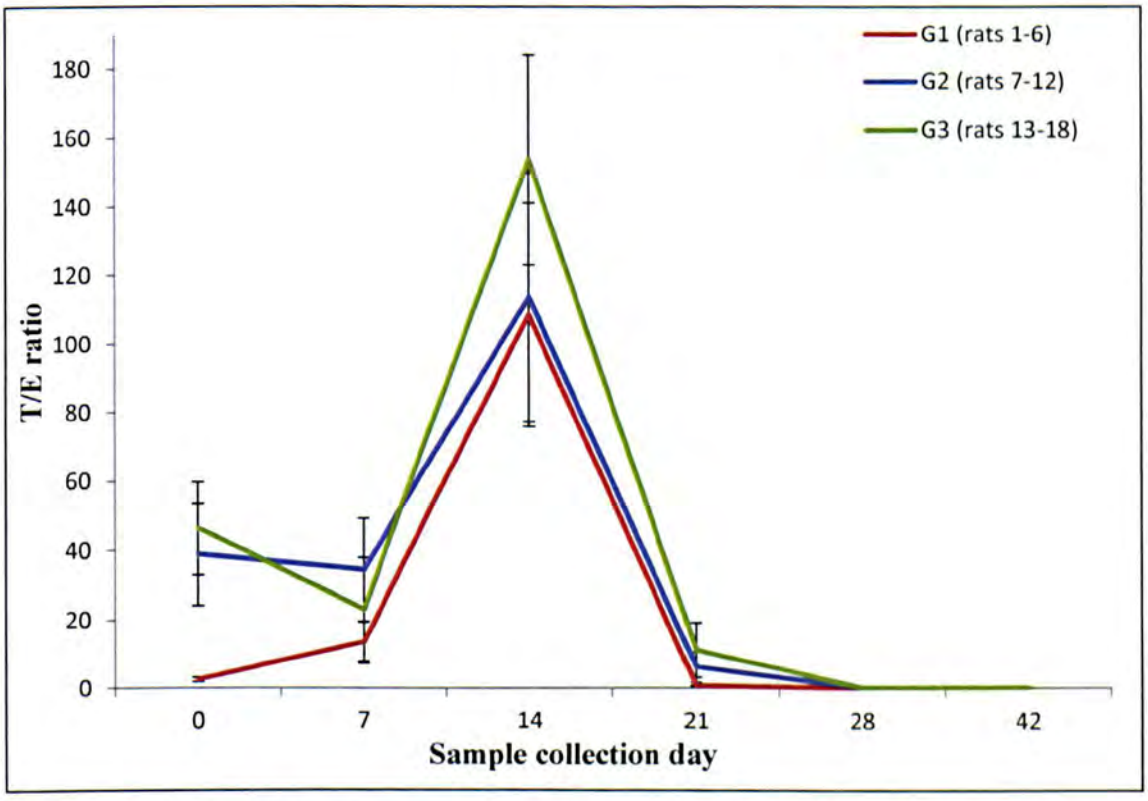


Figure 5.8 Urinary T/E ratios of rat groups 1, 2 and 3 at time points throughout the study when samples were collected before stanozolol administration. All groups were administered with 5 mg/kg stanozolol. Dliclofenac admistration was G1 – 0 mg/kg diclofenac, G2 – 1 mg/kg diclofenac, G3 – 5 mg/kg diclofenac. Data are presented as the mean ± SEM of each groups T/E ratio.

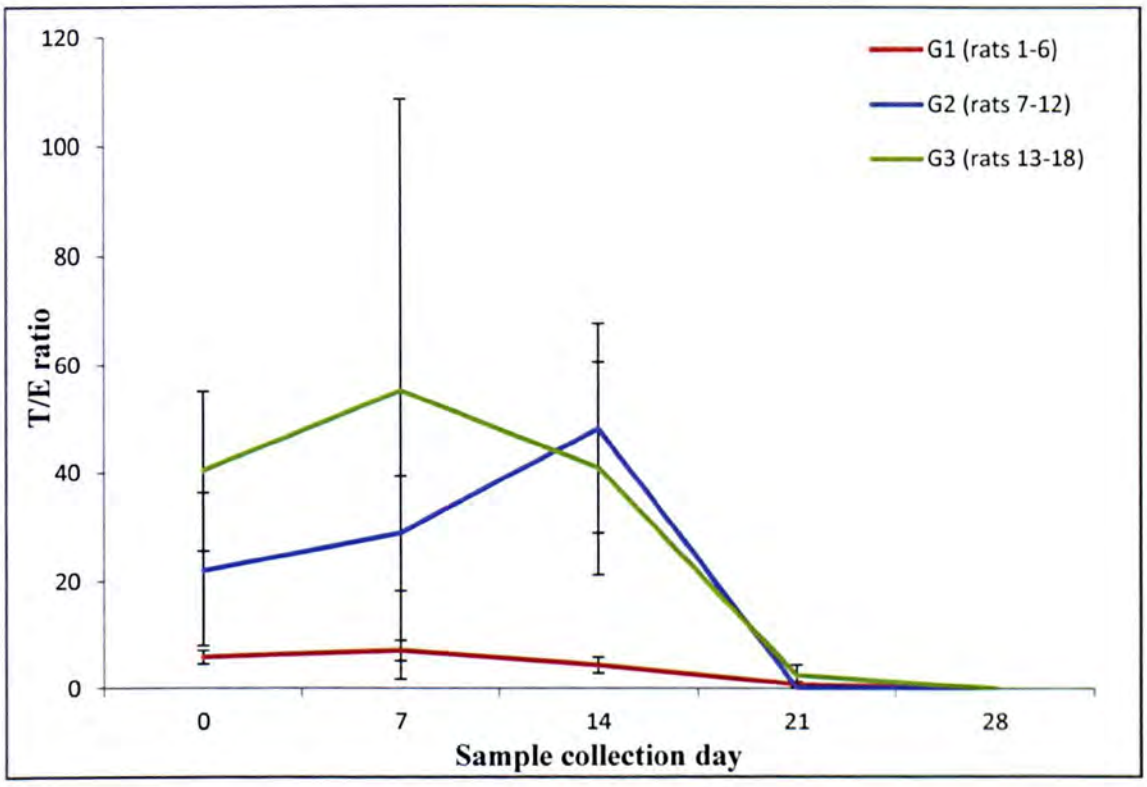


Figure 5.9 Urinary T/E ratios of rat groups 1, 2 and 3 at time points throughout the study when samples were collected after stanozolol administration. All groups were administered with 5 mg/kg stanozolol. Dliclofenac admistration was G1 – 0 mg/kg diclofenac, G2 – 1 mg/kg diclofenac, G3 – 5 mg/kg diclofenac. Data are presented as the mean ± SEM of each rat groups T/E ratio.

5.4.5 Testosterone glucuronidation activity of individual rat microsomes

Testosterone glucuronidation assays were performed with the microsomes of individual rats used during the study, following collection of microsomes samples at the termination of the study. The testosterone glucuronidation rates for each individual rat microsome sample are shown in Figure 5.10 using initial testosterone concentrations of 13.90 and 27.36 µg/mL. At the initial concentration of 13.90 µg/mL, a low amount of testosterone remained in a number of samples following termination of the reaction, hence why an

increased initial testosterone of 27.36 µg/mL was used. The average rates between the three rat groups are displayed in Table 5.10. Testosterone glucuronidation rate varied between individual microsome and when the glucuronidation rates of rats between each group were averaged, there were no significant differences in rates between the groups.

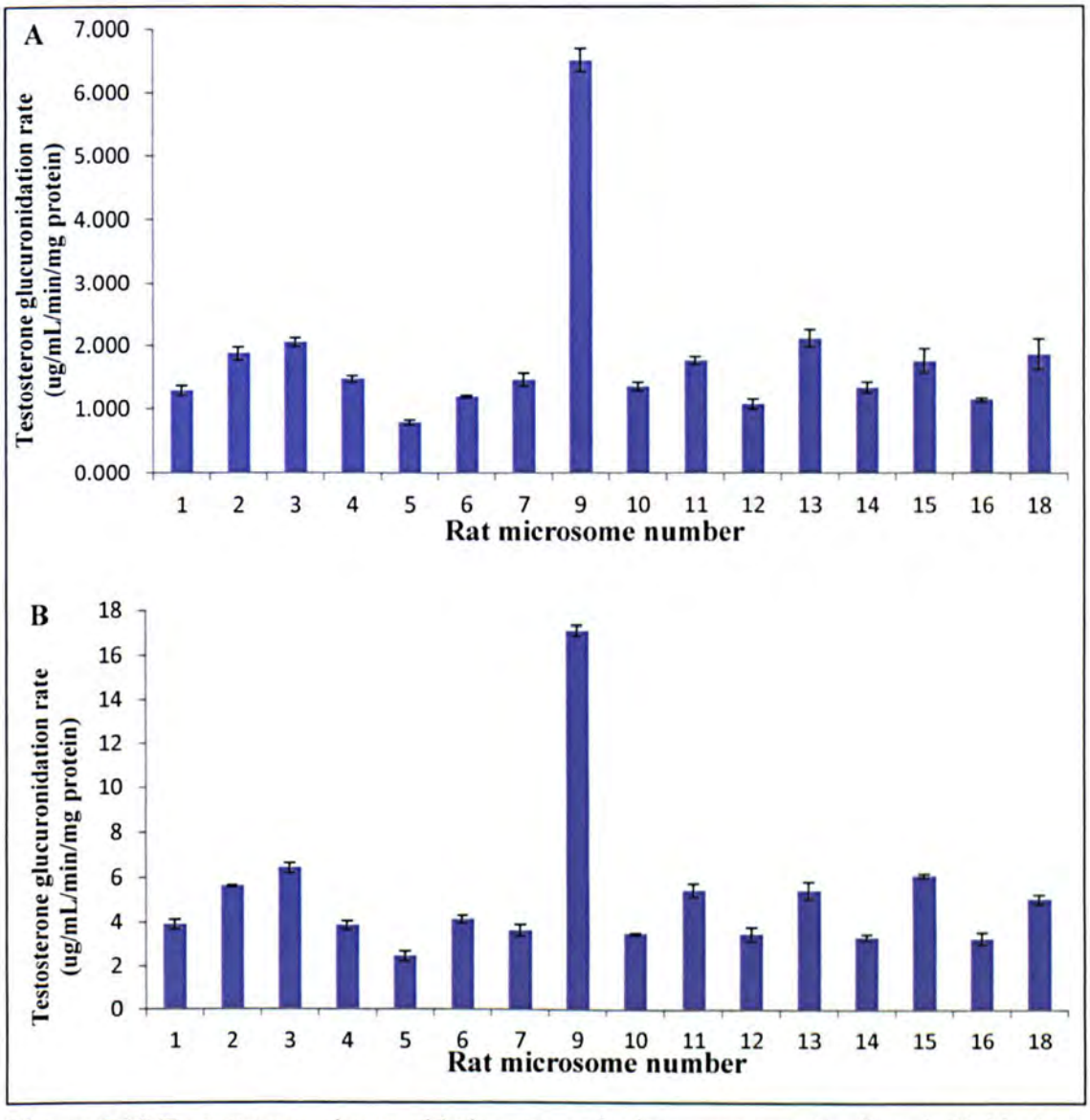


Figure 5.10 Testosterone glucuronidation rates of microsome samples from individual rats used in the study at A - 13.9 and B - 27.36 µg/mL initial testosterone concentrations. All rats were treated with 5 mg/kg stanozolol. Diclofenac administration was rat number 1-6 = 0 mg/kg diclofenac, rats 7-12 = 1 mg/kg and rats 13-18 = 5 mg/kg. Each value represents the mean ± SEM of duplicate samples.

Table 5.10 Average microsomal testosterone glucuronidation activity between rat groups in the study. Values represent the mean ± SEM of average values of the six rats in each group.

Rat groups	13.9 µg/mL initial Testosterone average rate	27.36 µg/mL initial Testosterone average rate
G1 (rats 1-6)	1.444 ±0.189	4.399 ±0.578
G2 (rats 7-12)	2.437 ±1.026	6.599 ±2.646
	(1.417 ±0.144 w/o rat 9)	(3.977 ±0.464 w/o rat 9)
G3 (rats 13-18)	1.653 ±0.180	4.571 ± 0.558

5.4.6 *In vitro* effects of stanozolol and 3'hyroxystanozolol on UGT2B17 testosterone glucuronidation

Dixon plot analyses were used for determining if stanozolol and 3'hydroxystanozolol expressed any inhibitory effect on UGT2B17 testosterone glucuronidation *in vitro*, the results are displayed in Figure 5.11 and Figure 5.12. As stanozolol and 3'hydroxystanozolol concentrations were increased, 1/V testosterone glucuronidation increased at each of the initial testosterone concentrations analysed owing to the reduced testosterone glucuronidation reaction rate. The results revealed that both stanozolol and 3'hydroxystanozolol were competitively inhibiting UGT217 testosterone glucuronidation under these concentrations, as the intersection of the trendlines in the Dixon plot intersect above the X axis. Stanozolol was more effective in competitively inhibiting UGT2B17 testosterone glucuronidation owing to the greater reduction in testosterone glucuronidation in comparison to 3'hydroxystanozolol at equal concentrations. The Ki value was 1.180 µg/mL for stanozolol and 1.618 µg/mL for 3'hydroxystanozolol.

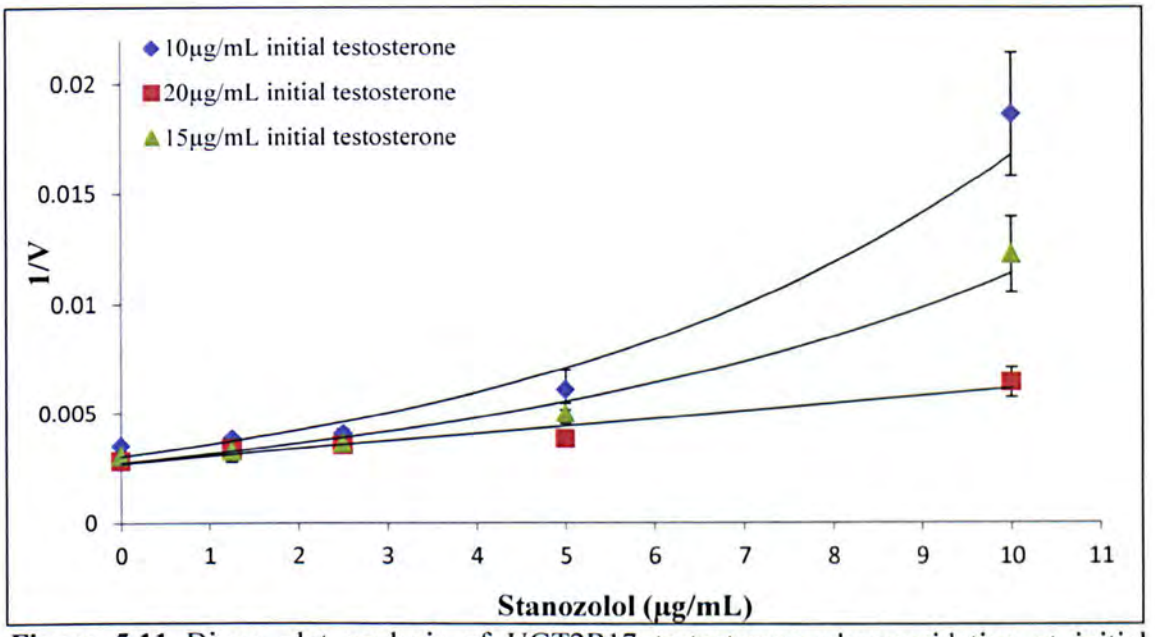


Figure 5.11 Dixon plot analysis of UGT2B17 testosterone glucuronidation at initial testosterone concentrations of 10, 15 and 20 µg/mL, following the addition of increasing stanozolol. The UGT2B17 protein concentration was 0.1 mg. Values are the mean ± SEM of duplicate values.

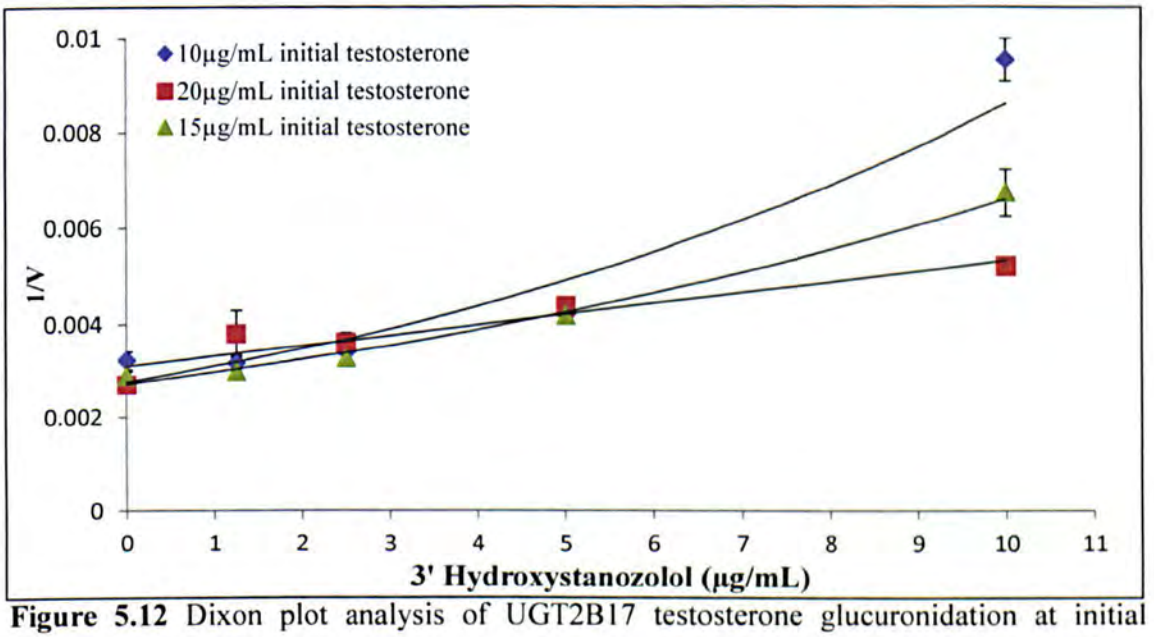


Figure 5.12 Dixon plot analysis of UGT2B17 testosterone glucuronidation at initial testosterone concentrations of 10, 15 and 20 µg/mL, following the addition of increasing 3'hydroxystanozolol. The UGT2B17 protein concentration was 0.1 mg/mL. Values are the mean ± SEM of duplicate values.

5.5 Discussion

5.5.1 LC-MS/MS methed for detection of testosterone and epitestosterone

An optimised LC-MS/MS method and validation has been described in this chapter for the analysis of testosterone and epitestosterone, following optimal deconjugation and extraction from rat urine and plasma. The method was capable of detecting testosterone at LLOQ 0.125 ng/mL and epitestosterone at LLOQ 0.250 ng/mL. All validation experiments for this method were in line with FDA guideline parameters (http://www.fda.gov/downloads/Drugs/.../Guidances/ucm070107.pdf). As this method was optimised for deconjugation with a good extraction recovery, this method could be applied for studies monitoring testosterone and epitestosterone concentrations in blood and urine *in vivo*. This method allows the determination of both epitestosterone and testosterone in blood and urine using 100 µL, in comparison to other based methods requiring 200 µL (French, 2013). A quicker extraction process that achieved good extraction recovery and greater separation of testosterone and epitestosterone in comparison to a similar LC-MS/MS method using solid-phase microextraction (Zhan *et al.*, 2011) was observed. This method also provides an LC-MS/MS method for the detection of testosterone and epitestosterone metabolites in urine along with determining the T/E ratio, as current WADA protocol uses gas chromatography tandem mass spectrometry for analysis of these metabolites (http://www.wada-ama.org/rtecontent/document/end_steroids_aug_04.pdf). Owing to developments in forensic toxicology software for LC-MS/MS, this method could be applied to software for advanced analysis and drug testing of compounds. This extraction method and LC-MS/MS analytical method could overcome some of the issues associated with sample preparation and analysis by GC-MS, which has a sample derivatisation step for analysis of steroids that is considered time consuming and

expensive. Thermal decomposition has been known to occur with steroid derivatisation analysis by GC-MS which can affect the method reliability (Deshmukh *et al.*, 2012a; He *et al.*, 2005).

5.5.2 Effects of stanozolol and diclofenac administration on urinary testosterone, epitestosterone and T/E ratios

A key aspect of this study was to investigate if stanozolol and diclofenac administration affected the levels of excreted urinary testosterone, epitestosterone and T/E ratios *in vivo*. Previous studies revealed that the NSAIDs diclofenac and ibuprofen competitively inhibit UGT2B17 testosterone glucuronidation *in vitro* (Sten et al., 2009b). A human *in vivo* study showed these NSAIDs have little effect on testosterone and epitestosterone, with the T/E ratio unaffected (Lundmark *et al.*, 2013). The study in this chapter describes the effect of diclofenac in a rat model. It was possible to analyse the effect of stanozolol and diclofenac as rats were grouped in four groups of 6 rats, each group received the same concentration of stanozolol, with increasing diclofenac administered into each of the groups of rats, apart from group one which did not receive diclofenac.

The analysis of urinary testosterone revealed that the excretion of free and deconjugated testosterone was high during the beginning of the study in all three groups between days 0-14. After 14 days treatment of stanozolol there was a significant reduction in excreted testosterone including groups that had been administered with diclofenac. The reduction in testosterone excretion continued between days 21-28 with samples collected on days 28 and 42 at the end of the study, having very little excreted testosterone. In several samples at the end of the study, free and deconjugated testosterone was lower than the LLOQ

detection for this method of 0.125 ng/mL. The reduction in excreted testosterone levels was observed in all groups in urine samples collected before and post stanozolol administration. Throughout the study, there were no significant differences in testosterone excretion between the three groups, indicating diclofenac did not impact on excreted testosterone levels. The partial eta squared values indicated a small effect size for these samples; this indicates the sample size did not effect the variation in values between groups.

As stanozolol was administered to all groups it is likely that the administration of stanozolol resulted in reduced excretion of testosterone. The effect of stanozolol on testosterone excretion levels could be from different factors. Anabolic steroids including stanozolol suppress negative feedback mechanisms that reduce LH and FSH hormone (Evans, 2004). In this study the administration of stanozolol could have led to suppression in the production of testosterone leading to less testosterone metabolised and excreted. Testosterone excretion could also be reduced by stanozolol through disrupting the metabolism of testosterone. Stanozolol has been found to undergo phase I hydroxylation and phase II glucuronidation (Stewart *et al.*, 2009; Schanzer *et al.*, 1990), testosterone also undergoes both of these metabolic processes (Sonderfan *et al.*, 1987; Sten *et al.*, 2009a; Schulze *et al.*, 2008b). The addition of stanozolol could lead to competitive inhibition with testosterone for enzyme active sites, if stanozolol is metabolised and glucuronidated by the same enzymes as testosterone. As glucuronidation is the final metabolic process before excretion of compounds (Radominska-Pandya *et al.*, 1999), a reduction in testosterone glucuronidation by stanozolol could impact levels of excreted testosterone. However little is currently known on the UGT enzymes involved in stanozolol glucuronidation and

whether stanozolol is glucuronidated, and to what extent, by the key UGT enzymes involved in testosterone glucuronidation such as UGTB17.

Diclofenac has previously been found to inhibit UGT2B17 mediated testosterone glucuronidation *in vitro* (Sten *et al.*, 2009b), in this study the reduction in testosterone excretion occurred in all groups regardless of diclofenac administration, including group 1 that was not administered with any diclofenac. This indicates that the effect of diclofenac, if any, on the changes in testosterone excretion levels would be minor.

Urine analysis of free and deconjugated epitestosterone revealed that initial epitestosterone concentrations were lower than testosterone concentrations at day 0 of sample collection. Epitestosterone concentrations remained at similar levels in all groups at the beginning of the study which was followed by a reduction to very low levels on days 14 and 21. An increase in excreted epitestosterone was then observed at day 28 in all groups. Epitestosterone excretion continued to increase further at the end of the study on day 48 were the highest concentration of epitestosterone excretion was observed. This increase in epitestosterone excretion was observed in all groups in the urine samples collected before and post stanozolol injections. The levels of epitestosterone were similar in each of the groups with no significant differences between groups at each sample collection day. The role of diclofenac on the changes observed in epitestosterone excretion is likely to be minimal, given that the changes happened in all three groups and epitestosterone did not vary significantly between any of the groups. Diclofenac has a minimal effect in reducing microsomal glucuronidation of epitestosterone *in vitro* (Sten *et al.*, 2009b), whereas the excretion of epitestosterone was suppressed slightly following administration of diclofenac and ibuprofen (Lundmark *et al.*, 2013).

The increase in excreted epitestosterone could be explained by the stanozolol administered to rats or by the reduction in testosterone excretion observed towards the end of the study that could be linked with epitosterone excretion increasing. The administration of stanozolol may explain the increase in epitestosterone excretion. The increase in circulating anabolic steroids from stanozolol administered may have led to increases in the production of epitestosterone. Whilst testosterone administration has not been linked with increases in epitestosterone (Catlin *et al.*, 1997), little is known about the role of stanozolol, or other synthetic anabolic steroids, on epitestosterone secretion and production. An explanation for this rise in epitestosterone excretion could be an increase in production in response to the increases in stanozolol. Another reason for the increased excretion in epitestosterone could be from the reduced excretion of testosterone, as epitestosterone excretion began to increase at the same sample collection day that testosterone excretion began to reduce. The increase in epitestosterone production could be in response to reduced testosterone. Although epitestosterone levels do not alter with testosterone administration (Catlin *et al.*, 1997), little is known on the effects of epitestosterone levels when testosterone is decreased. The rate of epitestosterone glucuronidation could also be enhanced because of reduced testosterone. A number of UGT enzymes including UGT2B7 are both substrates of testosterone and epitestosterone (Sten *et al.*, 2009a), the reduced testosterone would mean there is less competition for the active site of the enzyme giving a greater turnover of epitestosterone glucuronide for excretion.

The urine samples taken at day 0 before stanozolol was administered had been treated with diclofenac, apart from group 1 which had no diclofenac administered. These samples

revealed that the T/E ratios were higher in groups 2 and 3 than in group 1 ($p = 0.098$ between group 1 and group 2), ($p = 0.040$ between groups 1 and 3). This indicates that there was a significant increase in T/E ratios between the first group that had no diclofenac administered and group 3 that was administered with 5 mg/kg diclofenac. The average urinary T/E ratio was higher in group 3 than group 2 but this difference was not significant ($p = 0.957$). These results show that short term effects of diclofenac can increase urinary T/E ratios.

The urinary T/E ratios throughout the study for each group were in line with the excreted concentrations of testosterone and epitestosterone. There was an initial increase in the T/E ratios in all groups with the T/E ratios highest on day 14. This was due to the initial rise in testosterone and decrease in epitestosterone excretion. The T/E ratios in each group then lowered after day 14 until the end of the study. After day 21 T/E ratios were lower than 1 for most samples owing to testosterone reducing to very low undetectable levels and very high excreted epitestosterone levels. Apart from the initial sample collection at day 0 there was only small variations in T/E ratios between groups; with groups 2 and 3 having higher T/E ratios than group 1 on some sample days, although apart from day 0 this was not significant.

5.5.3 Testosterone glucuronidation activity of rat microsomes *in vitro*

Microsome samples were collected from each rat at the end of the study and *in vitro* testosterone glucuronidation assays were performed. The results from these assays indicated that the microsome samples had UGT enzymes present that glucuronidated testosterone. Whilst there was some variation in glucuronidation rates between individual

rat microsomes, when the rates were averaged into groups based on the concentration of diclofenac administered, there was no difference in glucuronidation rates between the three groups. Steroidogenic enzymes can be upregulated in response to androgen deprivation (Guillemette *et al.*, 1997). In this study the addition of diclofenac has not altered the activity of microsomal UGT enzymes towards testosterone glucuronidation as there is no difference in rates between the groups. The diclofenac administered to groups 2 and 3 does not appear to have had any effects on the levels of microsomal UGT enzymes in order to have an effect on the rate of testosterone glucuronidation at concentrations used in this *in vitro* study.

5.5.4 Interaction of stanozolol on UGT2B17 testosterone glucuronidation

An *in vitro* study was performed that demonstrated stanozolol and 3'hydroxystanozolol competitively inhibited UGT2B17 glucuronidation of testosterone; this was determined using Dixon plot analysis. Whilst glucuronide metabolites of stanozolol and 3'hydroxystanozolol have been detected in urine (Schanzer, 1996), the UGTs involved in their glucuronidation are unknown. As these compounds were both found to be competitive inhibitors of UGT2B17, this could provide an insight into the UGT2B17 being involved in stanozolol and 3'hydroxystanozolol glucuronidation as these compound could be competing for the active site of the enzyme with testosterone. Stanozolol was a more active inhibitor than 3'hydroxystanozolol owing to the lower Ki value. This indicates that stanozolol could be a stronger substrate of UGT2B17 than its metabolite 3'hydroxystanozolol. The results from this *in vitro* study support the observations made in the rat *in vivo* study were testosterone excretion decreased owing to stanozolol administered in rats. As UGT2B17 is the most active enzyme in testosterone

glucuronidation (Sten *et al.*, 2009a), the stanozolol present could be acting competitively to inhibit the glucuronidation of testosterone. This would lead to reduced levels of inactive testosterone ready for excretion. As stanozolol is used for long term medical conditions such as angioedema (Sloane *et al.*, 2007) and testosterone is used for treatments including hypogonadism (Makhsida *et al.*, 2005), any alterations to these steroid concentrations by drug-drug interactions and competitive inhibitions could lead to the altered bioavailability of these steroids used for clinical conditions.

5.6 Conclusions

To conclude, an optimised and validated LC-MS/MS method has was developed for the analysis of testosterone and epitestosterone in rat blood and urine, using stanozolol D3 as the internal standard. The administration of stanozolol in rats causes a short term increase in testosterone; however over the long term excreted testosterone levels were very low at the end of the study. Stanozolol did not appear to significantly alter epitestosterone levels initially, however over the long term duration of the study epitestosterone excretion increased. These changes in excreted testosterone and epitestosterone led to very low urinary T/E levels at the end of the study. Diclofenac appeared to have a short term effect on the urinary T/E levels; group 3 had a significantly higher T/E ratio than group 1. This could have implications on short term effects on T/E ratios for measuring and detetecting testosterone abuse in sport. However over the long term duration of the study, diclofenac did not appear to alter testosterone and epitestosterone excreted levels and only minor changes in T/E ratios were observed, as there were no significant changes between groups at each sample collection day. Testosterone glucuronidation assays *in vitro*, using microsome samples from each rat used in the study did not show any alterations in average

glucurondation rates between rat groups. This could demonstrate UGT enzymes were not up- or down-regulated in response to diclofenac. The diclofenac administered in groups 2 and 3 did not appear to alter the regulation of microsomal UGT enzymes to significantly alter glucuronidation rates. An *in vitro* study has shown that stanozolol and 3'hydroxystanozolol competitively inhibit UGT2B17 glucuronidation of testosterone; this could indicate the substrate specificity of stanozolol for UGT2B17. These results highlight the role of stanozolol and diclofenac on the excretion levels of testosterone and epitestosterone in rats over short and long term periods, to enhance previous *in vitro* and *in vivo* studies that assess the role of NSAIDs on testosterone glucuronidation and T/E ratios (Sten *et al.*, 2009b; Lundmark *et al.*, 2013).

Chapter 6. Discussion and future work

6.1 Discussion

The results and data presented in chapters 3 to 5 revealed a number of influences on the regulation of testosterone and epitestosterone through metabolism and excretion. The results focussed on the phase II enzymes involved in testosterone and epitestosterone glucuronidation, UGT2B17 and UGT2B7 respectively (Sten *et al.*, 2009a). Previous *in vitro* studies revealed the NSAIDs ibuprofen and diclofenac inhibit UGT2B17 testosterone glucuronidation (Sten *et al.*, 2009b). Although various substrates of UGT2B17 and UGT2B7 are known (Turgeon *et al.*, 2003; Court *et al.*, 2003; Belanger *et al.*, 2009), little is known on the roles of these substrates along with other dietary and pharmaceutical compounds, apart from the NSAIDs mentioned, have on the interactions with UGT enzymes that could impact on testosterone and epitestosterone glucuronidation rates, potentially influencing doping analysis.

Initial screening assays, followed by more detailed kinetic analysis, were performed to determine inhibitors of UGT2B17 testosterone glucuronidation. This study found several dietary samples including teas and red wine reducing testosterone glucuronidation rate. Analysis of these dietary compounds by HPLC revealed the presence of phenolic and catechin compounds in various abundances between samples. Kinetic analysis of these compounds revealed the level and type of inhibition being expressed by these compounds. Two known substrates of UGT2B17, 1-naphthol and 4-ethylphenol (Turgeon *et al.*, 2003) were found to competitively inhibit testosterone glucuronidation when combined together with testosterone, likely to be competing for the active site of the enzyme.

Some of the key inhibitors, including teas and catechin compounds, of UGT2B17 were then screened to determine if they had a similar inhibitory effect on epitestosterone glucuronidation by UGT2B7. These results revealed that the tea samples and several catechin compounds also inhibit epitestosterone glucuronidation by UGT2B7, although the inhibition of some compounds was at different efficacies than the effects on UGT2B17 testosterone glucuronidation. An example of this was the compound (-)EC having a significant inhibitory effect on UGT2B17, whereas there was no significant inhibition on UGT2B7, with the glucuronidation rate with (-)EC added being similar to the enzyme substrate control. Two anti-retroviral drugs, efavirenz and ziduvodine that are known substrates of UGT2B7, competitively inhibit the glucuronidation of epitestosterone when combined added to epitestosterone glucuronidation assays at increasing concentrations. Efavirenz was a more potent inhibitor of epitestosterone glucuronidation than ziduvodine, which correlates with previous findings indicating efavirenz as a stronger substrate of UGT2B7 (Belanger *et al.*, 2009).

In addition to the roles of dietary and pharmaceutical components on testosterone glucuronidation, the role of steroid interactions on UGT2B17 testosterone glucuronidation has been presented using epitestosterone and stanozolol. A previous study has demonstrated epitestosterone interacts with UGT2B17 to reduce the rate of testosterone glucuronidation at μM concentrations (Sten *et al.*, 2009a). The results presented here analysed the effects of testosterone glucuronidation at ng/mL by adding epitestosterone at similar ratios. The addition of epitestosterone reduced testosterone glucuronidation at ng/mL concentrations, however the impact of epitestosterone at these concentration levels was less than that found at μM concentrations. The synthetic anabolic steroid stanozolol along with 3'hydroxystanozolol was found to competitively inhibit UGT2B17

glucuronidation of testosterone. As stanozolol is glucuronidated (Pozo *et al.*, 2009), this finding could provide an insight the substrate specificity of UGT2B17 towards stanozolol, as the current UGT enzymes involved in stanozolol glucuronidation are not known.

The results from the rat study performed in chapter 5 revealed the administration of stanozolol depleted testosterone excretion over the course of the study to low levels whilst having an opposite effect on epitestosterone. Over the course of the study epitestosterone increased with the highest excreted levels being observed at the end of the study. These changes in excretion levels led to urinary T/E ratios being reduced to very low values over the course of the study. The increase in epitestosterone excretions could also be explained by the reduction in testosterone having a direct effect on epitestosterone rather than just the effects of stanozolol. The long term effect of diclofenac on any changes in excretion levels was not clear as there were no significant changes in excreted values between rat groups throughout the study. The only initial difference observed by diclofenac was between T/E ratios at the beginning of the study, indicating that diclofenac could have a short term effect in increasing urinary T/E ratios. As the *in vitro* testosterone glucuronidation activity of microsomes from the rats used in this study was not altered based on diclofenac administration, it is unlikely that diclofenac influenced the regulation of UGT levels in microsomes through the duration of the study.

These results have highlighted that the regulation of testosterone and epitestosterone can be effected by commonly consumed dietary compounds and clinically used pharmaceuticals. This could lead to implications where the circulating and excreted levels of these steroids could be altered. Altered enzyme activity could be further enhanced by other factors such as pharmacogenetic variations in key steroidogenic enzymes. If excreted levels of

testosterone and epitestosterone are altered it could affect the urinary T/E ratio used to measure testosterone doping in sport (Catlin *et al.*, 1997; Sten *et al.*, 2009b). Depending on the UGT enzyme that is inhibited, the suppression of testosterone excretion would lead to reduced T/E ratios whereas reduced epitestosterone excretion will enhance T/E ratios. This could lead to inaccurate false positive or negative readings of testosterone abuse. However any alterations to T/E levels from stanozolol interacting with testosterone glucuronidation would not affect doping tests as stanozolol is also listed as a banned substance by WADA (http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-Prohibited-list/2014/WADA-prohibited-list-2014-EN.pdf). Therefore stanozolol would be detected in urine at adverse levels meaning any reductions in testosterone excretion would be insignificant. A number of limitations of using the urinary T/E measurement to monitor testosterone doping have been discussed (Van De Kerkhof *et al.*, 2000) in addition to the results presented here. Owing to these limitations alternative methods of detecting testosterone abuse and accurate testosterone levels have been discussed. An example of this is using hair analysis to detect testosterone doping by measuring unconjugated testosterone before metabolism, this method would therefore not rely on a maintained metabolic system. (Van De Kerkhof *et al.*, 2000; Deshmukh *et al.*, 2010; Deshmukh *et al.*, 2012a). The analysis of human hair can also give an indication of steroid usage over a longer period than urinalysis based on the length of hair analysed (Deshmukh *et al.*, 2010; Deshmukh *et al.*, 2012a).

Alterations in testosterone glucuronidation by dietary, pharmaceutical and steroid interactions could lead to enhanced levels of circulating testosterone. A number of adverse clinical and endocrine effects discussed in detail in previous chapters are associated with testosterone. Inhibition of testosterone glucuronidation could lead to increases in

circulating testosterone levels or prolonged activity of testosterone; this could increase adverse testosterone effects. As testosterone is also used clinically for endocrine conditions such as hypogonadism as testosterone replacement therapy (Bang *et al.*, 2013; Velazquez and Bellabarba Arata, 1998), changes to testosterone glucuronidation rates could have implications on the correct dosages used in these conditions. The role of testosterone levels based on UGT2B17 and other UGT activities on prostate cancer has been discussed, domenstrating reduced UGT testosterone glucuronidation has been linked to exacerbating prostate cancer risk (Thind *et al.*, 2013; Cai *et al.*, 2012). These results highlight how dietary compounds could reduce UGT2B17 testosterone glucuronidation which could increase circulating levels of testosterone and enhance prostate cancer risk.

6.2 Limitations of experimentation

The results that focus on the inhibitory actions of dietary and pharmaceutical components on testosterone glucuronidation were performed *in vitro*. Whilst these studies can be advantagous in terms of being able to have a stronger control on experimental conditions such as concentration used in studies, all *in vitro* studies are limited in that the results obtained from these experiments do not necessarily mean the same effects will be observed *in vivo*. The greatest impact of the *in vitro* results would be an observed effect in humans owing to the impact on urinary steroid doping tests. A previous *in vitro* study that demonstrated inhibition of testosterone glucuronidation by NSAIDs (Sten *et al.*, 2009b) did not have the same effects when applied in a human *in vivo* study (Lundmark *et al.*, 2013). However the advantage of the results gained in this study is that a number of the inhibitors are commonly consumed dietary substances such as white tea; this means the

limitations in concentrations dosages of pharmaceuticals such as NSAIDs would not need to be as restricted with these samples in an *in vivo* study.

The use of a rat study in this project had some advantages over performing a human study. A rat study allowed for more control over diet and lifestyle for the duration of the study. These characteristics would have been difficult to control in a human study for the prolonged period of this study. The limitation of the rat study performed was that only the urine matrix samples were adequately available for analysis through the entire duration of the study. Whilst urinalysis provided detailed results as to changes in excreted steroid levels throughout the study, it was not possible to analyse the effects in other matrices such as blood that would have provided greater detail as to changes in steroid level production and circulating levels. These samples were collected by trained personnel as discussed in the methods section in chapter 2 and 5. Based on the number of rats available and grouping of rats, all groups of rats were administered with stanozolol throughout the study. Whilst the same effects were observed in each group, without a control group that contained no stanozolol present, it is difficult to make the comparison as to the extent of changes in steroid excretion levels observed during the study. The initial testosterone and epitestosterone levels varied within groups in the study leading to some average concentrations having a wide deviation. This study could have been improved through using rats with similar baseline testosterone and epitestosterone concentrations that may have reduced deviations in these concentrations within groups during the study; this may have also enhanced the statistical tests used for analysis.

6.3 Future work

For the *in vitro* results of dietary and pharmaceutical compounds on testosterone and epitestosterone glucuronidation inhibition, future work would involve human *in vivo* studies that would be useful for anti-doping detection. An initial rat study could be performed using a similar template to the rat study used in this project. This could provide an initial insight into the changes in these inhibitory effects from *in vitro* to *in vivo*. Future work with these results could involve analysing the effects of these inhibitory compounds with different pharmacogenetic variations in expression of UGT enzymes, particularly with UGT2B17 testosterone glucuronidation. As pharmacogenetic variations of UGT2B17 influence excreted testosterone levels (Schulze *et al.*, 2008b; Jakobsson *et al.*, 2006; Rane and Ekstrom, 2012), it would be useful to determine any further alterations in testosterone glucuronidation and excreted levels when combined with the inhibitors found in this study. An additional human *in vivo* study could be performed to determine the effects of these inhibitors when testosterone is used therapeutically in replacement therapies for endocrine conditions such as hypogonadism. Results from this investigation would indicate if these inhibitors impact the dosages used in replacement therapy. This could be performed using a similar method to a previous study that analysed the effects of UGT2B17 pharmacogentic variations on testosterone replacement therapy dosages (Bang *et al.*, 2013).

It has been demonstrated in this study that stanozolol competitively inhibits UGT2B17 testosterone glucuronidation *in vitro*, indicating synthetic anabolic steroids as potential substrates of UGT2B17. Future studies would involve investigating the inhibitory effects of the dietary inhibitors on the glucuronidation of other steroids such as stanozolol. If this

was applied as an *in vivo* study the effect of these inhibitors on stanozolol excretion levels could also be observed.

The results from the rat study performed revealed stanozolol decreases the long term excretion of testosterone whilst increasing epitestosterone excretion. Further studies using human *in vivo* studies could be preformed to enhance the knowledge on the effects of stanozolol and other synthetic anabolic steroids on testosterone and epitestosterone production and excretion levels. This would be beneficial for anti-doping knowledge.

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Appendices

Table 7.1 Chapter 5 Indivudual rat urinary testosterone and epitestosterone concentrations and T/E ratios in samples collected before stanozolol administration.

	Day 0			Day 7			Day 14		
Rat	Test	Epit	T/E	Test	Epit	T/E	Test	Epit	T/E
1	280.30	75.65	3.71	99.16	4.14	23.96	560.55	2.39	234.86
2	615.22	160.46	3.83	473.28	76.05	6.22	77.21	11.71	6.59
3	142.64	48.32	2.95	92.57	76.18	1.22	500.89	3.15	158.90
4	71.63	16.28	4.40	134.03	21.77	6.16	200.20	1.96	102.13
5	36.80	86.14	0.43	164.95	4.20	39.27	124.78	2.19	57.05
6	63.46	125.37	0.51	148.78	40.02	3.72	351.81	3.87	91.01
1-6 Ave-rage	**201.68 ±90.16**	**85.37 ±21.20**	**2.64 ±0.71**	**185.46 ±58.69**	**37.06 ±13.49**	**13.43 ±6.13**	**302.57 ±81.89**	**4.21 ±1.53**	**108.42 ±32.60**
7	245.01	2.66	92.07	1254.04	18.50	67.79	615.17	5.78	106.34
8	113.01	2.01	56.26	0bld	5.34	0.00			
9	1209.49	179.58	6.74	348.15	4.13	84.37	44.24	16.45	2.69
10	326.40	5.43	60.06	179.51	4.09	43.50	549.90	2.37	231.83
11	1026.33	168.04	6.11	1007.46	672.39	1.50	377.65	3.47	108.86
12	61.66	5.24	11.76	647.62	78.42	8.26	332.52	2.82	117.82
7-12 Ave-rage	**496.98 ±201.43**	**60.49 ±35.87**	**38.83 ±14.63**	**687.36 ±182.36**	**130.48 ±109.01**	**41.08 ±14.77**	**383.90 ±91.08**	**6.18 ±2.41**	**113.51 ±33.15**
13	118.43	1.60	73.89	655.73	77.73	8.44	342.66	1.19	287.02
14	477.12	76.96	6.20	1.35	6.84	0.20	219.14	3.72	58.91
15	92.40	17.78	5.20	77.22	2.74	28.19	784.10	4.83	162.21
16	115.60	1.68	68.75	31.62	6.37	4.97	291.23	1.90	153.46
17	98.94	1.97	50.13	1358.34	14.39	94.39	212.27	1.61	131.76
18	117.25	1.57	74.46	2.79	6.72	0.42	130.80	1.02	128.43
13-18 Ave-rage	**169.96 ±61.59**	**16.93 ±12.29**	**46.44 ±13.38**	**354.51 ±225.68**	**19.13 ±11.82**	**22.77 ±14.93**	**330.03 ±95.52**	**2.38 ±0.63**	**153.63 ±30.52**
19	355.60	27.47	12.94						
20	270.04	7.14	37.80	120.65	55.34	2.18			
21	115.61	3.98	29.05	688.74	187.61	3.67			
22	215.89	6.85	31.53	121.65	25.12	4.84			
23	132.59	2.73	48.62						
24	107.09	1.57	68.38	121.04	49.60	2.44			
19-24 Ave-rage	**199.47 ±40.69**	**8.29 ±3.94**	**38.05 ±7.72**	**263.02 ±115.87**	**79.42 ±36.65**	**3.28 ±0.50**			

Table 7.1 Continued

	Day 21			Day 28			Day 42		
Rat	Test	Epit	T/E	Test	Epit	T/E	Test	Epit	T/E
1	5.68	8.62	0.66	0bld	60.67		0bld	11.50	
2	8.62	14.84	0.58	0bld	106.40		0bld	429.77	
3	12.15	9.66	1.26	0.26	221.41		0bld	463.57	
4	13.03	14.93	0.87	0bld	110.68		0bld	509.92	
5	2.97	8.78	0.34	0bld	351.97		0bld	499.12	
6	7.32	9.86	0.74	0bld	133.44		3.94	510.62	0.0077
1-6 Average	**8.30 ±1.57**	**11.12 ±1.21**	**0.74 ±0.13**		**164.10 ±43.46**			**404.08 ±79.56**	
7	4.92	16.30	0.30	0bld	622.73		0bld	1034.50	
8									
9	35.11	19.98	1.76	0bld	121.87		0bld	966.72	
10	132.13	5.62	23.53	7.62	209.84	0.04	7.60	157.89	0.048
11	64.54	14.39	4.48	0bld	135.22		0bld	304.92	
12	5.43	8.03	0.68	0bld	148.20		0bld	331.42	
7-12 Average	**48.43 ±21.59**	**12.86 ±2.42**	**6.15 ±4.02**	**7.62**	**247.57 ±94.99**			**559.09 ±167.03**	
13	5.63	9.25	0.61	0bld	138.50		0bld	410.27	
14	5.15	11.02	0.47	0bld	143.73		0bld	852.88	
15	11.16	11.26	0.99	0bld	140.50		0bld	556.81	
16	116.64	11.24	10.37	0bld	140.36		0bld	139.74	
17	378.17	7.60	49.77	0bld	113.89		0bld	180.63	
18	33.83	7.70	4.39	0bld	145.28		0bld	220.63	
13-18 Average	**91.76 ±59.84**	**9.68 ±0.71**	**11.10 ±7.89**		**137.04 ±4.74**			**393.49 ±112.19**	

Table 7.2 Chapter 5 Indivudual rat urinary testosterone and epitestosterone concentrations and T/E ratios in samples collected post stanozolol administration.

	Day 0			Day 7			Day 14		
Rat	Test	Epit	T/E	Test	Epit	T/E	Test	Epit	T/E
1	326.07	28.52	11.43	12.89	19.73	0.65	12.98	11.37	1.14
2	648.71	156.33	4.15	155.79	12.21	12.75	2.53	5.61	0.45
3	152.90	61.00	2.51	14.90	1.31	11.36	557.53	75.00	7.43
4	76.30	15.11	5.05	92.05	15.49	5.94			
5	101.45	16.08	6.31	14.81	1.81	8.19	288.76	41.27	7.00
6	101.92	19.84	5.14	13.53	4.14	3.27	90.79	14.95	6.07
1-6 Average	**234.56 ±90.72**	**49.48 ±22.48**	**5.77 ±1.25**	**50.66 ±24.59**	**9.12 ±3.17**	**7.03 ±1.90**	**190.52 ±105.16**	**29.64 ±11.76**	**4.42 ±1.37**
7				109.21	2.62	41.70	1.33	5.49	0.24
8	20.07	21.11	0.95						
9	118.45	2.19	54.14	115.92	1.99	58.17	171.26	1.85	92.62
10	96.66	1.85	52.15	672.74	74.90	8.98	96.44	1.67	58.02
11	429.83	166.24	2.59	32.03	30.96	1.03	140.44	1.65	84.94
12	2.07	4.15	0.50	1146.22	33.24	34.48	31.81	5.85	5.44
7-12 Average	**133.42 ±70.57**	**39.11 ±29.20**	**22.07 ±12.70**	**415.22 ±196.93**	**28.74 ±12.17**	**28.87 ±10.55**	**88.26 ±29.17**	**3.30 ±0.97**	**48.25 ±17.73**
13	157.11	1.81	86.85	449.04	1.67	268.89	104.94	2.33	44.95
14	154.11	12.26	12.57				171.15	1.43	120.06
15	12.70	5.30	2.39	12.71	2.94	4.33	134.17	1.85	72.64
16	212.17	2.56	82.89	17.04	19.50	0.87	29.96	4.50	6.65
17	58.47	1.68	34.83	24.10	23.55	1.02	8.54	6.28	1.36
18	56.13	2.43	23.08	22.04	27.73	0.79	2.76	8.60	0.32
13-18 Average	**108.45 ±31.42**	**4.34 ±1.67**	**40.44 ±14.73**	**104.99 ±86.04**	**15.08 ±5.38**	**55.18 ±53.43**	**75.25 ±29.04**	**4.17 ±1.16**	**41.00 ±1973**
19	285.53	4.06	70.31						
20	129.00	2.96	43.62	638.06	398.36	1.60			
21	276.78	4.10	67.55	670.22	8.85	75.73			
22	469.02	8.81	53.24	422.47					
23	167.15	2.79	59.91	446.18	134.60	3.31			
24	138.29	1.89	73.15	496.15	60.60	8.19			
19-24 Average	**244.30 ±52.83**	**4.10 ±1.00**	**61.30 ±4.62**	**534.62 ±50.48**	**150.60 ±70.52**	**22.21 ±17.90**			

Table 7.2 continued.

	Day 21			Day 28			Day 42		
Rat	Test	Epit	T/E	Test	Epit	T/E	Test	Epit	T/E
1	4.22	13.14	0.32	0bld	171.20		0bld	11.50	
2	4.07	17.73	0.23	0bld	188.36		0bld	429.77	
3	51.30	14.19	3.62	0bld	231.59		0bld	463.57	
4	4.68	13.09	0.36	0bld	141.01		0bld	509.92	
5	0bld	58.35	0	0bld	216.28		0bld	499.12	
6	3.96	11.91	0.33	0bld	138.22		3.94	510.62	0.0077
1-6 Average	**13.65 ±8.59**	**21.40 ±7.43**	**0.81 ±0.56**		**181.11 ±15.69**			**404.08 ±79.56**	
7	4.58	12.17	0.38	0bld	368.03		0bld	1034.50	
8									
9	4.60	11.93	0.39	0bld	128.62		0bld	966.72	
10	1.23	9.72	0.13		237.18		7.60	157.89	0.048
11	0.64	7.05	0.09	0bld	242.30		0bld	304.92	
12	0.43	9.61	0.04	0bld	304.97		0bld	331.42	
7-12 Average	**2.30 ±0.86**	**10.10 ±0.85**	**0.21 ±0.07**		**256.22 ±39.82**			**559.09 ±167.03**	
13	0.64	8.71	0.07	0bld	224.56		0bld	410.27	
14	0.41	7.54	0.05	0bld	155.28		0bld	852.88	
15	35.02	12.23	2.86	0bld	148.22		0bld	556.81	
16	1.44	10.34	0.14	0bld	165.84		0bld	139.74	
17	67.38	5.56	12.11	0bld	240.35		0bld	180.63	
18	0.19	6.42	0.03	0bld	170.75		0bld	220.63	
13-18 Average	**17.51 ±11.44**	**8.47 ±1.02**	**2.54 ±1.97**		**84.17 ±15.74**			**393.49 ±112.19**	

List of publications

Refereed journal articles

Jenkinson C, Petroczi A, Barker J, Naughton DP. Dietary green and white teas suppress UDP-glucuronosyltransferase UGT2B17 mediated testosterone glucuronidation. Steroids, 2012, 77(6): 691-695.

Jenkinson C, Petroczi A, Naughton DP. Red wine flavonoids inhibit UGT2B17 and potentially increase circulating testosterone levels. Nutrition Journal, 2012, 11:e67.

Jenkinson C, Petroczi A, Naughton, DP. Effects of dietary components on testosterone metabolism via UDP-glucuronosyltransferase (UGT). Frontiers in Endocrinology, 2013, 4: e80.

Jenkinson C, Deshmukh NIK, Shah I, Zachar G, Szekely AD, Petroczi A, Naughton DP. LC-MS/MS-Based assay for free and deconjugated testosterone and epitestosterone in rat urine and serum. Journal of Analytical and Bioanalytical Techniques, 2014, S5: 006.

Thind J, **Jenkinson C**, Naughton D, Petroczi A. Modulation of UDP Glucuronosyltransferase 2B15 and 2B17 and prostate cancer risk: current perspectives. Advances in Cancer: Research & Treatment, 2013; ID 81212.

Conerence submissions

Jenkinson C, Petroczi A, Barker J, Naughton D. (2013) Effects of food omponents and the ratio of epitestosterone to testosterone on steroid glucuronidation. Endocrine Abstracts 2013, 32, P974. 15th European Congress Endocrinology.

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Red wine and component flavonoids inhibit UGT2B17 *in vitro*

Carl Jenkinson, Andrea Petroczi and Declan P Naughton*

Abstract

Background: The metabolism and excretion of the anabolic steroid testosterone occurs by glucuronidation to the conjugate testosterone glucuronide which is then excreted in urine. Alterations in UGT glucuronidation enzyme activity could alter the rate of testosterone excretion and thus its bioavailability. The aim of this study is to investigate if red wine, a common dietary substance, has an inhibitory effect on UGT2B17.

Methods: Testosterone glucuronidation was assayed using human UGT2B17 supersomes with quantification of unglucuronidated testosterone over time using HPLC with DAD detection. The selected red wine was analyzed using HPLC; and the inhibitory effects of the wine and phenolic components were tested independently in a screening assay. Further analyses were conducted for the strongest inhibitors at physiologically relevant concentrations. Control experiments were conducted to determine the effects of the ethanol on UGT2B17.

Results: Over the concentration range of 2 to 8%, the red wine sample inhibited the glucuronidation of testosterone by up to 70% over 2 hours. The ethanol content had no significant effect. Three red wine phenolics, identified by HPLC analyses, also inhibited the enzyme by varying amounts in the order of quercetin (72%), caffeic acid (22%) and gallic acid (9%); using a ratio of phenolic:testosterone of 1:2.5. In contrast p-coumaric acid and chlorogenic acid had no effect on the UGT2B17. The most active phenolic was selected for a detailed study at physiologically relevant concentrations, and quercetin maintained inhibitory activity of 20% at 2 μM despite a ten-fold excess of testosterone.

Conclusion: This study reports that in an *in vitro* supersome-based assay, the key steroid-metabolizing enzyme UGT2B17 is inhibited by a number of phenolic dietary substances and therefore may reduce the rate of testosterone glucuronidation *in vivo*. These results highlight the potential interactions of a number of common dietary compounds on testosterone metabolism. Considering the variety of foodstuffs that contain flavonoids, it is feasible that diet can elevate levels of circulating testosterone through reduction in urinary excretion. These results warrant further investigation and extension to a human trial to delineate the health implications.

Keywords: Red wine, Flavonoids, Testosterone, UGT2B17, Glucuronidation

Introduction

Numerous reports have attested to the health damaging effects of red wine and its components beyond excess alcohol consumption, for example - owing to pesticide and heavy metal content [1,2]. In contrast, many reports point to the health protective effects of red wine owing to the abundance of anti-oxidants [3,4]. Beyond modulating oxidative damage, one focus has been on the female endocrine system, following the reports that red wine has anti-aromatase properties [5]. This discovery broadened the debate regarding the link between alcohol intake and risk of developing breast cancer [6,7].

Equally, the associations of high and low testosterone levels with the development of various forms of prostate cancer have been subjected to considerable debate [8-10]. Given the inhibitory effects of red wine on aromatase it is conceivable that red wine also affects aspects of testosterone metabolism. Although recent epidemiological studies have suggested red wine consumption is not a potential risk factor

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for prostate cancer [11,12], the effects of red wine on testosterone metabolism warrant investigation.

Glucuronidation is a major metabolic pathway for the elimination of testosterone and numerous compounds from the body [13,14]. Glucuronidation of testosterone involves the transfer of a glucuronosyl group from UDP-glucuronic acid (UDPGA) to form the steroid conjugate, testosterone glucuronide, which is then excreted in urine [13]. The UDP-glucuronosyltranferase UGT2B17 enzyme is the main steroid glucuronidation enzyme of the UGT isotopes with more than double the glucuronidation activity compared to the second most active enzyme involved in glucuronidation of testosterone, UGT2A1 [15].

The metabolism of testosterone by UGT2B17 has been shown to differ between individuals owing to the variations in the expression of UGT2B17, which has been found to alter with ethnicity, affecting the excreted steroid concentrations [16]. In *in vitro* studies, the rate of testosterone glucuronidation has also been shown to be reduced with inhibitors of UGT2B17, such as non-steroidal anti-inflammatory drugs [15]. Whilst various drugs and compounds are glucuronidated as a substrate and inhibit UGT2B17 [13], little is known about the inhibitory effects common dietary substances could have on UGT2B17 and testosterone glucuronidation.

Recently, green and white teas and purified catechin constituents have been shown to inhibit the key testosterone glucuronidation enzyme UGT2B17 in a supersome-based assay [17]. Red wine is another rich source of phenolic compounds that have been found to exert anti-oxidant health benefits in humans [18]. Given the inhibitory effects of green and white tea on UGT2B17, along with the debate on red wine and prostate cancer, it is timely to investigate if phenolic compounds in red wine have an inhibitory effect on testosterone metabolism and excretion.

The aim of this study was to analyze the inhibitory effects of a dietary red wine sample and the common phenolic compounds found in red wine, independent of the effects of alcohol, on the glucuronidation of testosterone through the inhibition of UGT2B17. A further aim was to study the potential inhibitory effect of the common wine by-product 4-ethylphenol on testosterone glucuronidation.

Materials and methods

Materials

Testosterone, acetonitrile, ethanol, gallic acid, chlorogenic acid, caffeic acid and quercetin were purchased from Sigma Aldrich (Poole, United Kingdom). Dimethyl sulfoxide, methanol and high performance liquid chromatography (HPLC) grade water were purchased from Fisher Scientific. The UGT2B17 enzymes where purchased as human UGT2B17 supersomes from BD Biosciences. UDPGA was purchased as a UGT reaction solution (mixture A) from BD Biosciences. The $MgCl_2$ and Tris–HCl buffers, along with alamethicin were purchased together as a UGT reaction mixture (solution B) from BD Biosciences. The red wine sample used was a Cabernet-Syrah red wine purchased from a local supermarket (London). All solvents used where HPLC grade.

Methods

For general screening, HPLC analysis of testosterone glucuronidation was conducted on an *Agilent 1260* HPLC system using an Ascentis Supelco C18 column, 25 cm x 406 mm i.d., 5 μM at 25°C column temperature. The mobile phase was methanol and water (80:20) at a flow rate of 1 mL/min and a 100 μL injection volume. The remaining testosterone from the reactions was detected by UV detection at 246 nm using a diode array detection system. The results represent the SD of duplicate values.

To assay the effects of quercetin at low concentrations, an alternate highly sensitive HPLC method was adopted to analyze testosterone [19]. Testosterone was dissolved in acetonitrile and added as 1% v/v. The mobile phase was acetonitrile/water (39/61, v/v) at a flow rate of 1 mL/min. The injection volume was 50μL and detection at 245 nm. The results represent the SD of triplicate values.

The testosterone glucuronidation assay, described in the BD biosciences data sheet for the human UGT2B17 supersomes, employs a standard incubation mixture containing UDPGA (2 mM), alamethicin (25 μg/mL), magnesium chloride (8 mM) and pH 7.5 Tris–HCl buffer (50 mM) and deionised water comprising 50% of the overall reaction volume. Following incubation at 37°C for five minutes, the reaction was initiated by the addition of 0.2 mg/mL ice cold UGT2B17 supersomes. The reactions were stopped by the transfer of 100 μL aliquots to 100 μL ice cold acetonitrile, vortex-mixed with samples stored on wet ice. The samples were centrifuged at 10,000 x g for 5 minutes. The aliquots of the supernatants were then analyzed by HPLC.

In order to study the inhibitory effects of red wine, various volumes ranging from 2-8% of red wine were added to the reaction. The reactions were stopped after one or two hours and the remaining testosterone was analyzed to determine any increase in testosterone through the inhibition by red wine. The red wine sample had been evaporated to dry residue to remove the ethanol and reconstituted with the same volume of water and filtered by a Millex 0.45 μM filter device.

The phenolic compounds gallic acid, caffeic acid and quercetin that are present in red wine were analyzed for

the inhibition of UGT2B17. The phenolic standards (250 μM) were dissolved in ethanol and heated and mixed to aid dissolving where necessary and added to the reaction as 1% v/v of the reaction. The phenolic compound 4-ethylphenol was dissolved in ethanol and added to the reaction at 1% of the overall reaction volume at 750 μM overall concentration. The reaction duration was for 1 hour.

The red wine sample used in the glucuronidation assays was analyzed to determine the phenolic compounds present in the wine. The wine sample for HPLC analysis was prepared by evaporating the sample to dryness with the remaining dry residue dissolved in water to restore the original volume of the sample. The sample was then filtered by a Millex 0.45 μM filter device and injected into the HPLC. Quantification was performed as previously described [20] on an Agilent 1260 HPLC using a Kromasil C18 column, 250 mm x 406 mm 5 μM with detection at 280 nm. The mobile phase consisted of 0.1% orthophosphoric acid in water (A) and methanol (B). The mobile phase gradient elusions were (80% A: 20% B) 0 minutes, (60% A: 40% B) 10 minutes, (50% A: 50% B) 20 minutes, (45% A: 55% B) 30 minutes, (35% A: 65% B) 50 minutes. The flow rate was 1 mL/min with a 10 μL injection volume. Phenolic standards were dissolved in water at a concentration of 1 mg/mL and individually injected into the HPLC under the same conditions with the retention times compared to that of the red wine sample to indentify each phenolic compound.

Statistical analyses were conducted using SPSS v19. The significance of reduction in glucuronidation activity was tested for using a directional one-sample *t*-test (H_a: activity <100%) with the significance level set at 0.05. Mixed model ANOVA was used to test for statistically significant difference over time and between concentrations, including testing for interaction effect.

Results

Inhibitory effects of red wine on UGT2B17

The effects of increasing concentrations of red wine on the UGT2B17-mediated glucuronidation of testosterone were assessed as a function of the reduction in conversion of testosterone to testosterone glucuronide (Figure 1). The results show that increasing the concentration of red wine resulted in a lower conversion of testosterone to its glucuronide conjugate. A reduction in UGT2B17 activity was observed for all, ranging between ca 10% to over 70% over two hours for additions of 2% to 8% red wine ($p = 0.01$; 0.002; 0.008 and 0.014 over one hour and $p = 0.019$; 0.033; 0.008 and 0.007 over two hours, respectively for 2%, 4%, 6% and 8%). The percentage of ethanol present in the final assay was in the range of 0.26% - 1.04% corresponding to additions of red wine at 2% - 8% respectively. It is notable that during a 2 hour period the inhibition is more pronounced at higher concentration of the red wine. Statistically significant differences were evidenced between times ($p = 0.028$) and concentrations ($p < 0.001$), with significant interaction between the two ($p = 0.001$).

The effect on UGT2B17 activity by the addition of an evaporated red wine sample reconstituted with an equal volume of water at concentrations of 4% and 8% of the reaction volume resulted in a glucuronidation % of control at 59.18 ± 3.154 and 23.48 ± 4.405, respectively. These values, taken after a two-hour duration, resemble those from the intact wine samples (Figure 1) indicating minimal contributions from the ethanol content on the inhibition of UGT2B17 by red wine.

The effects of increasing concentrations of ethanol on the reduction of testosterone by UGT2B17 are shown in Figure 2. The results indicated that testosterone glucuronidation was only slightly altered by ethanol at a 1% concentration ($p = 0.353$); however as the concentration of ethanol was increased to above 2% of the reaction volume, testosterone glucuronidation was affected as shown but not reaching a statistically significant level ($p = 0.134$ and 0.110 for 2% and 3%, respectively).

Analysis of red wine

In order to identify individual inhibitors of UGT2B17, the phenolic content in the wine sample was investigated by HPLC. Analysis of the red wine confirmed the presence of gallic acid, chlorogenic acid, caffeic acid, p-coumaric acid and quercetin (Figure 3), which informed subsequent experiments.

The inhibitory effect of individual phenolic compounds

Initial experiments were performed to screen the phenolics found in red wine for their effects on UGT2B17. Table 1 shows the effect on the glucuronidation activity of testosterone when 250 μM of each of the three phenolic compounds found in the red wine sample were added separately to the reaction over 60 minutes. Apart from chlorogenic and p-coumaric acids glucuronidation activity was reduced by each of the compounds in varying degrees. The inhibitory action of 4-ethylphenol is also displayed in Table 1. The results show the reduction in testosterone glucuronidation at initial testosterone concentrations of 100 μM, 50 μM and 20 μM.

Quercetin was selected from the initial high concentration screening assay for further study as it exhibited the highest level of inhibition at 72%. Reducing the testosterone levels to 20 μM resulted in inhibition of 34-18% by a low concentration of quercetin, in a concentration dependent manner, despite the 10-fold excess in testosterone levels (Table 1). In addition, for a quercetin concentration of 2 μM, increasing testosterone levels to 30 μM resulted in a

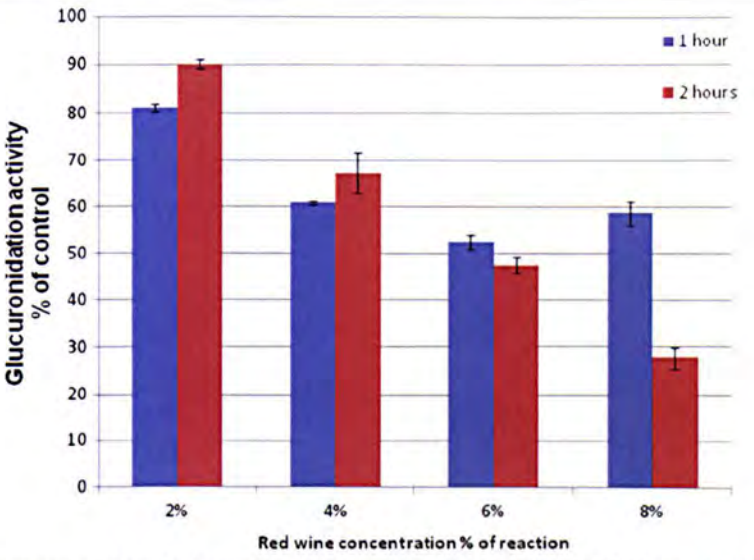

Figure 1 Reduction of testosterone glucuronidation in the presence of red wine at 100 μM initial testosterone. The results display the SD values of duplicate samples.

reduction in inhibition from 18% to 2% suggesting that the mechanism is by competitive inhibition.

Discussion

This report extends the previous study which demonstrated that tea and its component flavones competitively inhibit testosterone glucuronidation by UGT2B17. The results of this study showed that phenolic compounds commonly found in red wine, but also in many other foods, have comparable effect on testosterone glucuronidation. The rate of glucuronidation was similar on addition of the wine sample once the ethanol had been removed, indicating that it was likely to be the phenolic compounds that caused inhibition. Further studies revealed that ethanol had no effect at the concentrations found in the added wine sample. However, at higher concentrations of ethanol (>1%) the UGT2B17 enzyme activity was reduced. Ethanol has been linked to increased testosterone and aggression in male hamsters [21] and increased testosterone in rat brain [22]. From our results, the effects of alcohol on UGT2B17 are unlikely to account for the increase in testosterone, unless extremely high doses are consumed.

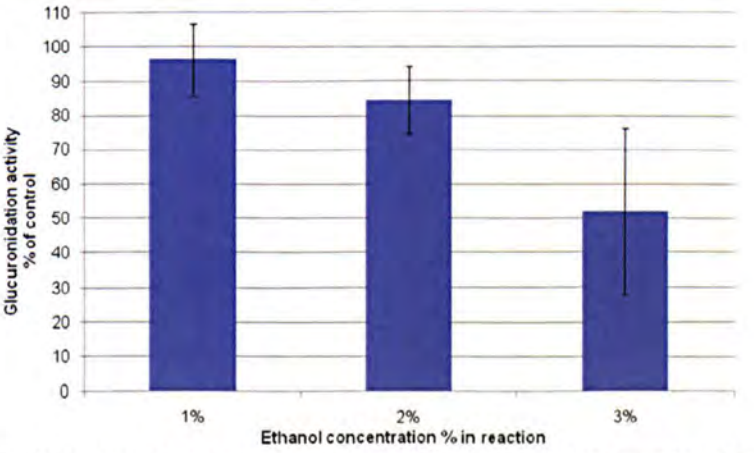

Figure 2 Effect of ethanol on the testosterone glucuronidation by UGT2B17, based on the concentration of testosterone. Concentration of testosterone was measured at the end of the reaction which was terminated after 30 minutes. The results display the SD values of duplicate samples.

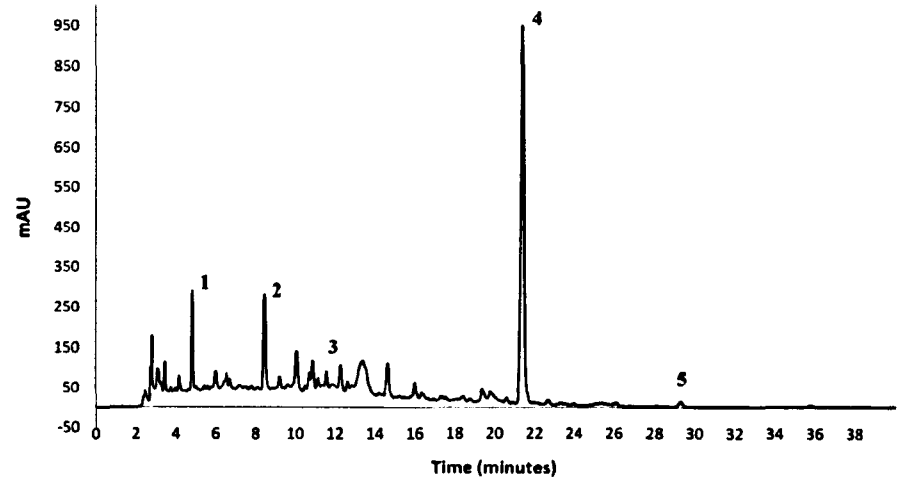

Figure 3 Chromatogram showing the phenolic compounds present in the red wine sample. 1. Gallic acid, 2. Chlorogenic acid, 3. Caffeic acid, 4. p-Coumaric acid, 5. Quercetin.

Several of the individual wine phenolic compounds inhibited the glucuronidation of testosterone at different efficiencies. The maximum inhibition was observed for quercetin, followed by 4-ethylphenol and caffeic acid. The serum concentrations of phenolic compounds that are commonly found in wine can be increased through supplementation such as with quercetin [23]. In one study, after supplementation, 1.5 μM quercetin levels in plasma were reported [24]. This concentration of quercetin affected a 9% reduction in UGT2B17 activity despite a high concentration of testosterone at 20 μM. The reported mean level of serum testosterone in adult males is 35.9 nM [25]. Given the inhibition is competitive; these much lower concentrations of testosterone should result in higher inhibition of UGT2B17 by quercetin. Future studies are warranted to investigate the effects of red wine and its components at physiological levels of testosterone.

In addition, plasma concentrations of caffeic acid have been shown to increase after the consumption of red wine [26]. Given these increases in serum concentrations through supplementation it may be possible to further reduce testosterone metabolism through glucuronidation.

The phenolic compound 4-ethylphenol has been found in red wines to produce distinct aromas [27], as well as being produced by the spoilage of yeast, hence it can be

Table 1 Reduction in testosterone glucuronidation activity of UGT2B17 by the addition of phenolic compounds

Initial testosterone concentration (μM)	Test sample concentration	Test sample concentration (μM)	Glucuronidation % of control (±SD)
Screening study			
100	4-Ethylphenol	750	78.9 ±1.131
50	4-Ethylphenol	750	57.3 ±13.548
100	Gallic acid	250	91.01 ±10.946
100	Caffeic acid	250	78.65 ±5.685
100	p-Coumaric Acid	250	NA
100	Chlorogenic Acid	250	NA
100	Quercetin	250	28.01 ±2.800
Low concentration study			
20	Quercetin	50	65.62 ±14.298
20	Quercetin	20	79.93 ±11.370
20	Quercetin	2	82.42 ±7.057
30	Quercetin	2	97.69 ±9.235
20	Quercetin	1.5	91.37 ±7.806

The results display the percentage change in the glucuronidation of testosterone over 60 mins by UGT2B17 in comparison to the uninhibited control.

found in other dietary substances [28] and enters knowingly or unknowingly into the diet. 4-Ethylphenol has also been shown here to inhibit the glucuronidation of testosterone through the UGT2B17 enzyme. The inhibition of UGT2B17 by 4-Ethylphenol was found to be greater at the lower 50 μM level of testosterone. However, at high initial testosterone concentrations above 150 μM there was very little or no inhibition showing that increasing the concentration of testosterone will overcome the inhibiting compound. 4-Ethylphenol has been shown to be a substrate for UGT2B17 with a glucuronide being formed [13]; therefore it is likely that these compounds are acting competitively with the UGT2B17 enzyme.

Whilst it has been found that no correlation exists between the variations of the UGT2B17 genotype with alterations in circulating serum testosterone levels [29-31], another study has found significant differences in circulating testosterone with an increase in individuals expressing no copy number of UGT2B17 compared to individuals with one or two copies of UGT2B17 [32]. It has yet to be determined if any direct inhibition of steroid glucuronidation enzymes could alter the levels of circulating serum testosterone in addition to altering the levels of testosterone excreted in urine. These results augment the previous study revealing that tea catechins can inhibit testosterone metabolism by supersomes containing UGT2B17 [17]. The ubiquitous presence of quercetin and other active flavonols, along with the catechins, in many foodstuffs indicate that any *in vivo* effects may be common. These effects, if found to occur *in vivo*, may have a pronounced effect on people with endocrine disorders or very low levels of endogenous testosterone, owing to high levels of receptor expression to compensate. A further aspect, although not studied here, is the potential interaction of quercetin-containing foodstuffs with drug metabolism as some drugs are metabolized via the action of UGT2B17 [33].

In conclusion it has been found that a commonly consumed dietary substance, red wine along with phenolic compounds present in red wine, inhibit testosterone glucuronidation. These results have also shown that although some of these compounds are not substrates of UGT2B17 they can inhibit the enzyme in supersomes.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
All authors contributed to the study design, data analyses and manuscript preparation. CJ completed the laboratory studies. All authors approved the final manuscript.

Acknowledgements
The authors thank Kingston University for funding for CJ.

Received: 2 May 2012 Accepted: 20 August 2012
Published: 7 September 2012

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doi:10.1186/1475-2891-11-67
Cite this article as: Jenkinson *et al.*: **Red wine and component flavonoids inhibit UGT2B17 *in vitro*.** *Nutrition Journal* 2012 **11**:67.

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Jenkinson et al., J Anal Bioanal Tech 2014, S5
http://dx.doi.org/10.4172/2155-9872.S5-006

Research Article Open Access

LC-MS/MS-Based Assay for Free and Deconjugated Testosterone and Epitestosterone in Rat Urine and Serum

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Abstract

Testosterone and epitestosterone are mainly excreted as glucuronides. The aim of this study was to develop and validate a method using liquid chromatography tandem mass spectrometry (LC-MS/MS) to analyse testosterone and epitestosterone in rat serum and urine to assist *in vivo* studies on steroid metabolism. The method was developed by spiking charcoal stripped rat plasma and urine with the analytes. The developed method was then applied to serum (n=6) and urine samples (n=6) from young male brown Norway rats to determine testosterone and epitestosterone concentrations. The assay showed linearity within quantification range coefficient (r^2) values above 0.991. Optimum conditions were determined for the deconjugation of glucuronidated testosterone and epitestosterone along with the internal standard stanozolol D3. Accuracy, precision and extraction recovery for both compounds was satisfactory in both matrices. The method was capable of quantifying 0.250 ng/mL concentrations of testosterone and epitestosterone in 100 µL of serum and urine. The average concentrations of free and deconjugated testosterone and epitestosterone found in the rat samples were: urine–201.68 ± 90.16 ng/mL and 85.37 ± 21.20 ng/mL; serum–363.40 ± 11.615 ng/mL and 1.75 ± 0.118 ng/mL, respectively. This method is sensitive, specific and reproducible for the determination of free and deconjugated testosterone and epitestosterone in rat serum and urine. The method can be used for *in vivo* analysis for further investigations of testosterone and epitestosterone concentrations in studies monitoring endocrine dysfunctions and doping.

Keywords: Bioanalytical methods; Biological samples; Clinical/biomedical analysis; HPLC

Introduction

The 2014 *World Anti-Doping Agency* (WADA) Prohibited List shows testosterone and epitestosterone listed as class S1.1a and S1.1b, with its use banned in and out of competition [1]. Doping with testosterone is suspected if the urinary ratio of testosterone over epitestosterone (T/E) exceeds 4 [2]. According to the 2012 (WADA) laboratory statistics, elevated testosterone based on the testosterone over epitestosterone (T/E) ratio accounts for 55.5% of atypical findings within the anabolic agents' drug class from a total of 2,279 for all anabolic agents, which constitute half of all adverse and atypical findings [3]. This T/E ratio approach to testing for testosterone abuse is only accurate if testosterone and epitestosterone are metabolised at the same rate and no other external factors or inter individual factors would alter the metabolism of these steroids [4].

One of the major metabolic pathways in testosterone and epitestosterone regulation and elimination is by glucuronidation through addition of glucuronic acid by UDP-glucuronosyltransferase (UGT) enzymes for excretion in urine. Variations in enzyme preference are observed with UGT 2B17 predominating for testosterone glucuronidation, whilst UGT2B7 is the most active UGT for epitestosterone glucuronidation [5]. A variety of effectors of steroid UGT glucuronidation have been identified, including pharmacogenetic variations resulting in different UGT2B17 expression which affects the urinary T/E ratios [6-8]. A number of *in vitro* studies have identified small molecule inhibitors of UGT2B17 and other UGTs toward testosterone metabolism. These inhibitors include non-steroidal anti-inflammatory drugs diclofenac and ibuprofen [5], flavonoids commonly found in red wine [9] and catechins in green and white tea [10]. As doping tests for testosterone abuse involve deconjugation by β-glucuronidase to determine total urinary T/E ratios, the doping test could be affected by the inhibitory effects of pharmaceuticals and dietary components. Inhibition of testosterone glucuronidation could lead to an increase in testosterone in serum leading to potential adverse health effects. Extending these potentially significant *in vitro* results to *in vivo, experimentation* calls for a streamlined analytical procedure that caters for analysing multiple biological samples, such as urine and blood without introducing intra-method variances owing to different sensitivity and specificity.

This report describes a method to determine testosterone and epitestosterone levels in rat serum and urine using liquid chromatography tandem mass spectrometry (LC-MS/MS). Rat models have a number of advantages for controlled clinical trials, including standardised conditions and diet, and testosterone is glucuronidated by phase 2 metabolism as is in humans [11,12]. This method could be applied to such rat studies, where the measurement of testosterone and epitestosterone in these matrices are required through the duration of study.

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Received December 16, 2013; **Accepted** January 15, 2014; **Published** January 17, 2014

Citation: Jenkinson C, Deshmukh NIK, Shah I, Zachár G, Székely AD, et al. (2014) LC-MS/MS-Based Assay for Free and Deconjugated Testosterone and Epitestosterone in Rat Urine and Serum. J Anal Bioanal Tech S5: 006. doi:10.4172/2155-9872.S5-006

Materials and Methods

Chemicals

Reference standards for testosterone and epitestosterone and testosterone glucuronide were purchased from Sigma Aldrich (Poole, UK). Epitestosterone glucuronide and stanozolol D3 (internal standard) were purchased from LGC Standards (Teddington, UK). All reference materials had a purity ≥ 98%. Sodium hydrogen phosphate heptahydrate, sodium phosphate monobasic dehydrate, HPLC grade pentane and LCMS grade acetonitrile and water were purchased from Sigma Aldrich (Poole, UK). β-Glucuronidase from *E. coli* (Cat No. 03707598001, Lot No. 13931722) was purchased from Roche Diagnostics (Burgess Hill, UK). Rat 4x charcoal stripped 0.2 μ filtered plasma and urine was purchased from Sera Laboratories. A SB C-18 column (2.1 mm, 50 mm, 1.8 μm) and 0.2 μm inline filter was purchased from Agilent (Stockport, UK). Syringe driven 0.2 μm PTFE filters were purchased from Millipore (Watford, UK). Silanized glass vials where purchased from Sigma Aldrich (Poole, UK).

Animals and samples

Urine samples were obtained from six male, brown Norway rats (weighing 280-340 g) as part of an independent study [13], purchased from Charles River laboratories (Sulzfeld, Germany). Rats were kept under the conditions and urine samples were collected as described previously [13]. Water and food was available *ad libitum*. Normal serum samples from six male brown Norway rats were purchased from Sera Laboratories. These rats where approximately 11-12 weeks old weighting between 250-275 g. The serum and urine samples were thawed at 37°C and vortex mixed. Aliquots (100 μL) were taken from each sample for analysis.

Enzyme hydrolysis of testosterone and epitestosterone glucuronide

The enzyme β-glucuronidase was used to deconjugate the glucuronide conjugates for the determination of total (glucuronide conjugated+unconjugated) concentration of testosterone and epitestosterone in serum and urine.

Sample purification

Liquid-liquid extraction was performed using pentane. The mixture was vortex mixed in a glass centrifuge for 20 seconds and centrifuged at 3220×g for 10 minutes at 4°C. The organic layer was transferred to a salinized vial and evaporated under incubation at 40°C using a gentle stream of compressed air. The sample were then reconstituted with 100 μL of acetonitrile and filtered through a 0.2 micron PTFE membrane filter for analysis by LC-MS/MS.

Liquid chromatographic-tandem mass spectrometry

Testosterone and epitestosterone analyses were carried out based on a previous method [14], with modifications, using an LC-MS/MS system comprised of a 1260 infinity LC system (Agilent, Workingham, UK) coupled to a 6430 triple quadruple mass spectrometer (Agilent, Workingham, UK). The LC system comprised of a binary pump, automatic degasser, column heater and 1290 infinity thermostated autosampler. Compounds were separated using a SB C-18 column (2.1 mm, 50 mm, 1.8 μm) that was heated to 45°C. An inline filter 0.2 micron was used to prevent the analytical column from blocking. The mobile phase consisted of water (solvent A) and acetonitrile (solvent B) with the gradient as follows: 0-5 min (53% A: 47% B), 5-9 min (53% A: 47% B), 9-11 min (0% A: 100% B), 11-25 min (53% A: 47% B). The flow rate was 250 μL/min and the injection volume was 4 μL.

The mass spectrometer was set using electrospray ionisation (ESI) operated in positive ion mode. The protonated molecules, $[M+H]^+$, of testosterone and epitestosterone (*m/z* 289.3) and stanozolol D3 (*m/z* 332.5) were used as precursor ions for collision induced dissociation (CID) for MS-MS analysis. Multiple reactions monitoring (MRM) mode was used to monitor the precursor ions and diagnostic product ions for each analyte and internal standard based on a previous method [14]. Table 1 details the MRM transitions, collision energy and retention times of testosterone, epitestosterone and stanozolol D3.

The mass spectrometer parameter optimisation was performed using Masshunter optimizer software (version B.03.01). The conditions applied for optimum ionisation were capillary voltage 4000 V, drying gas temperature 325°C, drying gas flow rate 10 L/min, nebulising gas pressure 35 psi and fragmentor voltage of 100 V. Masshunter workstation software (LC/MS data acquisition, version B.03.01) was used to control the LC-MS/MS system.

Method validation

Validation was performed in accordance with Food and Drug Administration (FDA) guidelines to establish sensitivity, specificity, selectivity, linearity, limit of detection, inter and intraday precision and accuracy, percentage recoveries, deconjugation and matrix effects. Steroid free rat plasma and urine were used for method validation.

Calibration curves of known standards were prepared by spiking charcoal-stripped plasma and urine with testosterone and epitestosterone and the internal standard (IS) stanozolol D3. Quality controls (QC) were prepared in the same manner over four concentration ranges of the calibration curve. The calibration curve was plotted for testosterone and epitestosterone levels in plasma and urine by using the analyte to IS ratio. The linearity of the calibration curve was determined using linear analysis.

The accuracy of the method was determined using replicate (N=6, per concentration level) QC samples at four concentration levels and comparing the mean calculated values to the nominal concentration values. Intra-day precision was determined by analysing the six replicated at each concentration level in one day and inter-day precision was performed by analysing 6 replicates at each concentration level over three consecutive days. The relative standard deviation (RSD %) was used for characterising precision. For the three concentration ranges, an acceptable variability was set at 15%, for the lower limit of quantification (LLOQ) this was set at 20%. LLOQ was determined as the lowest concentration of the analyte that gave a peak response which could be measured with precision and accuracy (RSD less than 20%).

The absolute extraction recoveries of testosterone and epitestosterone were determined by analysing a blank matrix of plasma and urine that was spiked with a known concentration of analyte and IS that had been extracted with standard solutions prepared in acetonitrile at the same concentrations. The peak area ratios were compared at four

Compounds	Retention Time (min)	MRM transitions	Collision energy (eV)
Testosterone	8.6	289.2>109	22
		289.2>97	26
Epitestosterone	10.58	289.2>109	21
		289.2>97	25
Stanozolol D3	11.64	332.2>81.2	50

Table 1: Analytical parameters for LC and MS.

concentration levels using six replicate samples at each concentration for the extracted and neat samples.

To analyse any effect of the matrix, blank plasma and urine were extracted and then spiked with known concentrations of testosterone, epitestosterone and IS, for comparison to a standard solution at equivalent concentrations in acetonitrile. These samples were prepared over four concentration ranges using replicates of six samples.

To assess the deconjugating activity of β-glucuronidase, the enzyme was incubated with standard concentrations of testosterone glucuronide, epitestosterone glucuronide and IS in blank plasma and urine over three concentration ranges using replicates of three samples. The samples consisted of blank plasma spiked with urine with 50 µL of β-glucuronidase added and incubated at 50°C for two hours. The samples were then cooled in wet ice prior to extraction. The concentration of testosterone and epitestosterone by de-conjugation was compared to samples spiked at the same concentration ranges with testosterone and epitestosterone and IS.

Analysis of testosterone and epitestosterone in rat serum and urine samples

Samples were prepared as previously described [15] and modified for serum and urine, by adding 100 µL of serum or urine to 1 mL of 0.2 M pH 7 phosphate buffer. The sample was spiked with 50 µL of IS stanozolol-D3 to give a concentration of 2 ng/mL. The internal standard was used in order to compensate for variations in instrument injection to injection response and loss of analytes during the extraction procedure. 50 µL of β-glucuronidase enzyme was added and samples where incubated for two hours at 50°C. The samples were cooled on wet ice and extracted as described above.

Results and Discussion

Method validation

The validation results are within the limits set by FDA guidelines. Injection of blank samples of charcoal stripped plasma and urine confirmed no components of the matrices were observed that would interfere with the analytes. Figure 1 shows the chromatogram for extracted analytes in plasma and urine at 0.25 ng/mL.

The assay for urine analysis was linear for testosterone and epitestosterone between concentrations of 0.250 ng/mL-1000 ng/mL. Owing to the high range of standards low and high concentration calibrations curves were created for testosterone and epitestosterone. The determination coefficient (r2) values were at or above 0.9917 for testosterone and 0.9997 for epitestosterone in urine and r^2 values were at or above 0.9919 for testosterone and 0.9997 for epitestosterone in plasma. Table 2 shows the accuracy, intra-day and inter-day precision results from the assay. The LLOQ for testosterone was found to be 0.250 ng/mL for testosterone and 0.250 ng/mL for epitestosterone.

The plasma assay was linear between 0.250 ng/mL-1000 ng/mL for testosterone and epitestosterone. The method was capable of detecting testosterone and epitestosterone at LLOQ of 0.250 ng/mL. The LLOD for testosterone was 0.125 ng/mL and 0.250 ng/mL epitestosterone. The extraction recoveries for testosterone and epitestosterone are shown in Table 3.

Table 4 shows the comparisons from the plasma and urine matrix effects between samples of plasma that have been extracted and spiked with standard concentrations in acetonirile.

The deconjugation assay confirmed the β-glucuronidase enzyme was effective in de-conjugating testosterone and epitestosterone with the concentration and incubation for the concentrations shown in Table 5.

Application of method for determination of testosterone and epitestosterone in real samples of rat serum and urine

The validated method was applied to determine the total (free and de-glucuronidated) testosterone and epitestosterone in rat serum and urine samples. Table 6 shows the total analyte concentrations in a selection of rat urine samples. The average urinary testosterone and epitestosterone concentrations were 201.68 ± 90.16 ng/mL and 85.37 ± 21.20 ng/mL, respectively. The average ratio of concentrations of testosterone to epitestosterone in urine was 2.64 (range: 0.42-4.40).

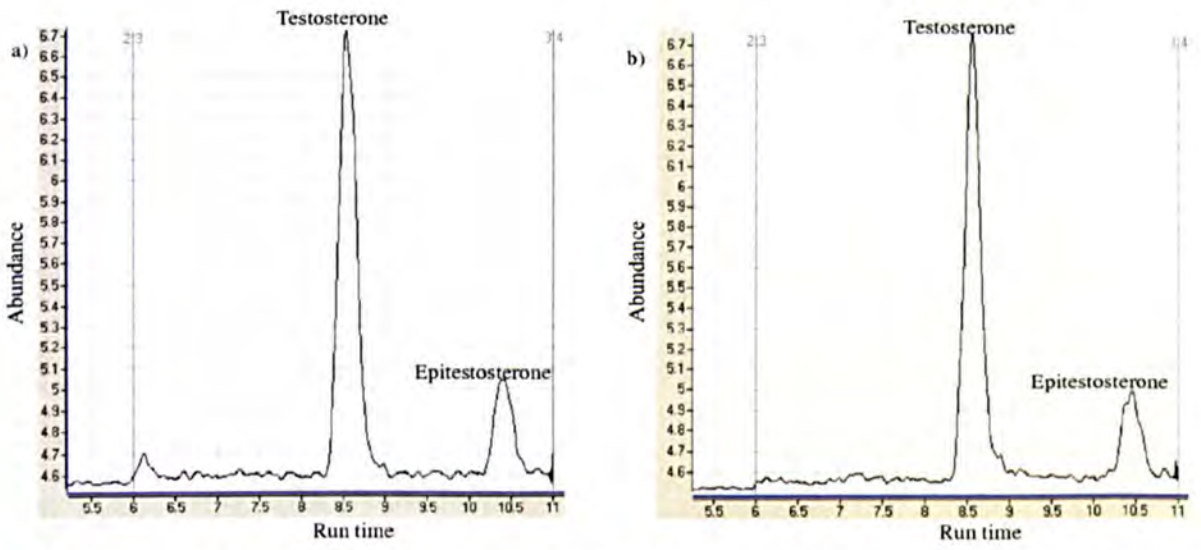

Figure 1: Chromatograms of testosterone and epitestosterone from a) plasma and b) urine at 0.250 ng/mL LLOQ concentration level for epitestosterone.

Matrix	Compound	Concentration (ng/mL)	Level	Precision RSD (%)		Accuracy (%)
				Intra-day N=6	Inter-day N=18	
Urine	Testosterone	0.25	LLOQ	10.01	10.11	107.03
		1.9	Low	2.66	5.51	96.61
		15.6	Medium	4.20	10.29	104.02
		250	High	1.13	3.39	97.11
	Epitestosterone	0.25	LLOQ	1.25	9.56	100.50
		1.9	Low	1.05	4.36	100.39
		15.6	Medium	8.17	8.94	96.45
		250	High	1.67	3.70	102.60
Plasma	Testosterone	0.25	LLOQ	8.14	8.21	109.14
		1.9	Low	3.94	4.26	98.26
		15.6	Medium	5.47	5.92	102.66
		250	High	1.86	2.29	96.55
	Epitestosterone	0.25	LLOQ	2.58	6.87	98.20
		1.9	Low	1.44	2.96	103.01
		15.6	Medium	7.05	6.50	98.29
		250	High	1.82	3.19	100.86

Table 2: Inter-day and intra-day accuracy and precision for the detection of testosterone and epitestosterone in urine and plasma.

Matrix	Compound	Concentration (ng/mL)	% Extraction recovery (N=6)
Plasma	Testosterone	0.25 (LLOQ)	94.49
		1	81.63
		4	85.60
		16	85.98
	Epitestosterone	0.25 (LLOQ)	92.77
		1	89.67
		4	84.72
		16	89.48
Urine	Testosterone	0.25 (LLOQ)	91.23
		1	87.63
		4	88.29
		16	89.16
	Epitestosterone	0.25 (LLOQ)	92.57
		1	91.44
		4	89.60
		16	90.16

Table 3: Absolute extraction recovery results for testosterone and epitestosterone in plasma and urine.

Compound	Concentration (ng/mL)	Extracted plasma (ng/mL) N=6	Extracted urine (ng/mL) N=6	Standard in acetonitrile (ng/mL) N=6
Testosterone	0.25 (LLOQ)	0.248	0.241	0.250
	1	0.977	0.952	0.957
	2	2.019	2.005	2.053
	8	8.089	8.129	8.455
Epitestosterone	1	1.002	1.094	0.992
	2	2.051	1.961	1.977
	8	8.038	8.122	8.110

Table 4: Matrix effect of plasma and urine on spiked testosterone and epitestosterone compared with standard concentrations in acetonitrile.

The results highlighted a variation in urinary testosterone and epitestosterone in rats. Albeit observed in a small sample, there were also variations in the ratios as the majority of rats had values higher than those which have been found in humans shown to be around a 1:1 ratio with variations [16]. A number of factors could be considered for the variations in testosterone and epitestosterone concentrations

Matrix	Compound	Concentration (ng/mL)	Extracted spiked steroid in matrix (ng/mL)	De-conjugated steroid concentration (ng/mL)
Urine	Testosterone	1	0.98	0.91
		2	2.08	2.03
		15	16.96	14.03
	Epitestosterone	1	1.09	1.15
		2	2.11	2.14
		15	16.18	14.85
Plasma	Testosterone	1	1.02	1.09
		2	2.04	2.07
		15	15.81	15.29
	Epitestosterone	1	1.08	0.96
		2	1.97	1.94
		15	15.87	15.06

Table 5: The concentration of testosterone and epitestosterone after de-conjugation by β-glucuronidase compared to standard concentrations of extracted testosterone and epitestosterone in plasma matrix.

Matrix	Animal	Testosterone	Epitestosterone	Ratio
Urine (ng/mL)	1	280.30	75.65	3.71
	2	615.22	160.46	3.83
	3	142.64	48.32	2.95
	4	71.63	16.28	4.40
	5	36.80	86.14	0.42
	6	63.46	125.37	0.51
	Average	201.68 ± 90.16	85.37 ± 21.20	2.64

Table 6: Concentrations of testosterone and epitestosterone in rat urine ± SEM of triplicate injections.

Matrix	Animal	Testosterone	Epitestosterone	Ratio
Serum (ng/ml)	1	386.39 ± 6.268	1.70 ± 0.286	227.89
	2	378.84 ± 17.143	1.64 ± 0.129	230.69
	3	318.52 ± 1.517	1.40 ± 0.025	227.12
	4	381.15 ± 15.214	2.11 ± 0.065	180.26
	5	378.80 ± 8.662	1.55 ± 0.028	244.54
	6	336.70 ± 1.835	2.08 ± 0.063	161.54
	Average	363.40 ± 11.615	1.75 ± 0.118	212.01

Table 7: Serum testosterone and epitestosterone in rats after the fourth week of sample collection ± SEM of triplicate injections.

between individual animals. One factor might be the alterations and differences in metabolic rate of these steroids. Another factor is possibly due to the amount of water consumed by the rats as this can change drug levels in the body. These variations, and the deviation from the ratio typically observed in humans can greatly affect clinical trials thus warrant attention.

Table 7 shows the testosterone and epitestosterone concentrations in the sera of six rats. The average concentration of testosterone was 363.40 ± 11.615 ng/mL and epitestosterone was 1.75 ± 0.118 ng/mL. The average testosterone to epitestosterone ratio was 212.01 (range: 161.54-230.69). The T/E ratio in the serum was a markedly higher than in urine owing to the high testosterone and low epitestosterone concentration in all animals.

These results showed that there was less variation in concentration in the serum samples compared to the urine samples probably due to individual differences in water consumption. Previous methods have been reported but with limitations such as validated applications to either urine [17-20] or serum [21] only; and with an extraction method taking greater than 1 hour [21]. In comparison, the new method benefits from improved resolution [19] and the reduced requirement for

sample volumes [20,21]. This report of a straightforward novel method of rapid analyses of testosterone and epitestosterone in both rat urine and sera benefits from a new rapid liquid-liquid extraction procedure, suitable for both biofluids. Unlike the predecessors, the proposed method streamlines experimental *in vivo* designs where simultaneous monitoring of testosterone and epitestosterone in both urine and blood is required, such as investigating the effect and consequences of altered metabolic function in testosterone and epitestosterone glucuronidation [7-10]. The new method also eliminates potential confounding effects from applying multiple methods to cater for multiple sample matrices.

Conclusions

A method has been described here for the validation and detection of the anabolic steroids testosterone and epitestosterone in rat serum and urine at low ng/mL concentrations using LC-MS/MS. This method shows optimal conditions for the de-conjugation of the steroid glucuronides to detect both free and conjugated steroids. In addition, this method has a good extraction recovery rate for both steroids allowing for accurate concentration determination by the use of the internal standard, stanozolol-D3.

Whilst methods have been described for the detection of testosterone and epitestosterone in humans [17,18], this method provides deconjugation and extraction procedures affording detection of testosterone and epitestosterone concentrations in rat serum and urine. This method could be used in future research to investigate testosterone and epitestosterone metabolism *in vivo*. Furthermore, it could also be extended to detect steroids in human serum and urine samples to confirm urinary levels of these compounds used in sports doping tests. This technique could also be applied to measure metabolism patterns of steroids *in vivo* in place of the current method used by WADA which specifies measurements acquired by gas chromatography tandem mass spectrometry [22]. An advantage is that the LC method could be incorporated into forensic toxicology software to take full advantage of drug testing by LC MS/MS.

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Citation: Jenkinson C, Deshmukh NIK, Shah I, Zachár G, Székely AD, et al. (2014) LC-MS/MS-Based Assay for Free and Deconjugated Testosterone and Epitestosterone in Rat Urine and Serum. J Anal Bioanal Tech S5: 006. doi:10.4172/2155-9872.S5-006

IBIMA Publishing
Advances in Cancer: Research & Treatment
http://www.ibimapublishing.com/journals/ACRT/acrt.html
Vol. 2013 (2013), Article ID 812129, 17 pages
DOI: 10.5171/2013.812129

Research Article

Modulation of UDP Glucuronosyltransferase 2B15 and 2B17 and Prostate Cancer Risk: Current Perspectives

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Received 18 December 2012; Accepted 31 January 2013; Published 17 March 2013

Academic Editor: Perihan Ünak

Abstract

UDP glucuronosyltransferases (UGTs) are a family of enzymes that glucuronidate a variety of exogenous and endogenous compounds, including androgens; which makes them more hydrophilic, changes biological activity, and aids biological excretion. UGT2B17 has been identified as the major enzyme for testosterone glucuronidation, where UGT2B15 has been suggested to play a minor role. A deletion polymorphism in the UGT2B17 gene and an amino acid change in the UGT2B15 gene at codon 85 (aspartate>tyrosine) has been described where the resulting reduced rate of glucuronidation was implicated in the increased risk of prostate cancer. This review aims to analyse the impact that UGT2B15 and UGT2B17 genetic polymorphisms have on the risk of prostate cancer, through the use of studies previously conducted assessing the level of expression of each independent UGT2B enzyme, whereby ethnicity was taken into account. Positive and negative outcomes of clinical studies have been outlined for both UGT2B15 and UGT2B17, where conflicting evidence has led to inconclusive results for determining the consequence of UGT2B enzymes in prostate cancer risk. Additionally, inhibitors of testosterone glucuronidation such as non-steroidal, anti-inflammatory drugs (diclofenac and ibuprofen), tea and red wine extracts have been identified to potentially affect the level of circulating testosterone by inhibiting UGT2B enzymes, thus potentially exacerbating prostate cancer risk. Future trials would involve a wider examination of other food substances and pharmaceuticals that can be attributed to the inhibition of UGT2B enzymes.

Keywords: Prostate Cancer, UDP-Glucuronosyltransferase, UGT2B15, UGT2B17.

Introduction

Prostate cancer *(PCa)* is the most prominent malignancy in western countries formulating one eighth of all male-specific cancers (Park et al., 2006). It is estimated that 75% of all new PCa cases arise in developed countries (Park et al., 2007). According to Crawford (2003), PCa is more prevalent in people of an African

Cite this Article as: Jasmin Thind, Carl Jenkinson, Declan P. Naughton and Andrea Petróczi (2013), "Modulation of UDP Glucuronosyltransferase 2B15 and 2B17 and Prostate Cancer Risk: Current Perspectives," Advances in Cancer: Research & Treatment, Vol. 2013 (2013), Article ID 812129, DOI: 10.5171/2013.812129

American background in comparison to any other ethnic minority. The incidence among African Americans is nearly 60% higher than among whites.

The aetiology of PCa has yet to be identified; however, risk factors such as race, age, family history and steroid hormone levels have been suggested (Gronberg, 2003). Differing androgen levels have been recognised as a potential risk factor for PCa, although results did not generate a coherent picture (Guillemette, 2003). The variation in androgen levels may be the outcome of differing activity or expression of the enzymes that are accountable for metabolism of androgens (Park et al., 2004; 2007).

The aim of this review is to summarise the available epidemiological evidence from the year 2000 onwards, regarding the association between UDP glucuronosyltransferase 2B15 *(UGT2B15)* and UDP glucuronosyltransferase 2B17 *(UGT2B17)* genetic polymorphisms and the risk of developing PCa. Additionally, the difference in the risk of developing PCa will be assessed in individuals of different ethnic backgrounds as well as the genotype expression of each independent enzyme in the prostate. Aside from genetic factors that predispose PCa, external triggers will also be discussed as potential risk factors of PCa. Data evaluated in this review will be from both *in vivo* and *in vitro* studies.

Genetic polymorphisms are associated with a variety of genes that are responsible for coding enzymes that participate in the metabolic activity or detoxification of carcinogens (Erichsen et al., 2008). Polymorphic variants in androgen metabolising enzymes vary the level of androgens and thus affect the risk of developing PCa (Park et al., 2004; Wilson et al., 2004; Gallagher et al., 2007). UDP glucuronosyltransferase *(UGT)* enzymes are a super family of enzymes situated on the internal membrane of the endoplasmic reticulum (Gregory et al., 2004). UGTs participate in a catalytic glucuronidation reaction of a variety of endogenous and exogenous chemicals. In addition to these compounds, steroids, toxins and almost all classes of drugs are substrates for UGT enzymes (Guillemette, 2003). Glucuronidation is one of many fundamental phase 2 reactions also known as a conjugation reaction, which involves the transfer of co-substrate uridine diphospho-glucuronic acid *(UDPGlcUA)* to hydrophobic molecules. This results in the formation of β-D glucopyranosiduronic acid. The carboxyl group from the glucuronic acid which is ionized at physiological pH values promotes the excretion by increasing the aqueous solubility of the glucuronide, which is otherwise highly hydrophobic. The glucuronide is recognised by the bilary and renal organic anion transport systems, which enable excretion into urine and bile (Guillemette, 2003). Glucuronidation acts as a clearance mechanism and detoxification process for such glucuronide-conjugated molecules whereby the more polar, water soluble, less toxic molecules are more readily excreted from the body (Timbrell, 2000; Ritter, 2000; Miners et al., 2004).

Expression and Metabolism of UGT2B Enzymes in the Prostate

The UGT enzymes exist as subfamilies and are grouped on the basis of evolutionary divergence and the sequenced similarity between the subgroups (Gregory et al., 2004). The two most familiar groups of UGTs are UGT 1 and UGT 2, where most known UGTs belong to either the UGT 1A, 2A or 2B sub-family (Mackenzie et al., 2005). Members of the UGT2B sub-family play a vital role in steroid metabolism to inactivate androgens (Turgeon et al., 2001). The enzymes are highly expressed in androgen-sensitive tissues such as the liver, kidney, skin, brain, breast, uterine and prostate (Turgeon et al., 2003). Two of them, UGT2B15 and UGT2B17 are expressed in the prostate which are concerned with the metabolism of androgenic steroids, such as testosterone, androsterone (ADT), androstane -3α-17β diol and dihydrotestosterone (DHT), which have been suggested to modify the risk of developing PCa (Bao et al., 2009). A study of human liver microsomes reported that UGT2B17 was expressed at some 4 fold higher in males with three fold higher activities (Gallagher et al., 2010).

Testosterone, secreted by the testis, is necessary in order to maintain and develop growth of the prostate. Testosterone, either from circulation or from the adrenal precursors, is the main steroid hormone produced in the prostate; it is rapidly converted into DHT and its metabolites by 5α-reductase activity. Testosterone is normally present in serum at a concentration approximately 10 times higher than that of DHT. However, in the prostate due to increased levels of 5α-reductase activity, the proportion of testosterone present is decreased. Such analysis led to the assumption that prostate growth is closely related to the tissue concentration of DHT. Additionally, the adrenals secrete dehydroepiandrosterone (DHEA), its sulphate (DHEAS) and androsteredione into the circulation. The adrenal androgens are converted to testosterone and then to DHT and its metabolites by a 5α-reduction (Barbier and Belanger, 2008).

In the human prostate, the alveoli form two types of epithelial cells: the basal and luminal cells. The two types of cells play an independent but complementary role in androgen formation and action (Barbier et al., 2000). The UGT2B17 isoform, expressed only in basal epithelial cells is concerned with the conversion of testosterone to DHT, which as a result, is metabolised to ADT and 3α-DIOL (Schleutker et al., 2011; Barbier et al., 2000). In contrast, the UGT2B15 isoform is expressed in luminal cells of the prostate where it is concerned with the inactivation of C19 steroid androgens, namely DHT (Olsson et al., 2008; MacLeod et al., 2000; Guillemette, 2003; Barbier et al., 2000). Although UGT2B17 shares more than 95% homology with UGT2B15 (Turgeon et al., 2003; Barbier et al., 2000), the UGT2B17 isoform is found to be the major enzyme implicated in testosterone metabolism within the prostate where it has the highest capacity for both ADT and testosterone (Barbier and Belanger, 2008). Alternatively, UGT2B15 plays a diminutive role (Sten et al., 2009a). Thus, it can be postulated that the intracellular concentration of DHT and its respective metabolites in the prostate can be modulated by both UGT2B17 and UGT2B15 in basal and luminal cells, respectively (Barbier et al., 2000).

According to Park et al., (2004; 2006) the potential mechanism of carcinogenesis of prostate tissue involves the interaction of the androgen receptor when accompanied with DHT. The DNA present in proliferating prostate epithelial cell has the potential to produce permanent genomic mutations resulting in carcinogenic prostatic tissue (Erichsen et al., 2008). Moreover, as both UGT2B15 and UGT2B17 play a role in the degradation of DHT, it is suggested that it is the incomplete inactivation of testosterone conversion to DHT by UGT2B enzymes that may be associated with PCa risk, resulting from increased levels of DHT (Park et al., 2007).

Modulation of UGT2B15 and UGT2B17 Enzymes and the Effect on Androgen Concentration and Activity in Prostate Cancer Lncap Cells

The existence of UGT2B15/B17 in the epithelial cells of the human prostate has been clearly established, where the presence of androstane-3α, 17β-diol (3α-DIOL) and ADT-glucuronide concentrations were observed in this tissue (Chouinard et al., 2007). The human androgen-dependent cancer cell line (LNCaP), expresses UGT2B15/17 and is also competent in conjugating androgens. The impact of both genes in the inactivation of androgens in LNCaP cells was examined by using RNA interference to inhibit their expression (Barbier and Belanger, 2008). The results showed that the glucuronidation of DHT, 3α-DIOL and ADT via LNCaP cell was decreased by more than 75% in UGT2B15/17 siRNA-transfected LNCaP cells in comparison to cells with a non-target probe (Chouinard et al., 2007).

Moreover, the UGT2B15/B17-deficient LNCaP cells were observed to respond more strongly to DHT than in control cells, as exemplified by cell proliferation and presence of known androgen-sensitive genes. Upon evaluation, it is evident that both UGT2B15 and UGT2B17 are key enzymes for the local inactivation of androgens and that glucuronidation is

critical for androgen action in prostate cells (Bao et al., 2009).

Genetic Polymorphism of the UGT2B15 Gene and Deletion of the UGT2B17 Gene

Polymorphisms associated with UGTs are suggested to influence steroid metabolism and cancer susceptibility (Lampe et al., 2000) as a result of a variation in the enzyme activity of the gene (Guillemette, 2003; Park et al., 2004). Polymorphic alleles have been identified in the coding region of the UGTB15 enzyme which results in an alteration at codon 85 from aspartate (D^{85}) to tyrosine (Y^{85}) (Hajdinkjak and Zagradisnik, 2004; Mononen and Schleutker, 2009; Miners et al., 2002; Lampe et al., 2000; Gsur et al., 2002; Guillemette, 2003;Park et al., 2004). Additionally, it was postulated that a homozygous deletion polymorphism of the UGT2B17 enzyme significantly increases the risk of PCa as a consequence of reduced glucuronidation rates (Lazarus et al., 2005).

UGT2B15 Genetic Polymorphism

In a study by MacLeod et al. (2000), a D^{85} to Y^{85} polymorphism was identified in the UGT2B15 enzyme causing a 50% reduction in enzyme activity. This study selected samples from different ethnic backgrounds where, according to Hajdinkjak and Zagradisnik (2004), selection may have only taken into account patients with poorly differentiated cancer, resulting in a poor reflection of the population as a whole. The UGT2B15 D^{85} allele is less proficient at glucuronidating DHT, and it is suggested that the presence of this low activity allele leads to higher intraprostatic DHT concentrations which is concerned with the increased risk of PCa (MacLeod et al., 2000; Park et al., 2004; Okugi et al., 2006; Barbier et al., 2000; Mononen and Schleutker, 2009; Miners et al., 2002). On this basis, people homozygous for the UGT2B15 D^{85} genotype have been suggested to be at a three-fold increased risk of developing PCa (MacLeod et al., 2000). Moreover, genotyping studies have revealed that the homozygous UGT2B15 D^{85} allele is more prevalent in individuals with PCa, where higher levels of DHT were implicated with PCa development (MacLeod et al., 2000; Park et al., 2004; Miners et al., 2002). The identification of the high-risk D/D genotype by Okugi et al. (2006) reinforces the association of this genotype and correlation with PCa. Hajdinkjak and Zagradisnik (2004) similarly highlight the significance of the D/D genotype frequency between the differentiated groups (OR=2.04, 95% CI=1.10-3.79).

Although UGT2B15 D^{85} and UGT2B15 Y^{85} have similar substrate specificities, the UGT2B15 Y^{85} allele was reported to have a two-fold higher Vmax than UGT2B15 D^{85} for C19 steroids (Okugi et al., 2006; Mononen and Schleutker, 2009; Lampe et al., 2000; Hajdinkjak and Zagradisnik, 2004; Turgeon et al., 2001), where the lower enzyme activity has the potential to increase the amount of DHT in prostate tissue as well as to increase exposure to androgens and possibly also increase the risk of PCa (Hajdinkjak and Zagradisnik, 2004). In contrast, Barbier and Belanger (2008) suggested the high efficacy of the Y^{85} enzyme may serve to protect the prostate from increased levels of DHT and consequently decrease the risk.

Furthermore, there is evidence to suggest that genotype distributions vary by ethnic groups. In one study by Lampe et al., (2000), the Y^{85} allele was more prevalent among Caucasians than Asians (32% vs. 18%, respectively). Therefore, we may postulate that PCa risk can also vary according to ethnicity. Additionally, studies have been identified in which the $D^{85}Y$ polymorphism was not associated with risk of developing PCa. A study by Gsur et al. (2002) revealed insignificant differences between cases and controls (UGT2B15 Y^{85}/Y^{85} genotype frequency for PCa cases and controls was 27% and 26%, respectively with an OR, 95% CI; 1.20). However, according to Wilson et al. (2004) the incorporation of recipients with benign prostatic hyperplasia may have negatively influenced the association. In accordance with the negative results outlined by Gsur et al. (2002), Hajdinkjak and Zagradisnik (2004) also found no correlation between PCa cases and controls. However, according

to Hajdinkjak and Zagradisnik (2004), the previous study (Gsur et al., 2002) failed to select a control group that reflected the frequency of alleles in the general population, perhaps confounding the final result.

Although the UGT2B15 allele plays a key role in conjugation and secretion of steroid hormones in the prostate, based on the conflicting evidence above it remains undecided if the UGT2B15 ($D^{85}Y$) polymorphism can be outlined as a marker for the risk of PCa (Table 1).

Ugt2b17 Deletion Polymorphism

Like UGT2B15, the UGT2B17 isoform is significantly associated with its active role in androgen glucuronidation (Gallagher et al., 2007; Chouinard et al., 2007; Turgeon et al., 2001; Barbier et al., 2000). The inter-individual variations of androgen inactivating enzyme activity may be implicated with the risk of developing PCa, as those with diminished enzyme activity were suggested to incur lower androgen levels in comparison to those who maintain normal activity (Schatzl et al., 2003). In contrast, Gallagher et al. (2007) and Park et al. (2007) claim that it is increased serum androgen levels that are associated with such risk. Genetic polymorphisms identified in UGT2B androgen metabolising enzymes were suggested to impact the level of enzymatic activity as well as expression (Park et al., 2006; Wilson et al., 2004; Gallagher et al., 2007). An entire polymorphic gene deletion of the UGT2B17 enzyme was indicated to cause a decrease in the glucuronidation activity (Lazarus et al., 2005), where a homozygous UGT2B17 gene deletion was implicated to be in risk of developing PCa (Gallagher et al., 2007).

Table 1. Summary of *in Vitro* and *in Vivo* Clinical Studies that Show the UGT2B15 Genetic Polymorphism and the Risk of Prostate Cancer

Clinical Study	Sample[a]	Number of cases/controls	Positive[b] or Negative[c] outcome	Findings
ASP85TYR polymorphism in the UDP-glucuronosyltransferase (UGT) 2B15 gene and the risk of prostate cancer (Park et al., 2004)	White Males	155/155	+	The UGT2B15 D^{85} allele exhibited a twofold decrease for DHT, which was indicated association with an increased risk of PCa UGT2B15 mRNA was found in all prostate tissues tested. An increased risk of PCa[d] was established in subjects with the homozygous $UGT2B15^{D85}$ genotype.
An Allele-Specific Polymerase Chain Reaction Method for the Determination of the D85Y Polymorphism in the Human UDP Glucuronosyltransferase 2B15 Gene in a Case-Control Study of Prostate Cancer (MacLeod et al., 2000)	Males	64/64	+	The distribution of the UGT2B15 polymorphism was examined in a small case-control group. The D^{85} to Y^{85} polymorphism was recognized where a 50% reduction in enzyme activity was found. PCa patients were found to be homozygous for the lower activity allele D^{85} UGT2B15 allele in comparison to control individuals (41% versus 19%), respectively. Increased risk of developing cancer in patients homozygous for the D^{85} low-activity allele was threefold higher.
Association of the polymorphisms of genes involved in androgen metabolism and signalling pathways with familial prostate cancer risk in a Japanese population (Okugi et al., 2006)	Japanese Males	102/117	+	Single polymorphisms with the existence of Y alleles showed a significantly lower risk of prostate cancer when compared to the D/D UGT2B15 genotype. The Y85 isoform was also found to be twice as efficient as the D85 in conjugating 3-DIOL and DHT.

Genetic polymorphisms of UDP-glucuronosyltransferases and their functional significance (Miners et al., 2002)	Caucasian Males	27 subjects	N/A[d]	The study stated that the Vmax for DHT glucuronidation by the Asp85 allele is reduced when compared to that for the Tyr85 allele. Those with the homozygous ASP85 allele were suggested to have increased androgen exposure.
A polymorphism in the UDP-Glucuronosyltransferase 2B15 Gene ($D^{85}Y$) Is Not Associated with Prostate Cancer Risk (Gsur et al., 2002)	Caucasian Males	190/190	-	The adjusted odds ratio (OR) for the genotypes were: UGT2B15 (Y^{86}/Y^{85}), UGT2B15 (D^{85}/Y^{85}), UGT2B15 (Y^{85}/Y^{85}) was 1.00, 1.25 and 1.20, respectively. The UGT2B15 ($D^{85}Y$) genotype frequencies showed that there was no association between prostate cancer risk and genotype.
Prostate cancer and polymorphism D85Y in gene for dihydrotestosterone degrading enzyme UGT2B15: Frequency of DD homozygotes increases with gleason score (Hajdinjak T and Zagradisnik B, 2004)	Males	206/178	N/A	Gleason scores were genotyped which enabled the recognition of both alleles in an individual. Controls: 16% DD; 52% DY; Prostate cancer patients: 23% DD, 49% DY. Subgroups of prostate cancer: well differentiated: 11% DD, 37% DY; moderately differentiated: 22% DD, 50% DY; Poorly differentiated: 34% DD, 50% DY. The difference in frequency of DD was significant among the groups. D85Y polymorphism in UGT2B15 corresponds with differentiation of prostate cancer.
Evaluation of genetic variations in the androgen and estrogen metabolic pathways as risk factors for sporadic and familial prostate cancer (Cunningham et al., 2007)	Males	499/493	-	The results in the study were not significant for the Try85Asp polymorphism, adjusted *p* values for multiple comparisons for sporadic prostate cancer cases versus control was 1.00.
Relative enzymatic activity, protein stability and tissue distribution of human steroid metabolizing UGT2B subfamily members (Turgeon et al., 2001)	HK293 Cell Line	N/A	N/A	There was a twofold higher glucuronidation rate of the UGT2B15 (Y^{85}) allele compared to the UGT2B15 (D^{85}) allele. UGT2B15 was shown to have a low activity towards testosterone and a higher activity towards DHT. The study demonstrates the relative activities and major substrates of human steroid-metabolizing UGT2B15 and UGT2B17 enzymes expressed in a *wide variety of steroid target tissue*
Cellular localization of uridine diphosphoglucuronosyltrans-ferase 2B enzymes in the human prostate by in situ hybridization and immunochemistry (Barbier et al., 2000)	Epithelial Cells	N/A	N/A	The $UGT2B15D^{85}$ enzyme was found to be less efficient in glucuronidating DHT, therefore it was proposed that the presence of the low activity allele would result in higher intraprostatic DHT concentration

[a] Sample refers to either a particular ethnicity being analysed or to a certain cell line

[b] A positive clinical study is where the results indicate a clear association between Prostate Cancer risk and UGT2B15 Genetic Polymorphism

[c] A negative clinical study is where the results establish no correlation between Prostate Cancer risk and UGT2B15 Genetic Polymorphism

[d] N/A= Not Applicable. The sub-heading of the column cannot be applied to the clinical study as the results of the study have not outlined conclusive results, or the nature of the study does not allow them to be applied, i.e. cell culture studies will not have cases or controls

Park et al. (2006) examined the prevalence of the UGT2B17 deletion polymorphism in Caucasians and African Americans with propensity to PCa risk. A strong association between UGT2B17 deletion polymorphism and the incidence of PCa was established. Stratified analysis by ethnicity exposed an increase in PCa risk amongst Caucasians with UGT2B17 deletion polymorphism, whereas the risk for African Americans

remained statistically insignificant (Table 2). However, based on this study it is unjustified to assume that those of the African American background are not susceptible to PCa. Additional population based studies in men of African American background would need to be carried out, where only by obtaining similar statistically insignificant results would a generalisation regarding the risk of PCa be of validity.

According to Lampe et al. (2000), polymorphisms often show distinctive population differences which result in different distributions amongst various ethnic groups. Wilson et al. (2004) demonstrated that the absence of the UGT2B17 gene is five times more frequent in Caucasians than in African Americans; 11% and 2%, respectively. The occurrence of the del/del genotype was also 11% with formerly reported genotype distribution amongst Caucasians (Park et al., 2006; Gallagher et al., 2007; Olsson et al., 2008). In addition, Jakobbson et al. (2006) demonstrated the UGT2B17 del/del polymorphism to be seven times more prevalent in the Korean (66.7%) than the Swedish population (9.3%), where Terakura et al. (2005) also reported higher prevalence among Asian men. The difference in genotype frequencies amongst the populations can be used to assume that the risk of PCa varies between ethnic minorities.

Table 2. Summary of *in Vivo* Clinical Studies that Show the UGT2B17 Genetic Polymorphism and the Risk of Prostate Cancer

Clinical Study	Sample[a]	Number of cases/controls	Positive[b] Or Negative[c] outcome	Findings
The UGT2B17 gene deletion polymorphism and risk of prostate cancer: A case-control study in Caucasians (Gallagher et al., 2007)	Caucasian Males	411/397	-	Using a novel real time PCR method was employed to differentiate between heterozygous (+/0) and homozygous (+/+) UGT2B17 genotypes. The study illustrated no distinctive difference in the occurrence of the UGT2B17 deletion genotype (0/0) amongst prostate cancer cases (10%) and controls (12%). Inconsistency in results may have arisen from a down-regulation of UGT2B17 transcription via androgens which are not modulated by androgens. The study found deletion of UGT2B17 to have little effect on glucuronidation rates and thus little impact on risk of PCa.
The UGT2B17 gene deletion is not associated with prostate cancer risk (Olsson et al., 2008)	Swedish Males	3,161/ 2,149	-	A cancer of the prostate Sweden (CAPS) population-based case-control study. A genotyping analysis which comprised of a 5o-nuclease activity assay was used to establish those with either one or two gene copies (ins/ins or ins/del) from individuals homozygous for the deletion (del/del) allele. The study found no significant association between the incidence

				of prostate cancer and deletion of the UGT2B17 gene for individuals who are UGT2B17 del/del carriers. (OR[d]=1.01, 95% CI 0.83-1.23).
Association Between Polymorphisms in HSD3B1 and UGT2B17 and Prostate Cancer Risk (Park et al., 2007)	White and African Americans Males	356/363	+	A significant association was found between the UGT2B17 null polymorphism and prostate cancer (OR= 3.0, 95% CI 1.0-9.2).
Characterization of a common deletion polymorphism of the UGT2B17 gene linked to UGT2B15 (Wilson et al., 2004)	Caucasians and African Americans Males	85 African Americans and 103 Caucasians	N/A[e]	The study screened participants for markers C and J, which specifically recognised the presence or absence of the UGT2B17 gene. DNA spanning the UGT2B17 gene is absent in 9 out of the 40 human DNA samples, while all samples contained the UGT2B15 gene. The absence of the UGT2B17 gene was more prevalent in Caucasians than African Americans (11% and 2%, respectively).
Deletion Polymorphism of UDP-Glucuronosyltransferase 2B17 and Risk of Prostate Cancer in African American and Caucasian Men (Park et al., 2006)	Caucasians and African Americans Males	420/487	+	The frequency of UGT2B17 distribution amid the Caucasian and African American ethnicities was 11% and 12%, respectively. An OR, of 1.7; 95% CI, 1.2-2.5 suggested an increased risk of prostate cancer for individuals with at least one UGTB17 allele. An increased risk of prostate cancer was observed amid Caucasian individuals with an OR, of 1.8; 95% CI, 1.2-2.8. The risk of prostate cancer among African Americans remained statistically insignificant with an OR, of 1.5; 95% CI, 0.7-3.1.
A UGT2B17-positive donor is a risk factor for higher transplant-related mortality and lower survival after bone marrow transplantation (Terakura et al., 2005)	Japanese Males	435 Subjects	N/A	Homozygous UGT2B17 deletion was observed in 85% of normal Japanese donors, which was found to be higher than that (11%) observed in normal White donors.
Deletion polymorphism of the UGT2B17 gene is associated with increased risk of prostate cancer and correlated to gene expression in the prostate (Karypidis et al., 2008)	Swedish Males	176/161	+	The study found that individuals homozygous for the insertion allele expressed 30 times more UGT2B17 mRNA in prostate tissue than the heterozygote's Carriers of the deletion had a significant increase in the risk of prostate cancer (OR=2.07; 95%CI=1.32-3.35). The study concluded that the UGT2B17 deletion polymorphism

				is associated with risk of prostate cancer.
Large Differences in Testosterone Excretion in Korean and Swedish Men are Strongly Associated with a UDP-Glucuronosyl Transferase 2B17 Polymorphism (Jakobsson et al., 2006)	Swedish and Korean Males	122 Swedes and 74 Koreans	N/A	Urine from subjects was analyzed for several androgen glucuronides, including *testosterone*. When the UGT2B17 genotypes were compared with urinary testosterone levels, all of the individuals of the UGT2B17 homozygous deletion/deletion genotype had little or negligible amounts of urinary testosterone. The deletion/deletion genotype was seven times more prevalent in the Korean (66.7%) than the Swedish population (9.3%).

[a] Sample refers to either a particular ethnicity being analysed or to a certain cell line

[b] A positive clinical study is where the results indicate a clear association between Prostate Cancer risk and UGT2B15 Genetic Polymorphism

[c] A negative clinical study is where the results establish no correlation between Prostate Cancer risk and UGT2B15 Genetic Polymorphism

[d] OR=odds ratio; CI= Confidence Interval

[e] N/A= Not Applicable. The sub-heading of the column cannot be applied to the clinical study as the results of the study have not outlined conclusive results or the nature of the study does not allow them to be applied, i.e. cell culture studies will not have cases or controls

Although the variation in the distribution of the UGT2B17 deletion polymorphism has been established, the results have not been consistent. For instance, the genotype distribution for African Americans in the study conducted by Park et al. (2006) was 11%, where Wilson et al. (2004) demonstrated an occurrence of a mere 2%. Such differences observed illustrate that UGT2B17 genotype frequency has the potential to vary in different regions of the United States. However, it also reinforces the need for additional studies to be conducted in order to confirm any potential trends which may lead to conclusive results regarding the risk of PCa amongst different ethnic minorities. Based on the findings of Wilson et al. (2004) and Park et al. (2006), it can be posited that Caucasians are at a greater risk of developing PCa when compared to the African American population in the studies. However, this generalisation from limited studies cannot be applied to the Caucasian population as a whole; as additional studies would need to confirm the greater risk of PCa in Caucasian males and in contrast to other ethnic groups.

A study carried out by Jakobsson et al. (2006) examined the effect of the UGT2B17 deletion polymorphism on the distribution and excretion of urinary testosterone. It was maintained that the deletion of the UGT2B17 gene is linked with low or negligible amounts of urinary testosterone, reiterating the importance of the enzyme for testosterone glucuronidation. Additionally, it was found that individuals who exhibit the del/del genotype undergo decreased levels of testosterone glucuronidation. PCa risk was previously implicated with reduced testosterone glucuronidation (Park et al., 2006), thus it can be assumed individuals who display the del/del genotype are at an increased risk. In contrast, other studies have shown the lack of association between UGT2B17 deletion polymorphism and PCa risk (Table 2).

In summary, there appears to be contradictory evidence surrounding the role of the UGT2B17 deletion polymorphism in PCa risk. Multiple studies that examine the role of the glucuronidating enzyme are required in order to produce conclusive results. Table 2 outlines in further detail the studies already discussed, as well as additional studies examining the UGT2B17 deletion polymorphism and risk of PCa. Both UGT2B15 and UGT2B17 bind testosterone and metabolites of 5-α reductase, where expression in steroid target tissues such as the prostate suggests a function in PCa (Wilson et al., 2004). Although both UGT2B15 and UGT2B17 have been identified with different genetic polymorphisms; they are both considered to be important in human liver microsomal (HLM) testosterone glucuronidation (Bowalgaha et al., 2007), where clinical studies have shown the two enzymes to independently effect intra-prostatic levels of DHT.

Factors Influencing the Metabolic Functions of UGT2B15 and UGT2B17

To further examine the consequence of UGT2B15 and UGT2B17 genetic polymorphism and the risk of PCa, it is important to identify other potential risk factors, such as diet, lifestyle and additional drugs that could impact the metabolism of such enzymes. Reduction in testosterone glucuronidation via the inhibition of UGT2B enzymes has been associated with elevated levels of circulating testosterone (Jacobsson et al., 2006; Sten et al., 2009b; Jenkinson et al., 2012a, 2012b), which was previously implicated in PCa risk (Park et al., 2007). Notably, UGT2B15 and UGT2B17 do not glucuronidate epitestosterone which are metabolised by alternate UGT enzymes (Sten et al., 2009a). Indeed, epitestosterone acts as a competitive inhibitor against UGT2B17, implicating a role for the known variations in epitetosterone levels in controlling testosterone concentrations (Sten et al., 2009a). However, further studies are warranted in this area as levels of epitestosterone that effected inhibition are considerably higher than those found in vivo (Havlikova et al., 2002).

Non-Steroidal Anti-Inflammatory Drugs (NSAIDS) Possibly Associated with Increased Risk

NSAIDs, such as ibuprofen and diclofenac, are commonly used drugs used in the prophylaxis of pain and inflammation and are metabolised by UGTs through glucuronidation. Both steroids and NSAIDs are substrates for UGT enzymes and it has been revealed that diclofenac and ibuprofen competitively inhibit UGT2B15 and UGT2B17 potentialy resulting in a reduced testosterone glucuronidation rate with an IC_{50} value for diclofenac inhibition of 64 ?M, and 213 ?M for ibuprofen (Sten et al., 2009b). UGT2B15 was more sensitive than UGT2B17 to the two drugs, particularly to ibuprofen (Sten et al., 2009b). Moreover, an increased sensitivity to ibuprofen signified that UGT2B15 is mostly responsible for testosterone glucuronidation in UGT2B17- deficient individuals. The inhibitory efficacy of ibuprofen was found to be reliant on the UGT2B17 genotype, where higher inhibition was illustrated in recipients with the del/del genotype compared to those with ins/ins and ins/del (Sten et al., 2009b). However, these results are for in vitro studies which have yet to be repeated in vivo.

Therefore, based on the inhibitory efficacy of both diclofenac and ibuprofen for HLM testosterone glucuronidation, the researchers can speculate that individuals who exhibit the UGT2B17 del/del polymorphism may be at a greater risk of developing PCa, as a result of the higher NSAID inhibition demonstrated in del/del subjects. However, the potential risk of PCa would only apply to those patients who are taking NSAIDs as a long-term prophylaxis. In order to evaluate the practical repercussions of such findings, future investigations are warranted to be conducted *in vivo,* where studies to examine the inhibitory effect of NSAIDs as well as UGT2B enzymes could be carried out. It would also be an advantage for future studies to be done in the context of PCa susceptibility to see if similar results could be obtained to the previous study by Sten et al. (2009b).

Diet and Risk of Prostate Cancer

To date, many studies have identified the effect of UGT2B genetic polymorphisms on the incidence of PCa risk. However, little has been investigated about the effect diet has on such UGT2B enzymes (Wolk, 2005). This is despite the numerous studies that testify to the high rate of self-medication with herbal and dietary supplements, particularly among cancer patients (Neuhouser and Rock, 2010). Although observational studies across cultures suggest that some dietary components and associated nutrients may have an effect on PCa risk (Lewis et al., 2009; Brasky et al., 2011; Ma and Chapman, 2009), caution is advised in promoting dietary supplements for said purpose, whilst concerns have been raised over potential herbal-drug interaction (Eichorn et al., 2011). Amongst the dietary components with putative prostate cancer preventive, teas (particularly green teas) received increased attention, but evidence for cancer preventive effects of dietary flavonoids such as those found in green tea is inconclusive (Boehm et al., 2009).

Dietary components such as flavonoids found in foods, such as fruits and vegetables have been suggested to have the ability to act as cancer preventative agents (Moon et al., 2006). Wolk (2005) suggested that diet plays a significant role in the initiation, promotion and progression of PCa.

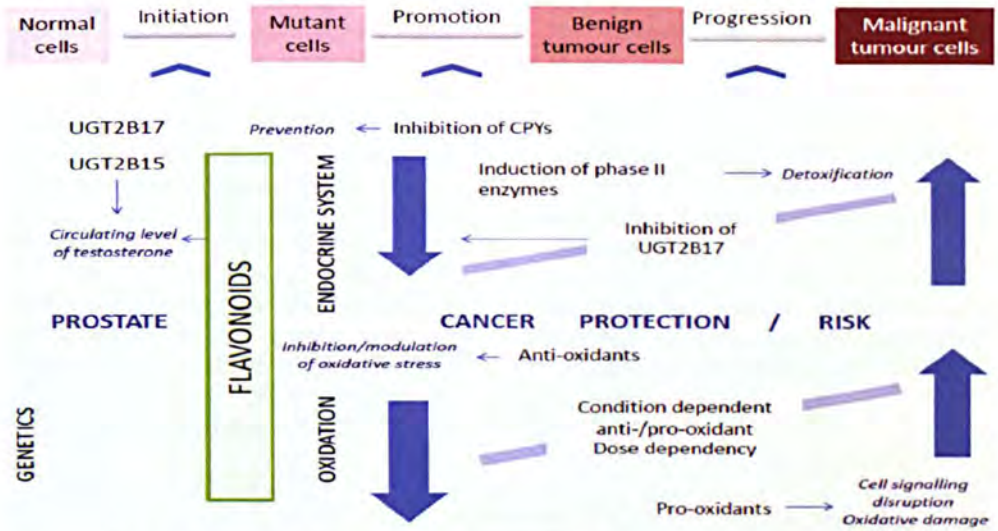

Figure 1: Multiple roles of flavonoids in the initiation and progression of stages of cancer development: UGT inhibition may affect toxin and endocrine levels including testosterone; while amelioration or augmentation of oxidative damage may occur in a dose dependent manner [adapted from Moon et al., 2006].

Figure 1 illustrates the stages at which dietary flavonoids are able to effectively block or suppress progression to carcinogenesis. Such flavonoids initiate the detoxification of carcinogens via the induction of phase 2 enzymes, resulting in their elimination from the body (Moon et al., 2006).

Advancing on this, a recent study by Jenkinson et al. (2012a) demonstrated that testosterone glucuronidation was inhibited by green and white tea extracts, alongside particular catechin compounds such as: epicatechin, epigallocatechin gallate and catechin gallate. Such compounds have been recognized as competitive inhibitors of the UGT2B17 enzyme through in vitro studies. Given the concentrations of catechins and testosterone used for these studies reflect those found in vivo, further studies in humans are warranted. Remarkably, the compounds identified as inhibitors were not substrates of the UGT2B17 enzyme, hence providing the potential to investigate the effect of other food substances containing catechins. Popular alcohol beverages such as red wine or cider are two of many substances containing catechins that could be an area for future investigation (Jenkinson et al., 2012b). Previous studies carried out by Park et al. (2007) confirmed the association between increased levels of circulating androgens and the risk of PCa. On this basis, the potential association between PCa risk and diet can also be suggested from the study carried out by Jenkinson et al. (2012a), whereby the inhibitory effects exerted by components of white and green tea extracts on the UGT2B17 enzymes, and the resulting potential increase in circulating testosterone levels is an indicator for the possible risk of PCa; however this is yet to be confirmed in vivo. Such assumptions warrant additional studies examining foodstuffs and their components for the inhibitory role against UGT enzymes in the perspective of PCa. The multiple roles of flavonoids in promoting cancer include contrasting effects on the tissue activity of detoxification enzymes, such as UGTs via upregulation or inhibition.

Athletes as More 'At Risk' Population

Studies investigating the consequences of modulated UGT2B15 and UGT2B17 activity to date have focused on linking PCa to androgen levels, as well as highlighting potential implications for the athletic

community (Figure 2), mainly involving competitive athletes and bodybuilders. Following the work by Schulze et al., (2008), concerns regarding the accuracy of the current doping testing for testosterone were raised and alternative approaches have been suggested (Kicman and Cowan, 2009; Deshmukh et al., 2010; 2012). More importantly from the public health point of view, athletes who are willing to manipulate the UGT2B enzyme activities in order to evade doping testing may put themselves in health risk beyond the currently know side effects of anabolic steroids (van Amsterdam et al., 2010). In addition to potential renal consequences for testosterone or synthetic anabolic steroid users with altered UGT2B17 activity (Deshmukh et al., 2010), the modulated UGT2B activity may also put sportsmen using testosterone boost to enhance their athletic performance or physical appearance at increased risk for developing PCa. Potential health-compromising consequences of testosterone and/or synthetic steroid doping may be further aggravated by habitual use of NSAIDS, diet and lifestyle choices.

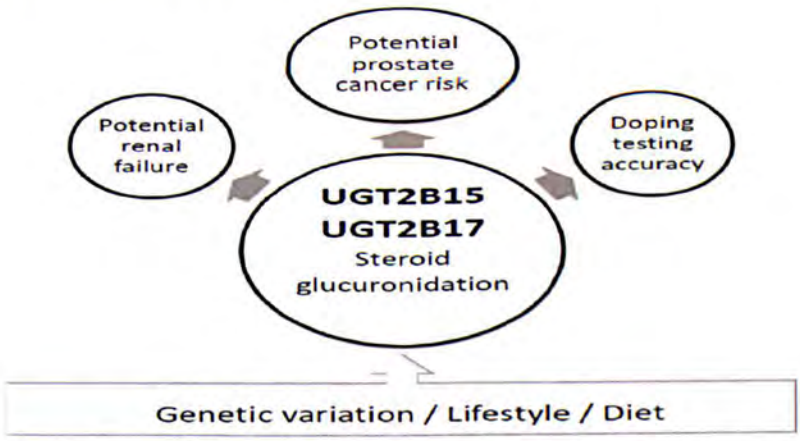

Figure 2: Potential implications of modulated UGT2B15 and UGT2B17 activity.

Conclusions and Future Work

In this review, the genetic polymorphisms of the UGT2B15 and UGT2B17 enzymes with respect to developing PCa have been discussed as well as the inhibitory effects exerted via UGT2B's on NSAIDs and dietary components. Both the UGT2B15 and UGT2B17 are expressed in the prostate where alterations in the activity may cause fluctuations in the concentration of active androgen levels present. Clinical data obtained during the last decade demonstrated that a reduction in testosterone glucuronidation by genetic alterations of their activity causes an increase in circulating levels of testosterone which has been associated with an increased risk of PCa. Conversely, a reduction in circulating androgen levels was also observed to be implicated with an increased risk. Thus based on the contradictory evidence accumulated, the consequence of UGT2B15 and UGT2B17 genetic polymorphisms remains inconclusive. In addition, a significant difference between the different ethnic minorities in the distribution of these polymorphisms was observed. Whether such genotypic differences constitute a greater risk of PCa amongst the ethnicities is yet to be determined. Furthermore, the assessment of the inhibitory effects of NSAIDs (diclofenac and ibuprofen) and foodstuffs brings to light the potential external influences that can affect the risk of PCa. However, the studies that confirmed such inhibitory effects were not carried out in the context of PCa. This creates a potential for future research where the effects of such inhibitory components on UGT2B enzymes can be assessed with regards to the risk of PCa.

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