Development and Application of Novel Methods for the Detection of Anabolic Androgenic Steroids in Hair and Other Matrices Using Liquid Chromatography Tandem Mass Spectrometry

Submitted in partial fulfilment for the degree of Doctor of Philosophy

By

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ABSTRACT

Owing to an increase in the number of doping cases with performance enhancing drugs (PEDs), mainly anabolic androgenic steroids (AASs), and the limitations of urinalysis and self-reports, there is an ever increasing need to develop new methods for detecting doping. This thesis reports the development and application of a series of novel analytical methods for the detection of frequently used AASs in hair and other matrices using liquid-chromatography tandem mass-spectrometry (LC-MS/MS).

Assays capable of detecting 0.5 pg/mg stanozolol and 3.0 pg/mg nandrolone (with 20 mg hair) were developed. Hair samples from 180 subjects (108 males, 72 females) were screened using ELISA, which revealed 16 subjects to be positive for stanozolol and 3 for nandrolone. LC-MS/MS analysis confirmed that only 11 subjects were positive for stanozolol (5.0 pg/mg to 86.3 pg/mg) and just 1 for nandrolone (14.0 pg/mg), thus showing the inaccuracy of using ELISA screening alone. The analytical findings were successfully employed to verify self-reported drug use data.

An assay capable of detecting 0.1 pg/mg testosterone (T) and 0.25 pg/mg epitestosterone (E) (with 50 mg hair) was developed. Seventy-five hair samples were collected from healthy volunteers (49 males, 26 females), with the natural levels of T 0.7-11.81 pg/mg and 0.33-6.05 pg/mg and the natural levels of E 0.63-8.27 pg/mg and 0.52-3.88 pg/mg, in males and in females respectively. This thesis reports for the first time the T/E ratio in hair, 0.5-3.37 in males and 0.56-1.81 in females.

Assays capable of detecting 0.125 pg/mg stanozolol/0.25 pg/mg 3'-hydroxystano-zolol (with 50 mg rat hair) and 0.063 ng/mL stanozolol/0.125 ng/mL 3'-hydroxystanozolol (with 100 μL of rat urine or serum) were developed. Diclofenac was found to significantly reduce the urinary excretion of 3'-hydroxystanozolol, but did not influence the concentration of both compounds in hair. This is the first *in vivo* study to report this effect.

Assays capable of detecting 0.25 pg/mL stanozolol and 0.5 pg/mL of 3'-hydroxystanozolol in 5 mL aqueous matrices were developed in order to investigate environmental contamination. Three out of six samples from the River Danube, collected from December '09 to November '10, were found to contain stanozolol, (upto 1.82 pg/mL). In contrast, stanozolol was detected only once (1.19 pg/mL) in tap water from Budapest city.

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DECLARATION

This thesis is based upon work conducted by the author in the **School of Pharmacy and Chemistry at Kingston University London** between March 2009 and February 2013. All of the work described herein is original unless otherwise acknowledged in the text or by references. None of the work has been submitted for another degree in this or any other universities. Part of this work has been published in the scientific literature.

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1. **Deshmukh N,** Hussain I, Barker J, Petroczi A, Naughton DP. Analysis of anabolic steroids in human hair using LC-MS/MS. *Steroids* 2010, 75(10):710-714.

2. **Deshmukh N,** Petroczi A, Barker J, Szekely AD, Hussain I, Naughton DP. Potentially harmful advantage to athletes: a putative connection between UGT2B17 gene deletion polymorphism and renal disorders with prolonged use of anabolic androgenic steroids. *Substance Abuse Treatment, Prevention, and Policy* 2010, 5:7, http://dx.doi.org/ 10.1186/1747-597X-5-7.

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LIST OF ABBREVIATIONS

3α-HSD	3α-hydroxysteroid dehydrogenase
5α-DHT	5α-dihydrotestosterone
17β-HSD	17β-hydroxysteroid dehydrogenase
19-NA	19-norandrosterone
19-NE	19-noretiocholanolone
AAF(s)	Adverse analytical finding(s)
AAS(s)	Anabolic androgenic steroid(s)
AKI	Acute kidney injury
BUN	Blood urine nitrogen
CID	Collision induced dissociation
COPD	Chronic obstructive pulmonary disease
DNA	Deoxyribonucleic acid
E	Epitestosterone
EG	Epitestosterone glucuronide
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
EPO	Erythropoietin
ESI	Electrospray ionisation
eV	Electron voltage
FDA	Food and drug administration
FSGS	Focal segmental glomerulosclerosis
FSH	Follicle-stimulating hormone
GC-HRMS	Gas chromatography high resolution mass spectrometry
GC-IRMS	Gas chromatography-isotope ratio mass spectrometry
GC-MS	Gas chromatography mass spectrometry

GC-MS/MS	Gas chromatography tandem mass spectrometry
HDL	High-density lipoprotein
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
IM	Intramuscular
IP	Intraperitoneal
IS(s)	Internal standard(s)
LC-MS	Liquid chromatography mass spectrometry
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LDL	Low-density lipoprotein
LH	Luteinising hormone
LLE	Liquid-liquid extraction
LLOD	Lower limit of detection
LLOQ	Lower limit of quantification
MEC	Mixed ethylcellulose
µg/mL	Microgram per millilitre
µL	Microlitre
mg/mL	Milligram per millilitre
MRM	Multiple reaction monitoring
ng/mL	Nanogram per millilitre
NHS	National health service
NSAID(s)	Non-steroidal anti-inflammatory drug(s)
OTC	Over-the-counter
PED(s)	Performance enhancing drug(s)
PET	Polyethylene teraphthalate
pg/mg	Picogram per milligram
PTFE	Poly tetra fluoro ethylene
QC	Quality control

r^2	Determination coefficient
RSD	Relative standard deviation
RNA	Ribonucleic acid
S/N	Signal to noise ratio
SPE	Solid phase extraction
SRM	Selective reaction monitoring
T	Testosterone
TG	Testosterone glucuronide
TMB	3, 3', 5, 5' tetramethylbenzidine
UDPGA	Uridine diphospho-glucuronic acid
UGT(s)	Uridine diphosphate-glucuronosyltransferase(s)
V	Voltage
WADA	World Anti-Doping Agency

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CHAPTER 1

INTRODUCTION

1.1. ANABOLIC ANDROGENIC STEROIDS (AASs)

Anabolic androgenic steroids (AASs) are synthetic derivatives of testosterone [1, 2], which is an endogenously produced male sex hormone capable of enhancing muscle growth (anabolic effect) and developing secondary masculine characteristics (androgenic effect) [3-5]. It has been claimed that the use of AASs increases lean body mass, strength and aggressiveness that may be beneficial for enhancing performances in sports. As a result of this, the use of AASs is officially banned since the mid-1970s by the sport authorities [4].

According to 'The 2013 Prohibited List' of the World Anti-Doping Agency (WADA) code, AASs belong to Class S1.1 and their use in- and out-of-competition is prohibited [6]. Despite the restrictions, AASs are commonly misused as performance enhancers by athletes, non-athletes, adolescents and recreational/competitive body-builders, as revealed by numerous adverse analytical findings (AAFs) during the past few years [7-13]. An AAF is a report from accredited laboratories which identifies the presence of prohibited substance (or its metabolite or markers) in a sample or which provides the evidence of use of prohibited substances or methods. According to the WADA report for the year 2011, AASs comprised of 59.4 % of all AAFs, and overall there is an increase in the proportion of positive results of AASs since 2005 (Figure 1.1).

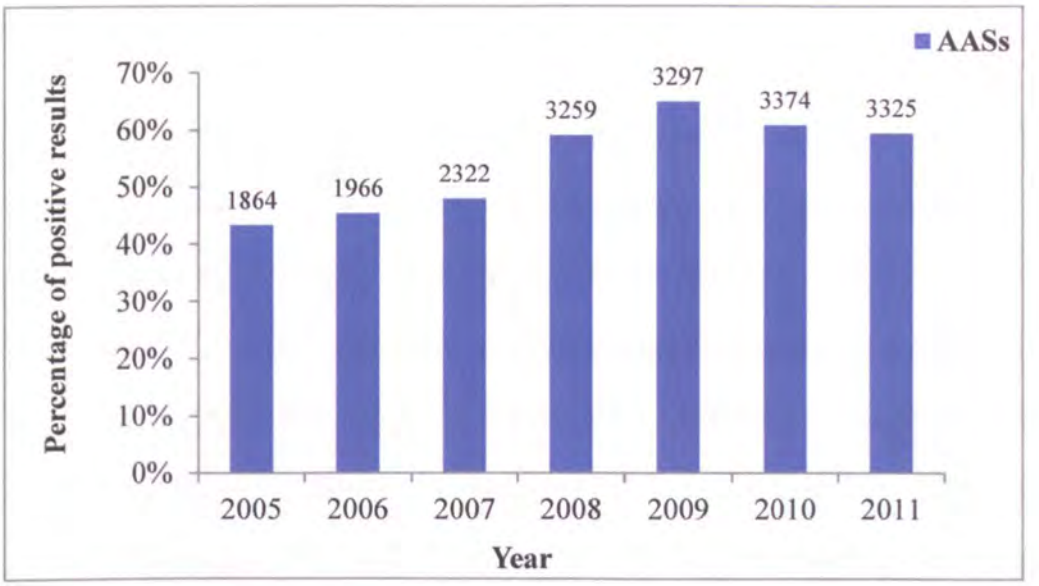

Figure 1. 1: Percentage of positive results of AASs amongst all AAFs (from year 2005 to 2011). Numbers above the bars represents the number of positive results of AASs reported per year [7-13]

Testosterone, stanozolol and nandrolone are three of the frequently abused AASs [7-13], where nandrolone and stanozolol are structural derivatives of testosterone (Figure 1.2) [4]. Nandrolone was synthesised for the first time by Birch in 1950 and by Wilds and Nelson in 1953 [14], whereas stanozolol was first synthesised in 1959 by Clinton *et al* [15].

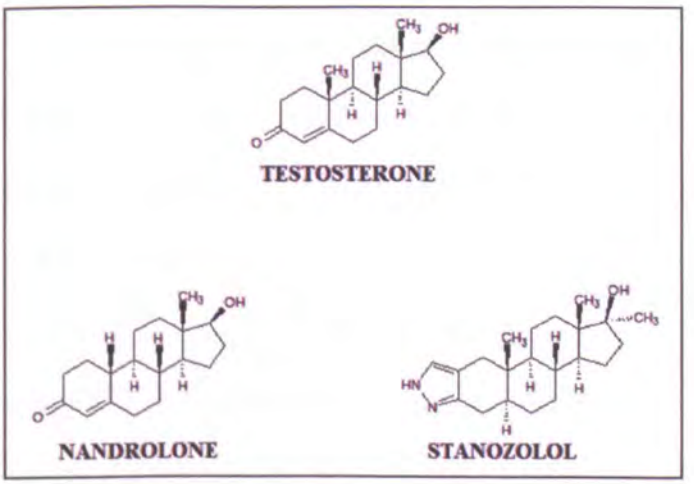

Figure 1. 2: Structures of testosterone, nandrolone and stanozolol

1.2. MECHANISM OF ACTION OF AASs

It has been reported that AASs induce enlargement of skeletal muscles by facilitating the hypertrophy of muscle fibres and stimulating muscle protein synthesis. However, the exact mechanism by which AASs stimulate muscle growth is not well understood. Generally, AASs act by binding to the intra-cellular androgen receptors located in skeletal muscles (satellite cells). AASs can either bind directly to androgen receptors or get converted to a more active compound first and then bind to receptors to form steroid-receptor complex in the cell nucleus [1, 3, 5, 16]. The resulting steroid-receptor complex interacts with RNA and DNA to stimulate protein synthesis [1, 17]. This is an important mechanism by which AASs can enhance the size of skeletal muscles [18]. However, some AASs like stanozolol have weak affinity to bind with androgen receptors and are thus weak androgens. Also there are some AASs like oxymetholone, which do not bind to the androgen receptors at all. Such AASs normally act after biotransformation to a more active compound or via alternative mechanism. Thus, the mechanism of action of AASs may differ between compounds owing to variations in the steroid molecule and affinity to androgen receptors.

The mononucleated myogenic cells called satellite cells, located between the basement membrane and sarcolemma of skeletal muscle fibres (Figure 1.3), contain androgen receptors [19-21]. Hence, satellite cells are generally considered to be targets of AASs for stimulating muscle growth. It has been observed that AASs induce a rapid proliferative response in satellite cells [20, 22-25]. At this stage, satellite cells are often referred to as myogenic precursor cells or myoblasts [24]. The majority of myoblasts start to differentiate into post-mitotic myotubes that eventually fuse with existing muscle fibres (Figure 1.3). This results in the hypertrophy of muscle fibres. Also, to a lesser extent, some of the myoblasts can fuse together to form new muscle fibres [21, 26, 27].

Adult muscle fibres contain several myonuclei, where each myonucleus sustains protein synthesis. AAS induced muscle fibre hypertrophy is known to be associated with incorporation of new myonuclei into the existing muscle fibre, which results in an increase in the myonuclei number. When the transcriptional activity of existing myonuclei reaches maximum and they are no longer able to sustain protein synthesis, the enhancement of number of myonuclei has been reported to get involved in protein synthesis. This is an important pathway by which satellite cells mediate the myotrophic action of AASs on skeletal muscles [22, 23, 28, 29].

A few myoblasts can escape differentiation and can return to quiescence [27]. This ultimately leads to the generation of new satellite cells, which replenish the satellite cell reserve pool in the skeletal muscle [23]. Satellite cells are normally quiescent and can get activated in response to a wide range of events such as resistance exercise, injury, androgens, overuse and denervation, to enter the cell cycle and thus to regenerate functional muscle fibres [21, 22, 29-31]. This indicates that satellite cells play a vital role in the repair, maintenance and post-natal growth of skeletal muscles (according to the demands of human body) [32]. Thus, AAS induced enhancement of satellite cell pool has important clinical implications.

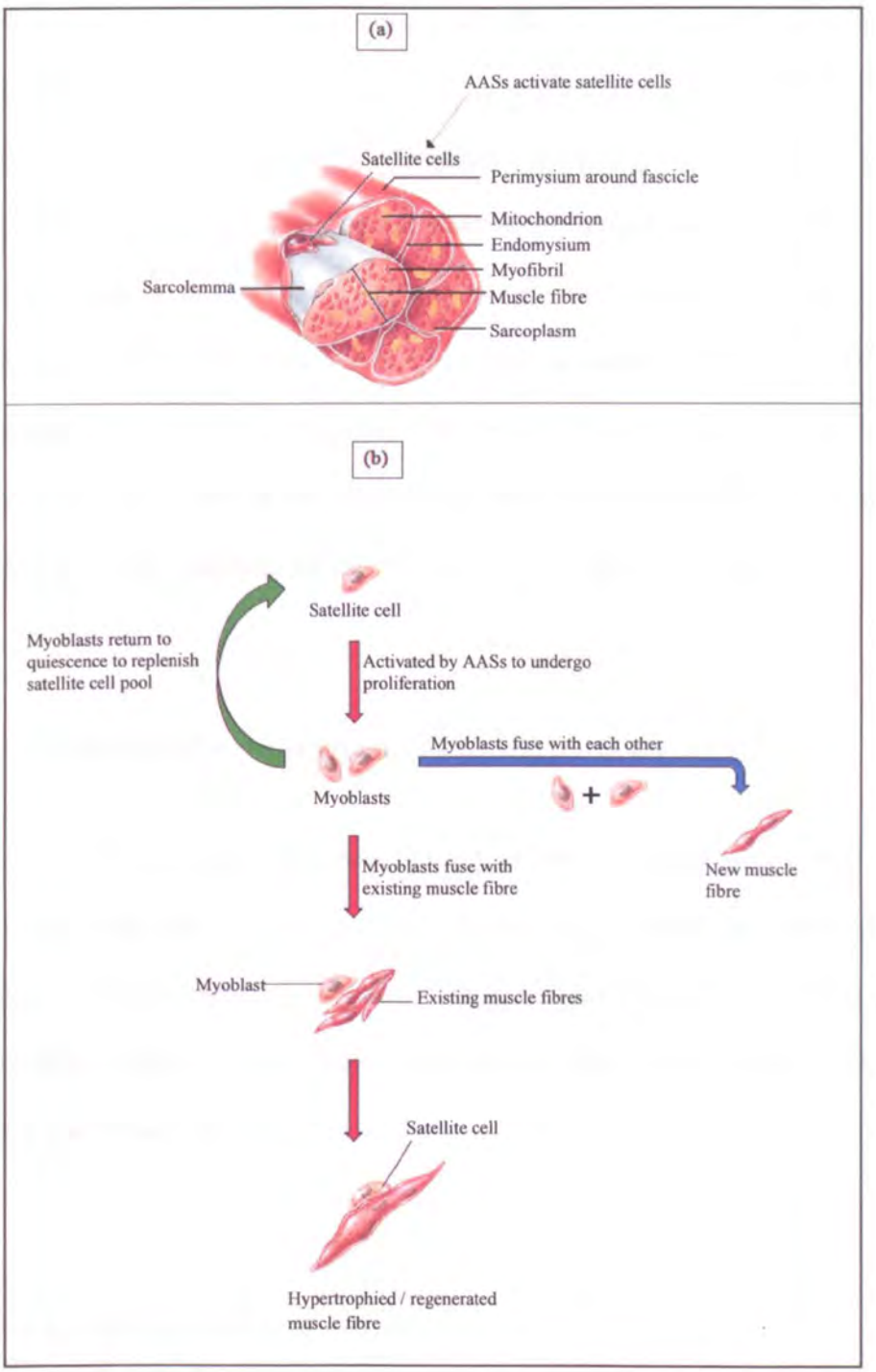

Figure 1. 3: (a) Microscopic organisation of skeletal muscle (b) Predicted mechanism by which AASs can induce enlargement of skeletal muscles [21, 24]

AASs have also been reported to induce an up-regulation of androgen receptors in satellite cells, which could also be an important mechanism by which AASs exert their effect on muscles [24, 33]. Muscular hypertrophy is associated with an increase in the production of myofibrils, the contractile element of skeletal muscles. Owing to this, hypertrophied muscles are capable of more forceful contractions [16, 21]. Intake of AASs supplemented with exercise can have an additive effect in stimulating muscle growth [16, 17]. This enlargement of skeletal muscles increases lean body mass and strength, which can be beneficial for enhancing the performance of sportsmen participating in body-building, weight-lifting and other sports activities.

1.3. OTHER PHARMACOLOGICAL ACTIONS OF AASs

The most prevelant reason for sportsmen initiating AAS abuse is to promote muscle mass and strength. However, in addition to stimulating muscle growth by interacting with androgen receptors (in skeletal muscles and other parts of body), AASs are capable of exerting other effects that can be beneficial for improving performance in sport activities [1-3, 34].

1.3.1. Anti-glucocorticoid activity

Glucocorticoids are catabolic substances that are released in the serum in response to physical or mental stress and are usually associated with heavy exercise, surgery and psychological problems. AASs are glucocorticoid receptor antagonists (competitive type) [1, 3, 34]. After binding to glucocorticoid receptors, AASs decrease the glucocorticoid mediated breakdown of proteins and thus reduce muscle atrophy (anti-

catabolic effect). An alternative hypothesis is that AASs may interfere with the expression of glucocorticoid receptors at the gene level. Thus, AASs can also exert anabolic effects indirectly by their anti-glucocorticoid action [1-3, 34].

1.3.2. Erythropoietic activity

Erythropoietin (EPO) is a glycoprotein hormone produced primarily in the adult kidney. EPO regulates the production of red blood cells or erythrocytes. The production of erythrocytes is called erythropoiesis. The haemoglobin present in the erythrocytes delivers oxygen from lungs to various body tissues. Thus, the amount of oxygen delivered to the tissues depends on the amount of erythrocytes in blood stream. EPO acts by binding to the cognate receptors expressed on the surface of erythroid progenitor cells. It is well known that kidneys play a key role in the production of erythrocytes and they respond according to the oxygen demand of the body. AASs are known to stimulate the production of erythropoietin in the kidney and promote erythropoiesis directly [1]. AASs enhance the sensitivity of erythroid progenitor cells to EPO. They also stimulate erythropoietic stem cell differentiation into mature enucleated erythrocytes. Thus in athletes, erythropoietic effect of AASs can lead to improved speed, endurance and performance beyond what is natural [3, 34].

1.3.3. Behavioural effects

Intake of AASs has been observed to influence an individual's behaviour such as aggression, cognitive abilities, mood and sexual behaviour [1-3, 34, 35]. AASs enhance the sense of well-being and joyfulness and reduce depression and anxiety. Cognitive functioning like attention, alertness and memory can play a key role in boosting performance of people involved in sports.

Owing to such performance enhancing effects associated with the intake of AASs, there is always an unfair advantage for AAS dopers over individuals who are not involved in doping with AASs.

1.4. STRUCTURAL MODIFICATIONS OF AASs

The structure of AASs influences their interaction with receptor proteins and various steroid metabolising enzymes [1, 3, 16, 34, 36]. Generally, for achieving performance enhancing effect it is desirable to use an AAS with maximum anabolic activity and minimum androgenic effects [34]. However, it has been reported that the anabolic effects of AASs cannot be completely dissociated from their androgenic effects and hence they are referred to as anabolic-androgenic steroids [37]. The desired effects of AASs can be obtained by modifying their chemical structure [2]. Several structural modifications of testosterone have been developed which improves its bioavailability and anabolic activity and reduces the androgenic adverse effects. For instance, structural derivatives of testosterone like nandrolone and stanozolol possess improved anabolic and minimised androgenic activity, than testosterone [38, 39]. The anabolic:androgenic ratio for testosterone, nandrolone and stanozolol are reported to be 1, 10 and 30 respec-

tively [38]. Thus, the androgenic adverse effects associated with the use of nandrolone and stanozolol are less than testosterone.

Figure 1.4 represents a few important structural modifications that can be made to the steroid nucleus to get a synthetic AAS with desired activity [3, 16, 36]. Modification of A, B, or C ring, 17β and 17α position of testosterone can achieve a number of goals, including i) slow metabolism, ii) enhanced affinity for the androgenic receptors, and iii) decreased binding of metabolites to androgenic receptors [16]. The 3-keto group on the A ring and 17β-OH group on the D ring of steroid nucleus (Figure 1.4) are known to form hydrogen bonds with the androgen receptor.

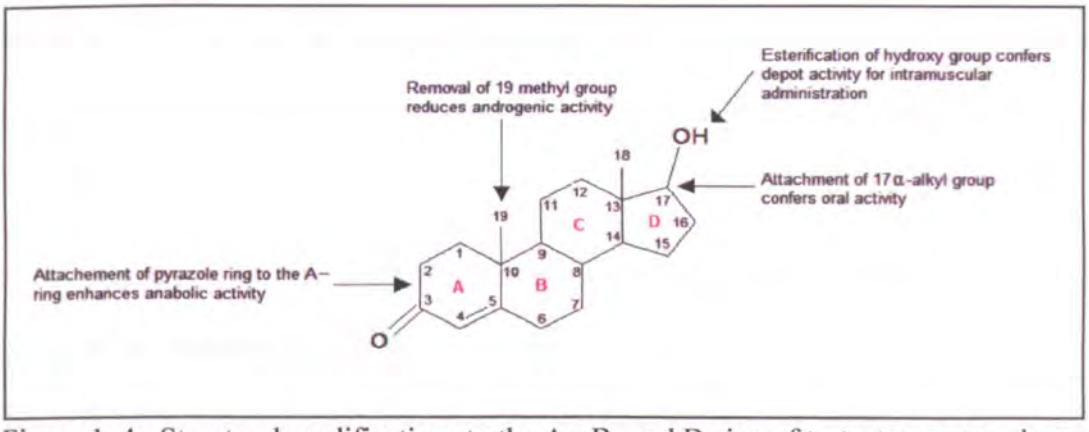

Figure 1. 4: Structural modifications to the A-, B- and D-ring of testosterone to enhance anabolic activity, reduce androgenic activity and to improve bioavailability [34]

Dosage forms and route of administration of AASs can also play a vital role in the overall activity of AASs. It is well known that testosterone is virtually inactive when administered orally [40]. Approximately 90% of the dose of orally ingested testosterone gets rapidly metabolised before reaching systemic circulation [36]. The plasma half-life of testosterone is reported to be 30 minutes. Owing to its extensive metabolism, supraphysiologically administered testosterone does not exert significant effects in the

human body. To prolong its pharmacological activity, testosterone is preferred to be administered via alternative routes such as transdermal patches or intramuscular injections [34, 36].

Replacing the 17α-H on the steroid nucleus with either a methyl or an ethyl group prevents the deactivation of steroid from first-pass metabolism in liver by sterically hindering the oxidation of 17β-hydroxy moiety [36]. Thus, such alkyl substitution enhances oral bioavailability of AASs by preventing their hepatic degradation [35]. However, further increasing the length of side chain is reported to interfere with the hydrogen bonding of 17β-hydroxy group to the receptor. It is also observed that 17α-alkylated steroids are more likely to cause hepatotoxicity which is one of the most serious side effects of AASs [3]. Examples of synthetic AASs with a methyl group introduced at 17α position are methyltestosterone, stanozolol, oxymetholone and oxandrolone and those with an ethyl group at the 17α position are norethandrolone, ethylestrenol, and norbolethone [3].

Generally, parenteral route of administration avoids first-pass effects as it allows drugs to be absorbed directly into the systemic circulation [5, 34]. Parenteral preparations of AASs usually do not require such alkyl substitutions. However, esterification of the 17β-hydroxy group with an acid prevents their biodegradation to keto steroids (a phase I metabolic pathway) [34]. Thus, esterification may prolong the duration of action of AASs when administered by the parenteral route. In parenteral preparations with an oily vehicle, esterification prevents the rapid absorption of AASs from the oily vehicle into the systemic circulation. Once in the systemic circulation, blood esterase enzymes catalyse the hydrolysis of steroid esters thus releasing active steroids [34]. The type of acid used to acidify the 17β-hydroxy group determines the duration of anabolic effect. Short-chain esters (e.g. C_2-C_3) result in short acting AASs, whereas long-chain esters

(e.g. C_7-C_{10}) lead to long acting compounds.The commonly used esters for parenteral formulations of testosterone include acetate, cypionate, decanoate, enanthate, isocaproate, phenylpropionate, propionate and undecanoate [34].

In the case of testosterone, the reduction of the C4,5 double bond in the A ring by an enzyme called 5α-reductase is a key step in the mechanism of action [3]. This leads to the formation of 5α-dihydrotestosterone (5α-DHT) which is a more potent endogenous androgen (than testosterone) as it binds more avidly with the androgen receptor (Figure 1.5) resulting in the formation of 5α-DHT-receptor complex. It is reported that the activity of 5α-reductase is negligible in skeletal muscles and is markedly prominent in androgenic tissues [34]. Hence, the intracellular level of 5α-DHT is low in skeletal muscle and may be further diminished by other enzymes. In contrast, the intracellular level of testosterone is high in skeletal muscles and other tissues where 5α-reductase is not expressed or is expressed at low levels. Thus, testosterone directly regulates skeletal muscle growth [34, 36].

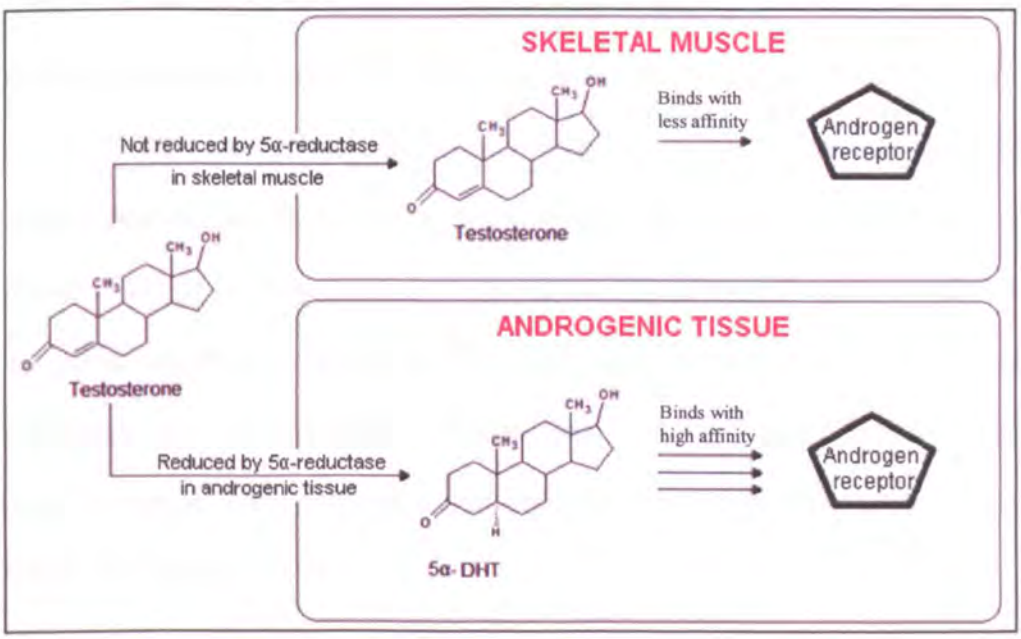

Figure 1. 5: In skeletal muscle testosterone binds directly to androgen receptors, whereas in androgenic tissues it gets converted to a more potent androgen, 5α-DHT [34]

The anabolic effects of an AAS can be partially dissociated from its androgenic effects by modifying the structure of AAS in such a way that it binds with high affinity to the androgenic receptor, but, upon undergoing 5α-reduction, binds with less affinity. Hence, in androgenic tissues the AAS will get converted to a less potent metabolite, whereas the parent AAS will predominate in the skeletal muscle tissue [1, 3, 16, 34, 36] to exert its anabolic activity.

Nandrolone is formed by substituting a hydrogen atom in the C19 methyl group of testosterone [3, 34]. Removal of 19-methyl group results in the reduction of androgenic activity but retention of the anabolic activity, compared to testosterone This may be due to a new asymmetric centre created at C10 position owing to removal of C19 methyl group [3]. Nandrolone does not suffer steric hindrance of the C19 methyl group and hence binds with greater affinity to androgenic receptors, than testosterone. Similar to testosterone, nandrolone also has an unsaturated C4,5 double bond which gets reduced by the 5α-reductase enzyme. This leads to the formation of 5α-dihydro-19-nortestosterone which binds with weaker affinity to androgenic receptors than its parent steroid nandrolone (Figure 1.6) [34]. In contrast, the 5α-reduced metabolite of testosterone, 5α-DHT, has greater binding affinity to the androgenic receptor than its parent steroid testosterone. Hence, in androgenic tissues, testosterone is converted to a more potent androgen whereas nandrolone is converted to a less androgenic metabolite [34]. As nandrolone predominates in the skeletal muscles, it possesses more anabolic and less androgenic activity, compared to testosterone [34]. Unlike testosterone, nandrolone does not undergo an aromatisation reaction, thus lacks the feminising side effects associated with testosterone [16].

Esterification of the 17β-hydroxy group of nandrolone with cyclohexylpropionic acid or phenylpropionic acid yields more stable and more anabolic products [3]. Other esters of nandrolone include laurate and decanoate (a long-chain fatty acid). Intramuscularly (IM) administered nandrolone decanoate is released slowly into the circulation and exerts its optimal anabolic activity over 6-7 days [3].

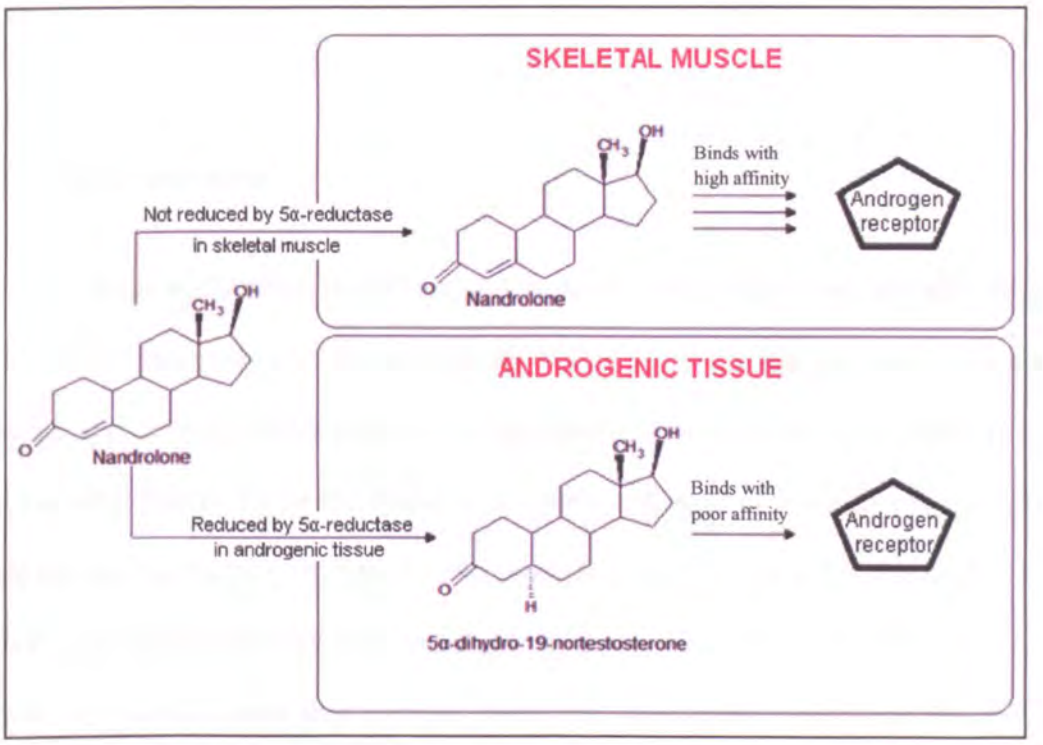

Figure 1. 6: In skeletal muscles, nandrolone binds directly with androgen receptors, whereas in androgenic tissues it gets reduced to a less potent androgen, 5α-dihydro-19-nortestosterone [34]

Stanozolol

Stanozolol is a 17α-methylated, 5α-reduced AAS with a pyrazole ring fused to the ring A of the steroid nucleus [3]. The 17α-methyl group enhances oral activity. Stanozolol is found to bind with lesser affinity to androgen receptors and thus possess less androgenic side effects, compared to testosterone. Owing to the pyrazole ring, stanozolol is found to be more anabolic than testosterone [34]. However, the exact mechanism by which it acts is not well understood.

1.5. FATE OF AASs

Once in the blood circulation, AASs rapidly reach equilibrium amongst different body fluid compartments. The availability of free and biologically active AASs at local target sites is regulated by protein binding and enzymatic conversions of AASs [41, 42]. Generally, depending on the chemical structure and route of administration, AASs are deactivated in the body by phase I and phase II metabolic reactions. The phase I metabolic pathway usually involves enzymatically catalysed reactions like oxidation, reduction, or hydroxylation, that convert AASs into more polar compounds that facilitate their inactivation and elimination from the body [41]. The phase II metabolic pathway involves enzymatically catalysed conjugation reactions like glucuronidation or sulphatation [43]. The resulting conjugates of AASs and their phase I metabolites are water soluble and get excreted from the body in urine. In the glucuronide conjugation reaction, various uridine diphosphate-glucuronosyltransferase (UGT) enzymes catalyse the transfer of glucuronosyl group from the uridine diphospho-glucuronic acid (UDPGA) to AASs and their phase I metabolites.

Glucuronidation is an important detoxification and deactivation pathway of AASs. Amongst the UGTs, UGT2B17 is the main enzyme responsible for catalysing the glucuronidation reaction of majority of AASs and their phase I metabolites [43]. However, other UGTs like 1A1, 1A3, 1A4, 1A8, 1A9, 1A10, 2B4, 2B7, and 2B15 may also contribute to the glucuronidation step of AASs [44-47]. It is well known that there are inter-ethnic and inter-individual variations in the prevalence of deletion mutations in the gene coding of UGT2B17 enzyme [48, 49]. Such variations are reported to influence the urinary excretion patterns of steroids [50-52].

Testosterone

One of the important metabolic pathways of testosterone involves the reduction of the C4,5 double bond, which is catalysed by 5α-reductase or 5β-reductase enzyme [36]. The reduction results in the formation of 5α-dihydrotestosterone (5α-DHT) or 5β-dihydrotestosterone with the H atom at C5 configured below or above the planar molecule, respectively [41]. The two isomers will be formed in a ratio depending on the relative catalysing effects of the two enzymes. As soon as the double bond is reduced, the 3-keto moiety gets reduced predominantly by 3α-hydroxysteroid dehydrogenase (3α-HSD). Testosterone can also undergo an aromatisation reaction, which is catalysed by the aromatase enzyme. This leads to the formation of estradiol which binds with estrogen receptors to exert estrogenic effects [41]. The aromatisation reaction mainly occurs in the adipose tissue. Both, aromatisation and reduction by 5α-reductase are undesirable metabolic pathways in anabolic drugs that may be intended to be used for doping purposes. Aromatisation is responsible for feminising side effects and 5α-reduction may reduce the ratio of anabolic:androgenic activity (depending on the structure of AAS) [16]. Figure 1.7 depicts the major phase I metabolism of testosterone.

Besides, 5-reduction and aromatisation, testosterone can also get metabolised through oxidation of the 17β-hydroxy group on the D ring to form androstenedione [3]. This reaction is catalysed by the 17β-hydroxysteroid dehydrogenase (17β-HSD) enzyme [41]. Androstenedione can further undergo 5β- and 3-keto reduction to form etiocholanolone. Alternatively, androstenedione can get converted to androsterone by undergoing 5α- and 3-keto reduction [53].

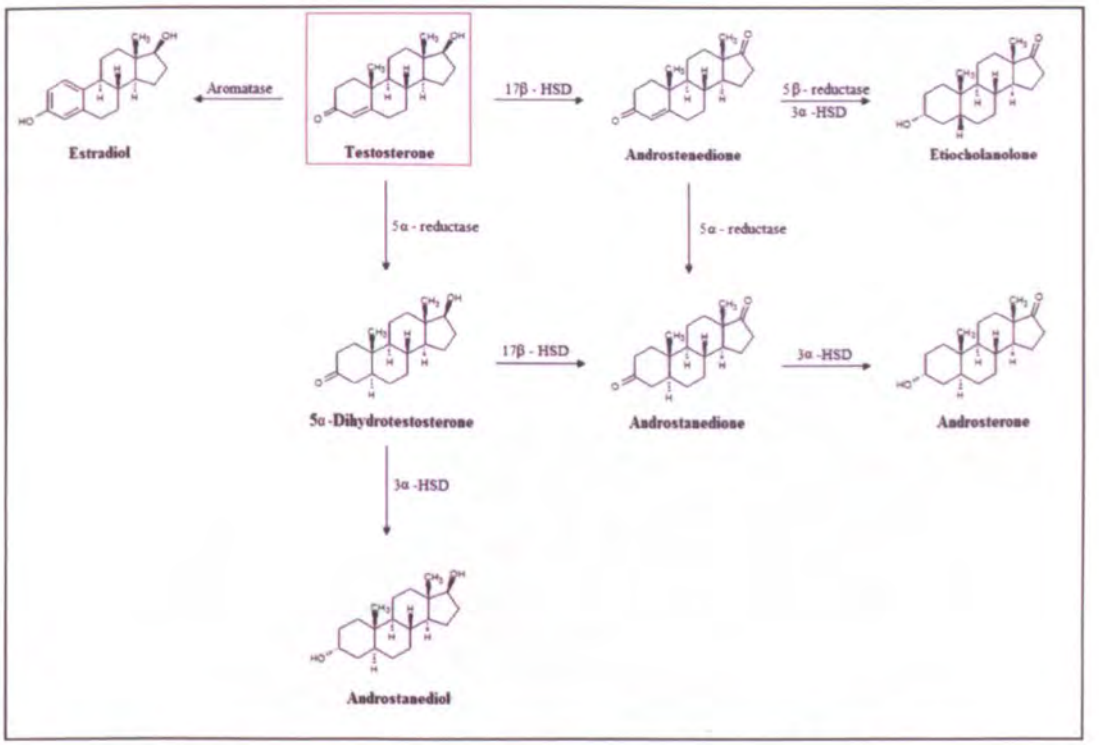

Figure 1. 7: Major phase I metabolic pathways of testosterone [3, 41, 54]

The main metabolites of testosterone that are excreted in urine mainly as glucuronide conjugates are androsterone (3α-hydroxy-5α-androstan-17-one), etiocholanolone (3α-hydroxy-5β-androstan-17-one) and androstanediol (5α-androstane-3α, 17β-diol) [36, 53]. Depending on the structure, most of the synthetic AASs follow metabolic paths similar to that of testosterone.

Testosterone is also excreted in urine in the form of the glucuronide conjugate. The UGT2B17 enzyme plays a major role in the glucuronidation reaction of testosterone, whereas the other UGTs like UGT2B15 play a minor role [55]. The resulting testosterone-glucuronide (TG) conjugate is an important urinary metabolite that is generally tested for controlling testosterone doping. However, for determining testosterone doping it is essential to distinguish endogenously produced testosterone from supraphysiologically administered testosterone. This can be achieved by determining the urinary ratio of testosterone-glucuronide to epitestosterone-glucuronide (EG). Epitestosterone is not a metabolite of testosterone, but, it is an endogenously produced inactive epimer of testosterone [56]. Epitestosterone is also excreted in urine mainly as epitestosterone-glucuronide. The glucuronidation reaction of epitestosterone is chiefly catalysed by the enzyme UGT2B7 [55]. The concentration of epitestosterone is not affected by the intake of testosterone and hence the urinary ratio of TG/EG can indicate illicit use of testosterone [55, 56]. According to the WADA, if the urinary ratio of testosterone-glucuronide to epitestosterone-glucuronide (TG/EG) exceeds 4, then doping with testosterone is suspected [57-61].

Several studies have been carried out which indicate that there are inter-ethnic and inter-individual variations in the occurrence of deletion polymorphisms in the gene coding of the UGT2B17 enzyme, and the excretion rate of testosterone-glucuronide is dependent on the genotype of UGT2B17 enzyme [48, 49, 52, 57]. It has been observed that the excretion rate of testosterone-glucuronide in individuals with *del/del* genotype is significantly less than those individuals carrying *ins/del* or *ins/ins* genotypes. As the major enzyme involved in the glucuronidation of epitestosterone is UGT2B7, deletion mutations in the UGT2B17 gene may not significantly affect the excretion rate of epitestosterone-glucuronide [62]. Thus, the accuracy of TG/EG test is challenged, as such genetic variation may lead to false doping results.

Wilson *et al.* reported that the absence of the UGT2B17 gene was five times more frequent in Caucasians than in African Americans [52]. In another study carried out by Sjoqvist *et al.*, it was observed that the occurrence of UGT2B17 deletion genotype was seven times more frequent in Koreans than in the Swedish population [49]. They also investigated the association between deletion polymorphism and urinary levels of testosterone and epitestosterone. They observed that the excretion of testosterone was 16 times higher in Swedish people compared to the Koreans [49]. There was no relation found between the UGT2B17 genotype and the excretion rate of epitestosterone. Jakobsson-Schulze *et al.* reported that, when testosterone was administered to individuals deficient in the UGT2B17 gene (*del/del* genotype), their urinary TG/EG ratio [48] did not reach the population based ratio of 4. Thus, false negative doping results were obtained. In addition to testosterone, UGT2B17 is also reported to be involved in the glucuronidation of some of its major metabolites like DHT and androstanediol and hence such genetic variations may also influence their glucuronidation rate. All these findings indicate the importance of this enzyme in the excretion of testosterone and other AASs.

Nandrolone

The metabolic pathway of nandrolone (19-nortestosterone) strongly follows that of testosterone [41]. It undergoes 4-ene-3-oxo reduction and 17β-hydroxy oxidation (Figure 1.8). The major metabolites of nandrolone are reported to be 19-norandrosterone (19-NA) and 19-noretiocholanolone (19-NE) [53, 63, 64]. These metabolites are excreted in urine predominantly in the form of glucuronide conjugates [65]. Generally, doping with nandrolone is controlled by testing the presence of 19-NA in urine [66]. According to the WADA, if the urinary concentration of 19-NA exceeds 2 ng/mL then doping with nandrolone is suspected [67].

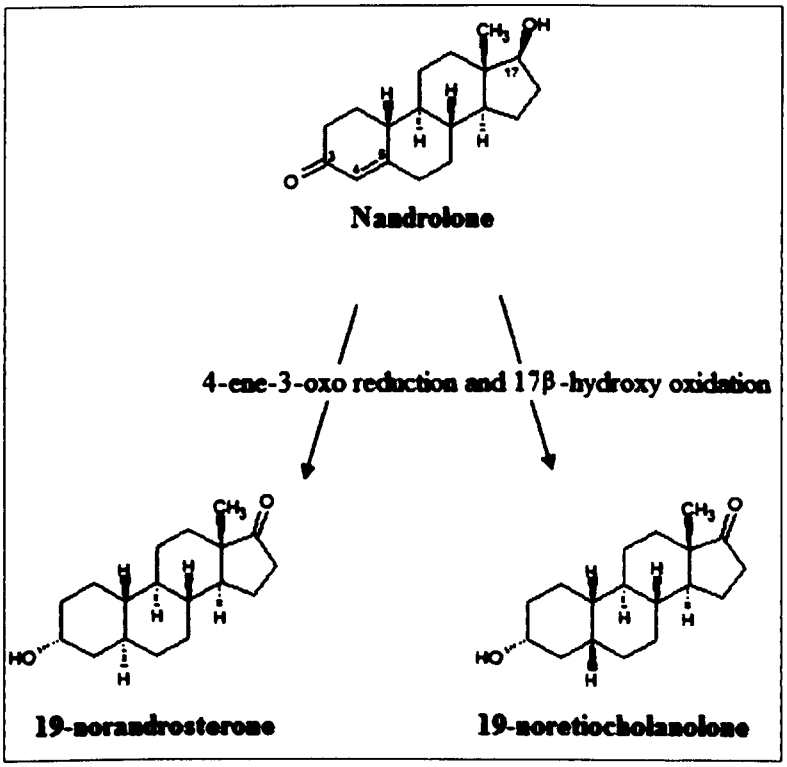

Figure 1. 8: Major phase I metabolic pathways of nandrolone [63]

Stanozolol

Once in the body, stanozolol gets rapidly metabolised leading to low concentration levels of parent compound found in urine [68]. Hydroxylation is an important phase-I metabolic pathway of stanozolol [69]. Fifteen urinary metabolites of stanozolol have been reported [70]. Amongst them the major metabolites are 3'-hydroxystanozolol, 4β-hydroxystanozolol, and 16β-hydroxystanozolol that are excreted in urine mainly as glucuronide conjugates (Figure 1.9) [3, 53, 68, 70]. In addition to these, other metabolites include mono/di/ poly-hydroxylated analogues or analogues epimerised at C17 position [70]. It has been reported that less than 5% of the metabolites are excreted in the unconjugated form [70]. According to the WADA, if stanozolol and/or its metabolite 3'-hydroxystanozolol is detected in the urine sample of individuals at a concentration above 2 ng/mL then stanozolol doping is suspected [60, 71].

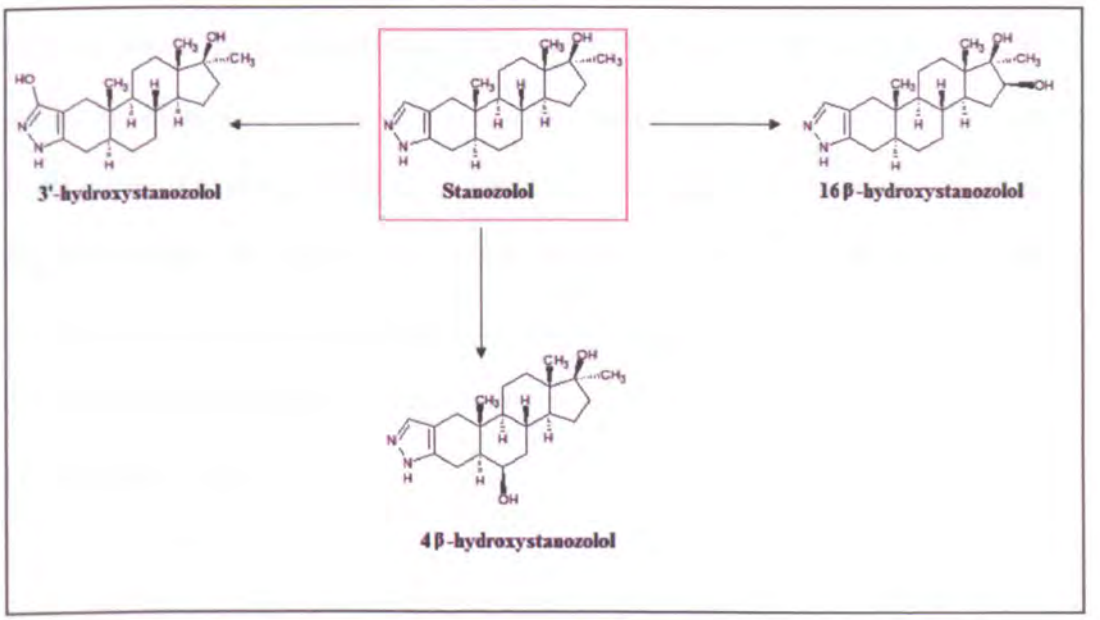

Figure 1. 9: Major phase I metabolic pathways of stanozolol [15, 41, 53, 68, 70]

1.6. CLINICAL APPLICATIONS OF AASs

Apart from being misused as performance enhancers in sports activities, AASs are also known to have therapeutic benefits [3, 72]. Owing to their ability to increase lean body mass, AASs can be useful in the treatment of muscle loss and cachexia associated with conditions like HIV infection, renal failure, severe burns and chronic obstructive pulmonary disease (COPD) where nutrition and standard care could be ineffective [1, 34, 73]. A number of studies have been carried out to determine the effectiveness of AASs in improving the condition of patients suffering from such muscle wasting syndromes [72, 74-80]. It has been demonstrated that AASs like oxymetholone, oxandrolone, nandrolone, and testosterone are effective in increasing the body weight of cachectic HIV infected patients [3]. Stanozolol, as well as nandrolone are demonstrated to be successful in increasing the mean body weight of patients suffering from COPD [3]. Nandrolone has been reported to be efficient in countering sarcopaenia in patients receiving dialysis [81]. Oxandrolone, either alone or in combination with growth hormones has been known to promote growth in children with retarded growth and puberty [3, 82]. Turner's syndrome, which is associated with significant shortness of stature, can also be treated with oxandrolone [83, 84]. Owing to their erythropoietic effects, AASs can be used in a variety of haematological disorders. For instance, oxymetholone is demonstrated to be useful in the treatment of sickle cell anemia, hairy cell leukemia and aplastic anemia [34].

1.7. ADVERSE EFFECTS OF AASs

Prolonged use of AASs has been known to be associated with severe adverse effects. The extent of undesirable effects may vary depending on the AAS administered, dosage and the duration of AAS use [2]. Once the intake of AAS is stopped, recipients may recover from some of the undesirable effects.

1.7.1. Liver disorders

Amongst the AASs, the 17α-alkylated AASs are most common to cause hepatotoxicity, including cholestatic jaundice, peliosis hepatitis and neoplastic lesions [1-3, 34]. Elevated levels of liver enzymes like aspartate aminotransferase, alanine aminotransferase and lacto dehydrogenase are also common in individuals using AASs [1, 3].

1.7.2. Reproductive effects

Depending on the dose, AASs can lead to virilisation (masculinisation) in females and feminisation in males [1-3, 34]. In females, use of AASs is known to cause acne vulgaris, clitoral hypertrophy, menstrual irregularities or amenorrhoea, breast atrophy, deepening of voice, male-pattern baldness and hirsutism [1, 3].

In males, administration of AASs is known to disturb the endogenous production of hormones like follicle-stimulating hormone (FSH), luteinising hormone (LH) and testosterone [1]. Both, FSH and LH are essential for spermatogenesis. Suppression of FSH and LH production in males can lead to testicular atrophy, abnormal sperm morphology, reduced sperm motility, decline in sperm density and sperm count. Such

reductions in the quality and quantity of semen production can predispose users to infertility. Furthermore, gynaecomastia, enlarged nipples and acceleration of baldness has also been observed in male dopers [1, 34]

1.7.3. Psychiatric effects

Aggressive behaviour and mood disturbance have been associated with the intake of AASs [1-3]. Furthermore, AAS users also experience heightened irritability, anxiety, insomnia, nausea, vomiting, changes in libido, destructive impulses, self-destructive impulses, and increased feeling of well-being [1, 34]. Athletes involved in AAS doping are more likely than non-users to misuse other drugs and alcohol. There are data suggesting that AAS administration can cause addiction [85, 86]. Furthermore, depression and craving for steroids may occur after withdrawal of AAS intake.

1.7.4. Cardiovascular effects

Elevated and prolonged use of AASs can lead to detrimental abnormalities in the blood lipid profiles [1]. An increase in the low-density lipoprotein (LDL) and decrease in the high-density lipoprotein (HDL) has been noted [3]. Owing to this, the users are predisposed to increased risk of atherosclerotic heart disease. Elevated blood pressure has also been reported to be linked with the use of AASs [1]. Furthermore, AASs can also cause coronary artery vasospasm, stimulate platelet aggregation, and enhance coagulation enzyme activity. Thus, owing to such unfavourable effects, AAS users are predisposed to thrombus formation. In addition, other cardiovascular events that are observed in healthy young athletes are atrial fibrillation, QT dispersion, cerebrovascular accident, acute heart failure and development of cardiomyopathy [1].

1.7.5. Renal effects

Prolonged use of AASs are reported to cause renal diseases such as acute renal failure, Wilm's tumor, acute kidney injury (AKI), nephropathy, focal segmental glomerulosclerosis (FSGS), proteinuria and membranoproliferative glomerulonephritis [1, 87-90]. However, throughout the last decade, the literature sporadically presented cases of severe renal disorders among AASs users, especially with elevated and prolonged use [90-95]. This suggests that renal complications are infrequent among AASs users in comparison to other, more prevalent disorders. The number of incidents presented is well below the estimated number of AAS users, however, the user profiles described in these case studies do not differ significantly from those AASs abusers who do not develop renal complications. Thus, it can be assumed that the observed renal disorders among AAS users are connected to the genetic profiles of these users.

It is well known that there are genetic variations in the occurrence of UGT2B17, which is an important enzyme responsible for AAS glucuronidation reaction. Since, glucuronidation is an important elimination and deactivation pathway for AASs, individuals lacking the enzyme UGT2B17 excrete less than normal amounts of AASs. Thus, the serum levels of biologically active AASs may get elevated. As a result of this, it is assumed that in UGT2B17 deficient individuals, chronic intake of AASs may lead to surplus increase in muscle mass and circulating levels of proteins, compared to individuals with a functional enzyme [96]. The resulting increase in body mass index can cause renal injuries owing to sustained elevated glomerular filtration rate and glomerular pressure. Owing to this, it has been hypothesised that individuals devoid of functional UGT2B17 enzyme are susceptible to suffer from renal complications with chronic use of AASs [96]. Furthermore, it can also be assumed that individuals devoid of the UGT2B17 gene are more susceptible to other adverse effects associated with chronic

use of AASs, compared to individuals with a functional enzyme. The enzyme deficient athletes who are aware of their own genetic profile may benefit from evading doping testing, but they may be vulnerable to serious health consequences [96].

1.8. DOPING CONTROL

Since, AASs are generally excreted in urine, mainly in the form of glucuronide conjugates of parent steroid as well as their phase I metabolites, the standard procedure for testing doping with AASs involves carrying out urinalysis in accredited laboratories [58, 71, 97, 98].

1.8.1. Drawbacks of urinalysis in detecting doping with AASs

Though urinalysis is an important method for controlling doping, it has a number of shortcomings. A single urine sample provides only a short term history of an individual's drug use [99]. Generally, drugs or their metabolites can be detected in urine for a few days to weeks [40]. However, this depends on the pharmacokinetics and the route of administration of drugs. AASs are training drugs and are efficient only when taken for a prolonged period of time accompanied with strength exercise [2, 4, 38, 85], whereas intake of AASs for a few days before competition may not have significant performance enhancing effects. Athletes can evade urinalysis by getting involved in chronic use of AASs during training period and deliberately stop taking AASs at a safe period before the doping test. In a case study [99], it was observed that during a doping test, urinalysis failed to detect doping by a suspected bodybuilder. However, hair analysis results confirmed doping. This corresponded to the claim of the suspected bodybuilder

who stated to have stopped the use of AAS about 8 weeks before the test. Hence, the accuracy of urinalysis is often challenged.

Urinalysis is reported to be unsuccessful in distinguishing between chronic use and single accidental exposure [99]. Nutritional supplements, which are generally taken by athletes, are reported to be deliberately adulterated with AASs (and other stimulants) in an attempt to improve the efficacy of the supplement products or accidentally contaminated (during manufacturing, transport or packaging) [100-109]. It has been reported that single exposure to such contaminated or adulterated nutritional supplements can lead to positive doping result after urinalysis [100]. AASs are also known to be misused in veterinary practices for promoting growth of meat producing animals and as a result their use is prohibited since 1981 by the European Union (Directive 81/602/EEC) [68, 110-114]. In line with previous reports [100], it can be assumed that urinalysis of individuals who consume meat from animals that are administered with steroids may also lead to false doping results. However, this will depend on several factors like time between steroid administration and animal slaughter, metabolic pathway, dosage and route of administration of steroids in animals and individuals diet. Furthermore, AASs and other banned substanes have been reported to be detected in environmental waters [115-122]. Thus, athletes who are accidently exposed to such contaminated food or water [123] are vulnerable to get falsely tested positive for doping. This may result in innocent athletes getting banned from competition.

As mentioned previously, deletion polymorphism in the gene coding of UGT2B17 enzyme influence the urinary excretion pattern of AASs and such genetic variations can lead to false doping results. Thus, the enzyme deficient dopers who are aware of their own genetic profile will thus have a potential advantage of evading urine based doping tests. Some commonly used over-the-counter (OTC) non-steroidal anti-

inflammatory drugs (NSAIDs) like diclofenac and ibuprofen are known to competitively inhibit the testosterone glucuronidation activity of UGT2B17, UGT2B15 and some other UGTs, *in vitro* [45, 124-129]. Furthermore, common dietary substances such as red wine [130], white tea and green tea [131] have also shown similar inhibitory effects in *in vitro* studies. Although the inhibitory effect is yet to be examined and reported *in vivo*, these *in vitro* results indicate that concomitant use of AASs along with such commonly used medication or dietary products may lead to impaired urinary excretion of AASs and their metabolites. Hence, owing to the drawbacks associated with urinalysis it may be essential to employ additional matrices which can either be used alone or in combination with urinalysis [132-134].

1.8.2. Hair analysis

For the past three decades, hair analysis has been employed for determining chronic drug use. Owing to its unique potential in determining the retrospective history of drug use depending on the length of hair analysed, it is gaining attention in controlling drug doping [4, 59, 134-151]. It is capable of providing complementary information to urinalysis and thus can support the current anti-doping regimes. Normally, AASs are detected in hair in picogram range.

1.8.2.1. Mechanism of incorporation of drugs in hair

Generally, drugs can get incorporated into the hair matrix, at various times during the hair growth cycle [152], by one or more of the following three ways: i) endogenous pathway, ii) endogenous-exogenous pathway and iii) exogenous pathway [153, 154]. However, the exact mechanism is not fully understood. Figure 1.10 presents the structure of hair and the three pathways by which drugs can get incorporated into hair [155].

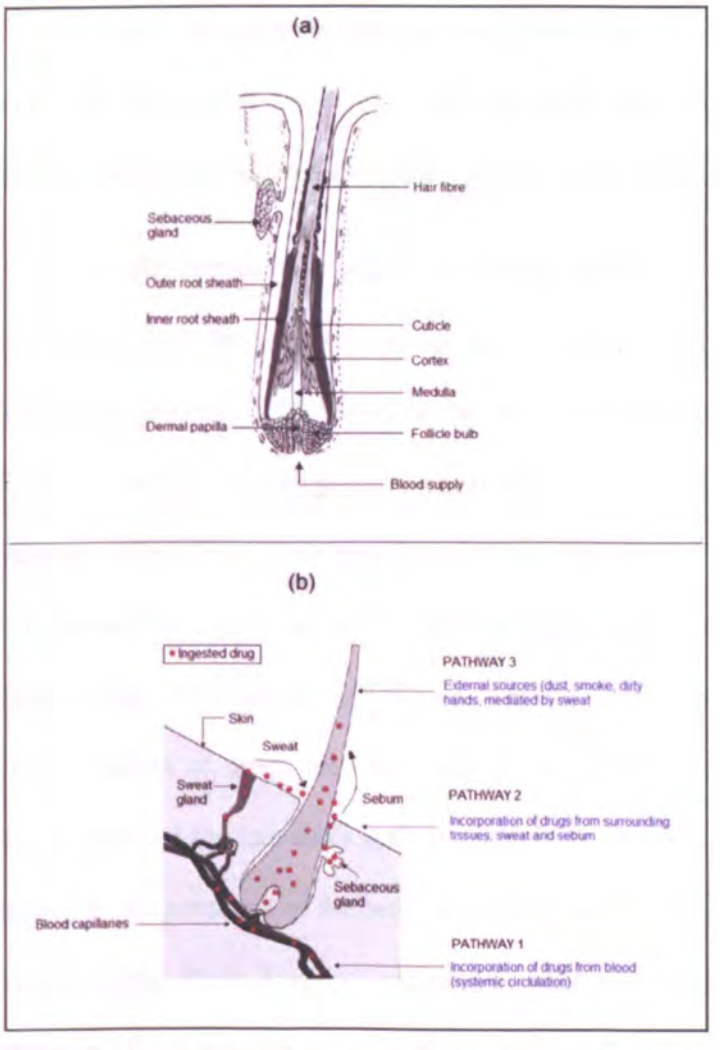

Figure 1. 10: (a) Structure of hair, (b) pathways by which drugs can get incorporated into hair [155]

Endogenous pathway

This involves active or passive diffusion of drug molecules from the blood-stream into the growing hair (histogenesis) via the hair follicle [156, 157]. The hair follicle is provided with a good uniform blood supply and thus the ingested drugs that are present in the systemic circulation can be delivered to the hair follicle. However, in order to get incorporated in the hair matrix cells, drugs should be able to diffuse across the cell membrane. For this, the drugs should have sufficient lipophilicity so that they can diffuse through the cell membrane and thus get incorporated into the hair matrix [158]. It has been demonstrated that the cell membrane is less permeable to polar compounds. In most cases, drug metabolites are more polar than the parent drug. Hence, metabolites enter hair to a lesser extent than their lipophilic parent drugs. As a result of this, hair predominantly favours the detection of parent drugs in comparison to their metabolites.

In addition to the physicochemical properties of drugs, the pH gradient between the plasma and the cells also influence the rate of diffusion of drug molecules [157]. The pH of plasma is 7.3, whereas the pH of melanocytes and keratinocytes varies between 3 and 6. This pH gradient predominantly facilitates the diffusion of basic drugs into the cells [157]. Once the drugs get accumulated in the cell cytosol, drugs get protonated and hence are not able to diffuse back into the plasma. However, protonation of drugs in the cell depends on the pKa of drugs. It has also been suggested that the passive diffusion of drugs into hair may be augmented by drug binding to intracellular components of the hair cells such as melanin and keratin. It has been demonstrated that owing to the presence of melanin, incorporation of drugs into the pigmented hair is favoured [154]. Basic drugs are reported to have a significant affinity for melanin. Thus, melanin affinity and low pH of melanocytes can facilitate the incorporation of lipophilic and basic drugs (in contrast to acidic drugs) into pigmented hair.

In a study carried out by Hold *et al.*, [159] stanozolol was administered (20 mg/kg/day intraperitoneally for 3 days) to male, hooded Long Evans rats. The concentration of stanozolol in pigmented and non-pigmented hair 14 days after dosing was determined. It was observed that the concentration of stanozolol in pigmented hair was higher than in non-pigmented hair. The mean ratio of concentration of stanozolol in pigmented hair to that in non-pigmented hair (from same animal) was found to be 3.42 ± 2.19. This suggests that stanozolol gets preferentially incorporated into pigmented hair, which is predominantly owing to the basic nature of its pyrazole ring. Similarly, other basic drugs like cocaine, phencyclidine, codeine, amphetamine, methamphetamine and nicotine have been reported to have an inclination in getting incorporated into pigmented hair [160, 161]. In another study carried out by Kronstrand *et al.*, [162] pigmented and non-pigmented hair samples were obtained from grey haired patients who were on a long term selegiline medication. The hair samples were analysed for determining the concentrations of selegiline metabolites methamphetamine and amphetamine. The mean ratio of concentration of methamphetamine in pigmented hair to that in non-pigmented hair (in same subject) was found to be 3.69 ± 1.88, whereas for amphetamine it was found to be and 2.95 ± 1.16. Neutral AASs like nandrolone and testosterone have also been reported to have affinity for melanin and thus get preferentially incorporated into pigmented hair. It was reported by Gleixner *et al.*, [163] that the concentration of testosterone in bull hair was around 4 times higher in pigmented hair than in non-pigmented hair.

In another study carried out by Höld *et al.*, [164] nandrolone decanoate was administered to Long-Evans rats (60 mg/kg/day intraperitoneally for 10 days) and it was observed that nandrolone was detectable in the pigmented hair. However, nandrolone was not detectable in the corresponding non-pigmented hair. These findings indicate

that in addition to basic drugs, neutral AASs like nandrolone and testosterone also have affinity to bind with melanin.

In contrast, acidic drugs are reported to show no hair pigmentation bias. Gygi *et al.* [165] administered codeine and phenobarbital to Long Evans rats (40 mg/kg/day intraperitoneally for 5 days). Concentrations of codeine (basic drug) in hair were 44 times greater in pigmented than non-pigmented hair, from the same animal. In contrast, concentrations of phenobarbital (acidic drug) were identical in both pigmented and non-pigmented hair. Similarly, N-acetylamphetamine, a non-basic analogue of amphetamine, does not exhibit a hair colour bias in humans [161]. Thus, it can be stressed that the basic as well as neutral AASs (opposed to acidic drugs), with sufficient lipophilicity get preferentially diffused across the cell membrane and thus get incorporated in hair. Since drugs have been demonstrated to be incorporated into non-pigmented hair it implies that, in addition to melanin, other components of hair like keratin also play a key role in accumulation of drugs. Hence, although some drugs may have an affinity to bind with melanin, they may still get incorporated into non-pigmented hair.

Endogenous-exogenous pathway

It is well known that drugs and their metabolites in the systemic circulation can get excreted in sweat. Drugs and metabolites can get absorbed or transferred from sweat (sebum, transdermal excretion via stratum corneum) to the keratinised hair [154, 156]. This represents the endogenous-exogenous pathway. It has been found that drugs like alcohol, amphetamine and cocaine have been found in sweat, often in concentrations greater than found in blood. Also substances like cortisol and cortisone have been suggested to get accumulated in hair mainly through diffusion from sweat and not through

the bloodstream [154]. It has also been suggested that lipophilic drugs can get incorporated in hair from deep compartments of skin.

Exogenous pathway

This is the least common pathway and it involves the deposition of drugs in keratinized hair by absorption from external environment (cosmetic hair treatments, pollution) [156].

1.8.2.2. Advantages of hair analysis in detecting doping with AASs

Hair grows around 1-1.5 cm per month and thus depending on the length of hair analysed, a retrospective history of drug use, well beyond than that obtained by urine, can be determined [4, 59, 141]. Owing to this, hair analysis can identify dopers who attempt to evade urine based doping tests by intentionally stopping the use AASs before competition, resulting in a sufficient drug free period to give false negative results.

To ensure doping-free sport, out-of-competition doping testing regimes are carried out by sport organisations to ensure that athletes are not using drugs like AASs [166]. Such doping control regimes require top elite athletes to be available for doping tests on a very regular basis. However, on a regular basis this can be cumbersome and time consuming for athletes. Hence, hair specimens that are obtained with less intrusion than urinalysis can be used to determine retrospective information on an individual's drug use. Thus, hair can be an ideal matrix for detecting out-of-competition doping [99]. If a urine sample is positive for a particular drug, hair can discriminate between single exposure and chronic use. Hair testing is tamper resistant in comparison to urinalysis.

For instance, athletes can drink plenty of water before the urine sample collection, which can dilute the urine leading to misleading results, whereas the concentration of drugs in hair may not be affected by deliberate intake of excess of water [99, 142]. Furthermore, hair favours the incorporation of parent AASs compared to the relatively more polar metabolites, which are mainly excreted in urine in contrast to the parent AASs [4, 59, 68, 96, 111]. Hence, when hair analysis and urinalysis are employed together, both, the parent AAS and its metabolites can be detected.

As discussed earlier, individuals devoid of the functional UGT2B17 enzyme excrete less than normal amounts of AASs in urine. However, there are no reports suggesting that there is suppression in the incorporation rate of AASs in hair in UGT2B17 deficient individuals. Impaired urinary excretion of steroids may lead to elevated plasma steroid levels and hence potentially greater amounts of steroid will be available to get incorporated into hair [96]. Thus, if urinalysis fails to determine doping due to such genetic variations, then hair analysis may assist in determining use of AASs. Although hair analysis may not completely replace urinalysis, it can be used in combination with urinalysis to reinforce the current anti-doping regimes.

1.8.2.3. Arguable drawbacks of hair analysis

Sceptics may say that athletes may use harsh chemical treatments for styling their hair or to deliberately compromise the analytical results, but hair testing is not limited to scalp hair [167]. For instance, AASs can be incorporated into body hair other than scalp hair [155], particularly into androgen sensitive hair [167, 168]. Also, it is more likely that harsh treatments to hair may affect the concentrations of endogenously produced steroids (example testosterone) in hair. Thus, testing of testosterone or any other endogenous steroids in hair can be considered as a marker to identify athletes who have treated their hair. Some might also suggest that devious athletes may also take drastic actions by shaving all body hair to evade doping tests. However, such failure or refusal to provide samples without convincing justification constitutes anti-doping rule violations [169].

1.8.2.4. Hair analysis for supporting social science research studies into doping

For anti-doping purpose, it is also important to advance in social science research predominantly focused on investigating the doping prevalence rate and understanding athletes' attitude and behavioural intentions towards doping in the society. The majority of social science research studies into doping rely on self-reports [170-176]. Anti-doping prevention programmes are also evaluated via self-reported changes in attitudes and inclination towards using doping substances. However, the data obtained from self-reports are susceptible to potentially distorting effect of socially desirable responding [170, 174, 176]. Owing to the fear of being caught, respondents can hesitate to provide compromising information about them (i.e. taking prohibited substances) and may

manipulate their answers on all related measures in order to maintain their image they wish to project. Furthermore, studies investigating proximal and distal factors of doping behaviour whilst relying on self-admission (of doping) could yield a misleading behavioural model. This is owing to the reason that those who deny doping have the tendency to respond to questions about factors assumed to underlie doping behaviour (attitudes, norms, beliefs, pressure, expectation) in such way that creates the impression of a 'clean athlete' [175]. Therefore their expressed views may not represent their true thoughts and opinions, thus distort the overall outcome of the study (i.e. these users and their answers are erroneously grouped with the non-users).

In addition to social pressures there are other sources that can attribute to errors in self-reporting. For instance, respondents can find it difficult to understand the survey questions and may face problems in recalling information necessary to accurately answer these questions [177]. Inaccurate reporting can lead to erroneous assumptions about the severity of doping problems in the society and thus influence the decisions regarding the need for treatment and prevention services [178, 179]. Hence, researchers using self-reported questionnaires or surveys are concerned about the validity of the responses. It has been proposed that self-reported information, when feasible, should be verified with objective methods at least during the pilot study phase [170, 175]. Researchers have previously reported the use of biochemical analysis for the validation of self-reported drug use information [170, 175, 177-181]. As biochemical analysis can be expensive, such approach may not be feasible for all types of studies (mainly large scale). However, biochemical analysis can be used on a small representative sample size prior to or as part of main data collection. This can assist in determining the degree to which the self reports are distorted and accordingly measures can be taken to make self-report methods more reliable before applying them to large scale studies.

In the past, self-reported drug use information has been verified by analysing respondents' urine, blood, saliva or hair samples for the presence of social drugs [177-182]. The majority of such studies have indicated that the participants who dishonestly deny drug use can be identified by appropriate biochemical tests. This highlights the importance of independent chemical analysis in validating self-reported drug use information and its ability to support social science research. Furthermore, in some cases discrepancies between chemical analysis and self-reports have been observed in the unexpected direction, i.e. some participants who reported substance use were tested negative by urinalysis [182]. This highlights the potential drawbacks of urinalysis in corroborating self-reported drug use informations. Compared to urine, blood and saliva, hair has many advantages in detecting drug use. The key advantages include its ability to determine the retrospective history of drug use and the ease with which samples can be obtained. Hence, it can be suggested that the self-reported performance enhancing drug (PED) use information can be reliably validated by hair analysis (either alone or in combination with urinalysis).

1.9. CONCLUSIONS

Owing to the limitations of urinalysis and self-reports, a requirement exists for employing additional biological samples like hair, which has the potential to reliably determine doping with AASs and thus verify self reported doping information, covering prolonged periods depending on the length of hair analysed. Hence, for anti-doping purposes, this thesis presents the development of new highly sensitive analytical methods that are capable of detecting widely used exogenous and endogenous AASs in human hair.

Further research is required to investigate the effects of UGT substrates, *in vivo*, on the metabolism and urinary excretion of AASs, for anti-doping purposes. To take a step forward, this thesis also focuses on further investigating the metabolism of one of the widely used exogenous AASs, stanozolol. Also, the potential application of hair analysis when the detection of AAS use via urinalysis is constrained by metabolic variations is investigated.

1.10. AIMS OF THE RESEARCH PROJECT

- To develop methods for the detection of frequently used exogenous AASs, nandrolone and stanozolol in human hair using ELISA and LC-MS/MS.
- To apply these novel analytical methods for analysing hair samples that were collected as part of social science research studies.
- To develop a method for the detection of testosterone and epitestosterone in hair using LC-MS/MS.
- To apply this method for determining the natural levels of testosterone (T), epitestosterone (E) and T/E ratio in human hair. Also, to investigate the relationship between hair colour and the concentration of T and E, using a rat model.
- To develop methods for the detection of stanozolol and its metabolite, 3'-hydroxystanozolol in rat hair, urine and serum using LC-MS/MS.
- To employ these methods to investigate the inhibitory effect of diclofenac on the urinary excretion of stanozolol and 3'-hydroxystanozolol, *in vivo*, using a rat model. Also, to investigate the potential application of hair analysis when excretion of drugs in urine is impaired.
- To develop methods for the detection of stanozolol and its urinary metabolite, 3'-hydroxystanozolol in environmental water samples.
- To apply these methods for the analysis of drinking and environmental water samples collected from Budapest.

1.11. PREFACE

The work presented in this Ph.D thesis mainly involves the development and application of a series of novel analytical methods to detect widely used AASs using liquid chromatography tandem mass spectrometry (LC-MS/MS). The work is reported over seven chapters.

- Chapter one provides the general introduction.
- Chapter two provides the details of the materials and methods employed in this project.
- Chapter three represents the development and application of methods for the detection of frequently used exogenous AASs, nandrolone and stanozolol in human hair using ELISA and LC-MS/MS.
- Chapter four reports the development and application of methods for the detection of stanozolol and one of its major metabolite 3'-hydroxystanozolol in drinking and environmental water samples collected from Budapest.
- Chapter five details the development and application of the method for the detection of testosterone and epitestosterone in human and rat hair.
- Chapter six represents the development and application of methods for the detection of stanozolol and 3'-hydroxystanozolol in rat hair, urine and serum.
- Chapter seven provides the concluding remarks of the project and informs the potential future work arising from this work.

CHAPTER 2

MATERIALS AND METHODS

The following chapter describes the materials and general methods used for the entire project. All the chemicals (mainly analytical grade) and consumables were purchased from Sigma Aldrich (Poole, UK), LGC standards (Teddington, UK), Roche Diagnostics (Burgess Hill, UK), Agilent (Stockport, UK), Fischer Scientific (Loughborough, UK), Capital analytical (Leeds, UK) and Millipore (Watford, UK). Detailed description of methods employed in this project is provided in individual chapters (3 to 6).

2.1. MATERIALS AND INSTRUMENTS

Table 2. 1: General chemicals used

Chemicals	**Lot number/size**	**Grade**	**Source**
Sodium hydroxide	1401534/1kg	Analytical	Sigma Aldrich
Hydrochloric acid	1397813/500mL	Analytical	Sigma Aldrich
Sodium hydrogen phosphate heptahydrate	1445623/250gm	Analytical	Sigma Aldrich
Sodium phosphate monobasic dihydrate	BCBB7743/1kg	Analytical	Sigma Aldrich
Formic acid	09232523/500mL	HPLC	Sigma Aldrich
β-glucuronidase	12438921/5mL	Bioreagent	Roche Diagnostics
Melanin	109K1414V/100mg	Bioreagent	Sigma Aldrich
Reference standards			
3'-hydroxylstanozolol	F012011-01/1mg	Analytical	LGC standard
3'-hydroxylstanozolol D3	F021711-02/1mg	Analytical	LGC standard
3'-hydroxylstanozolol-glucuronide	00-S-14/1mg	Analytical	LGC standard
Nandrolone	FE092107-02/1mg	Analytical	LGC standard
Stanozolol	FE051509-01/1mg	Analytical	LGC standard
Stanozolol D3	FE090409-01/1mg	Analytical	LGC standard
Testosterone	5117X/250mg	HPLC	Sigma Aldrich
Epitestosterone	079K1250/250mg	Analytical	Sigma Aldrich

Table 2. 2: Solvents used

Solvents	Grade	Source
Water	HPLC	Sigma Aldrich
Water	Chromasolv for LC-MS	Sigma Aldrich
Water	Ultra chromasolv for LC-MS	Sigma Aldrich
Acetonitrile	HPLC	Sigma Aldrich
Acetonitrile	Chromasolv for LC-MS	Sigma Aldrich
Methanol	Chromasolv for LC-MS	Sigma Aldrich
Pentane	Chromasolv for HPLC	Sigma Aldrich
Chloroform	Chromasolv for HPLC	Sigma Aldrich
Ethyl acetate	Chromasolv for HPLC	Sigma Aldrich
Hexane	Chromasolv for HPLC	Sigma Aldrich
Dichloromethane	Chromasolv for HPLC	Sigma Aldrich

Table 2. 3: Essential consumables used

Consumables	Description	Source
Zorbax SB C-18 Columns	2.1 mm, 1.8 µm, 50mm	Agilent
	2.1 mm, 1.8 µm, 100mm	
	2.1 mm, 1.8 µm, 150mm	
Inline column filter	0.2 µm	Agilent
Syringe driven micro-filter	0.2 µm, PTFE membrane	Millipore
Syringe driven micro-filter	0.45 µm, MEC membrane	Millipore
Silanised amber glassware	4 mL with PTFE lined screw caps	Sigma Aldrich
Silanised glass inserts	200 µL	Capital Analytical
Amber vials	1.5 mL with screw caps	Fisher Scientific
Glass centrifuge tubes	10 mL with PTFE lined screw caps	Fisher Scientific
Plastic centrifuge tubes	15 mL, polyethylene teraphthalate	Fisher Scientific

Table 2. 4: Instruments used

Instruments	Brand	Model	Source
LC-MS/MS	Thermoscientific	Please see section 2.5	Thermoscientific
LC-MS/MS	Agilent	Please see section 2.5	Agilent
Centrifuge machine	Thermoscientific	Labofuge 400 R	Thermoscientific
Vortex mixer	Fischerbrand	ZX Wizard with infrared sensor	Fischer Scientific
Ball mill	Fritsch	Mini-mill pulverisette 23	Fritsch
Sample concentrator	Techne	Dri-block DB-3D	Fischer Scientific
pH meter	Mettler Toledo	Seven Easy S20	Fischer Scientific
Microplate reader	Varian	Cary 50	Varian

2.2. HUMAN HAIR SAMPLES

This project was a collaborative work between the School of Pharmacy and Chemistry (Kingston University, London, UK), and the School of Life Sciences (Kingston University, London, UK). Hair samples were obtained via the School of Life Sciences as part of different studies [4, 59, 170, 174, 175, 183, 184]. Method development, validation and the analysis of hair samples was carried out by the author (School of Pharmacy and Chemistry). For this project, hair samples were obtained from 210 European subjects (131 males, 79 females). Participants' age ranged between 18 and 55 years. All the participants recruited were healthy as determined by their questionnaire response. Each hair sample consisted of approximately 50 hair strands, minimum 3 cm in length and weight *ca.* 100 mg. Hair samples collected were untreated, i.e. free of harsh chemical treatment such as dyes, bleach, perming agents. Hair was cut directly at the skin surface at the vertex posterior of the head. This area was preferred as it shows less variability in hair growth rate compared to other areas. Also, the number of hairs in active growth is larger in this region. The hair samples were stored individually in labelled, sealable, paper envelopes until analysis.

From the available 210 hair samples, 180 (108 males, 72 females) were analysed for the determination of widely used synthetic AASs, nandrolone and stanozolol, as discussed in chapter 3 [4]. The findings were used to verify participants' self-reported questionnaire (drug use) results [170, 175, 176]. Out of the 180 hair samples analysed, 45 hair samples were available in surplus amounts and the left over hair samples were used in other studies. These 45 hair samples plus remaining 30 unused hair samples (210 - 180 = 30) were analysed for determining the natural levels of endogenous AAS, testosterone and its inactive epimer epitestosterone [59, 183]. This study is discussed in chapter 5. All studies were carried out according to the protocols established and approved by the Kingston University Faculty Research Ethics Committee.

2.3. ANIMALS USED IN THE PROJECT

2.3.1. Long Evans rats

Eleven male, hooded, Long Evans rats were purchased from Charles River laboratories, (Sulzfeld, Germany). Each animal weighed around 726-834 g and were approximately 11 months old. Each animal had pigmented and non-pigmented hair patches, which were shaved using an electric shaver. These animals were used to investigate the pattern of incorporation of testosterone and epitestosterone in pigmented and non-pigmented hair. Further details are discussed in chapter 5.

2.3.2. Brown Norway rats

Twenty four male, brown Norway rats were purchased from Charles River laboratories (Sulzfeld, Germany). Each animal weighed around 280-340 g and were approximately 5 months old. These animals were used to investigate the inhibitory effect of diclofenac on the urinary excretion of stanozolol and its metabolite 3'-hydroxystanozolol, *iv vivo*. Also, the potential application of hair analysis and blood analysis when the urinary excretion of these compounds is impaired was investigated. For this, the animals were administered with a fixed dose of stanozolol (5 mg/kg) and varying doses of diclofenac (0 to 25 mg/kg) over a period of time. Urine, hair and serum samples were obtained periodically and analysed to establish a relationship between the drug levels detected in the three matrices. This study is detailed in chapter 6.

All animals used in this project were kept in an animal house located in Semmelweis University, Budapest, Hungary. Animals were housed in groups of three individuals in standard laboratory cages. Rats were kept in a constant room temperature environment with an alternating 12-h light-dark cycle. Food and water were available *ad-libitum*. The administration of drugs and sample collection were conducted under the institutional license of Department of Anatomy, Histology and Embryology, Semmelweis University, Budapest, Hungary in accordance with the EC Council directives on laboratory animals (86/609/EEC) [185]. All the methods were developed and validated at Kingston University by the author. Sample analyses (human and animal) was also carried out at Kington University by the author.

2.4. METHODOLOGY

This project mainly involves the analysis of human hair, rat hair, rat urine, and rat serum samples using LC-MS/MS. Figure 2.1 outlines the general method employed in this project for the preparation of samples.

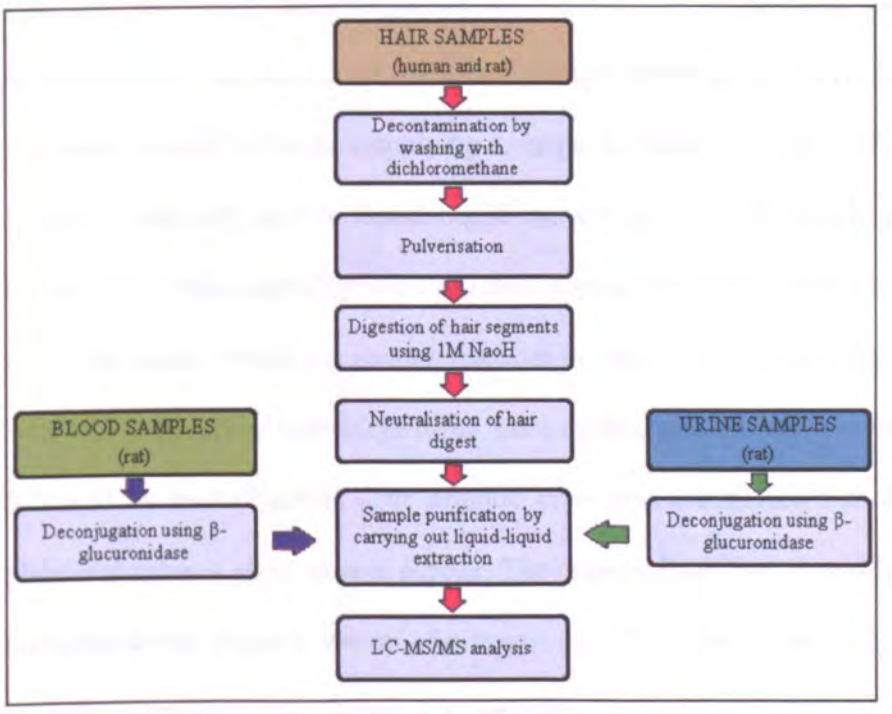

Figure 2. 1: Summary of sample preparation method employed in the project

2.4.1. General method used for the preparation of hair samples

In line with previously published reports [4, 59, 154, 184-186], all hair samples (human and animal) were initially decontaminated by rinsing them with 2 mL di-chloromethane for two minutes at room temperature. This step was repeated twice to ensure that hair is free from contaminants like sweat, sebum, shampoo etc. which may interfere with the analysis. The major advantage of using dichloromethane is that it is a

non-protic solvent and hence it does not swell the hair shaft. Thus, extraction of substances from hair itself is avoided and only external contaminants are eradicated. After decontamination, hair samples were air dried and then cut (using scissors or ball mill) into ca. 1 mm segments. The required amount of decontaminated hair segments were weighed in a clean vial and incubated with 1 mL of 1M sodium hydroxide solution at 95 ^{0}C for 10 to 15 minutes in the presence of a deuterated internal standard (IS) [99, 142, 150, 154, 187-193]. The homogenate was allowed to cool and then neutralised using 1 M hydrochloric acid and 2 mL of 0.2 M phosphate buffer (pH 7.0). The neutralisation step was standardised before applying to large number of samples. The neutralised homogenate was subjected to liquid-liquid extraction (LLE) for which it was transferred into a 10 mL glass centrifuge tube and then a suitable organic solvent (pentane or a mixture of pentane, chloroform and ethylacetate in ratio 3:2:1 v/v/v), depending on the analyte, was added. The centrifuge tubes were capped and the mixture was vortex mixed followed by centrifugation. The organic layer was transferred into a clean silanised glass vial using a glass pasteur pipette. The organic layer was then dried using heat accompanied with a gentle stream of nitrogen gas. The dried extract was then reconstituted with acetonitrile or methanol (depending on analyte) and injected into the LC-MS/MS system after filtering through syringe driven 0.2μm PTFE filters.

To determine the extraction recovery, decontamineted hair segments (drug free) were spiked with known amounts of analytes and IS. The mixture was subjected to alkali digestion followed by LLE and analysis. The ratios of peak areas of analytes to IS were determined and compared with the ratios of peak areas of analyte to IS of standard solutions at same final concentrations. The extraction recoveries obtained are comparable to previously published work [146, 194].

2.4.2. General method used for the preparation of serum and urine samples (brown Norway rats)

In line with previously published research work, deglucuronidation of rat serum and urine samples was performed using β-glucuronidase enzyme [70, 185, 195, 196]. For this, serum or urine samples were mixed with 1 mL of 0.2 M phosphate buffer (pH 7.0). The mixture was incubated with 50 μL of β-glucuronidase enzyme at 50 ^{0}C for two hours in the presence of deuterated ISs. After cooling, organic solvent was added to the mixture and LLE was performed (as described above) followed by LC-MS/MS analysis.

2.5. LC-MS/MS INSTRUMENTATION

Thermoscientific and Agilent LC-MS/MS systems were used separately for the entire project. Figure 2.2 represents photographs of the instruments used in the project. The Thermoscientific LC-MS/MS system comprised of an Accela LC system (Thermo Scientific, Hertfordshire, UK) coupled to a triple Quadrupole TSQTM mass spectrometer (Thermo Electron Corp, Hertfordshire, UK). The LC system comprised of a quaternary pump, automatic degasser, column heater and thermostated autosampler. The mass spectrometer comprised of an electrospray ionisation source (ESI) operated in positive ion mode. The Xcalibur software package, version 2.0 (Thermo Scientific, Hertfordshire, UK) was used for operating the LC-MS/MS system and data acquisition.

The Agilent LC-MS/MS system comprised of a 1260 infinity LC system (Agilent, Wokingham, UK) coupled to a 6430 triple quadrupole mass spectrometer (Agilent, Wokingham,UK). The LC system comprised of a binary pump, automatic degas-

ser, column heater and 1290 infinity thermostated autosampler. The mass spectrometer was equipped with an ESI source operated in positive ion mode. The LC-MS/MS system was controlled by the Masshunter workstation software version B.03.01 (Agilent, Wokingham, UK). The Agilent LC-MS/MS was found to be more sensitive than the Thermoscientific LC-MS/MS. Hence, depending on the project, appropriate LC-MS/MS was used.

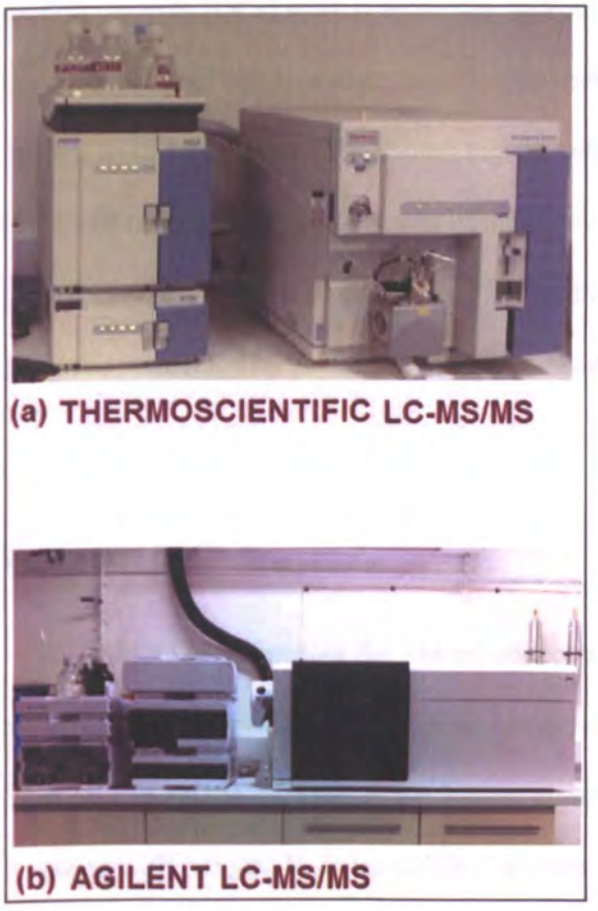

Figure 2. 2: Photographs of the two LC-MS/MS instruments used in the project

2.6. METHOD VALIDATION

The performances of all the methods described in the project were validated according to the Food and Drug Administration (FDA) guidelines for the following set of parameters: linearity, lower limit of quantification (LLOQ), lower limit of detections (LLOD), accuracy, intra-day precision, inter-day precision, selectivity and extraction recovery for each analyte [197]. In each study, a negative control representative matrix was used for method development and validation. Samples for calibration curves and quality controls (QCs) were prepared by spiking the negative control matrix with known concentrations of target analyte and IS. Calibration curves were obtained by plotting the analyte to IS ratio against the known concentrations of analyte in each sample. The analyte to IS ratio for each analyte was obtained by dividing the peak area of analyte by the peak area of the IS. The linearity of all the methods was assessed using linear regression analysis, performed by using LC-MS/MS software.

LLOQ and LLOD

LLOQ is defined as the lowest concentration of the analyte which can be reliably quantified and which gives a peak response with a signal to noise ratio (S/N) of at least 10. LLOD is defined as the lowest concentration of analyte which gives a peak response with a signal-to-noise (S/N) of at least 3 [197].

Accuracy and precision

Accuracy and precision of the analytical method is determined by analysing QC samples at three concentration levels, equally distributed over the linear range, in replicates (six per concentration level). For accuracy, the determined values are compared with the known concentration values. Accuracy is referred to as the closeness of the determined value to the known value of the analyte under the given analytical conditions. According to the FDA guidelines, the mean determined value should not deviate more than 15% from the known value except at the LLOQ where it should be within 20% of the true value [197].

Precision is referred to as the closeness of the individual measures of an analyte when the analytical method is applied repeatedly to multiple aliquots of samples. Precision is further divided into inter-day (repeated for three consecutive days) and intra-day precision (repeated on the same day) and is evaluated by calculating the relative standard deviation (RSD). According to the FDA guidelines, the precision calculated at each concentration level should not exceed 15% of the RSD except at LLOQ, where it should not exceed more than 20% of the RSD [197].

Selectivity

Selectivity of the method is referred to as the degree to which the analytical response of target analytes is unaffected by contributions from the components of the sample matrix. Such matrix components may produce measurable response at a retention time similar to the analytes and thus may challenge the selectivity of the analytical methods. The selectivity of the methods is confirmed by the absence of interfering peaks at the retention times of analytes and ISs in extracted negative control representa-

tive matrix samples (hair, serum, urine). Some matrix components do not produce detectable response, but its co-elution can suppress or enhance the intensity of analyte/IS response [197]. Such matrix effects were determined by extracting the biological matrix and then spiking it with the analytes followed by analysis. The loss of signal was calculated as follows:

$$\% \text{ Loss of signal} = (A_n - A_m)/A_n \times 100$$

where A_n is the peak area for the analyte in neat solution and A_m is the peak area for the analyte in matrix.

Extraction recovery

Extraction recoveries are determined by spiking the negative control matrix with analytes and IS and then extracting the samples according to the respective LLE methods. The peak area ratios of analyte to IS obtained by analysing these extracted samples are compared with the peak area ratios of analyte to IS obtained by analysing standards solutions (unextracted) of analytes and IS of same final concentrations [197].

CHAPTER 3

DETECTION OF NANDROLONE AND STANOZOLOL IN HUMAN HAIR USING LC-MS/MS

3.1. INTRODUCTION

Nandrolone and stanozolol are two of the most widely used exogenous AASs for performance enhancement [13]. According to 'The 2013 Prohibited List' of the World Anti-Doping Agency (WADA), both the AASs belong to Class S1.1.a and their use in and out of competition is prohibited [6]. Despite these restrictions, they are still commonly abused by athletes as revealed by numerous AAFs during the past few years. Figure 3.1 presents the percentage (and number) of positive results of nandrolone and stanozolol, within the class of anabolic agents as reported by accredited laboratories from the year 2005 to 2011 [7-13].

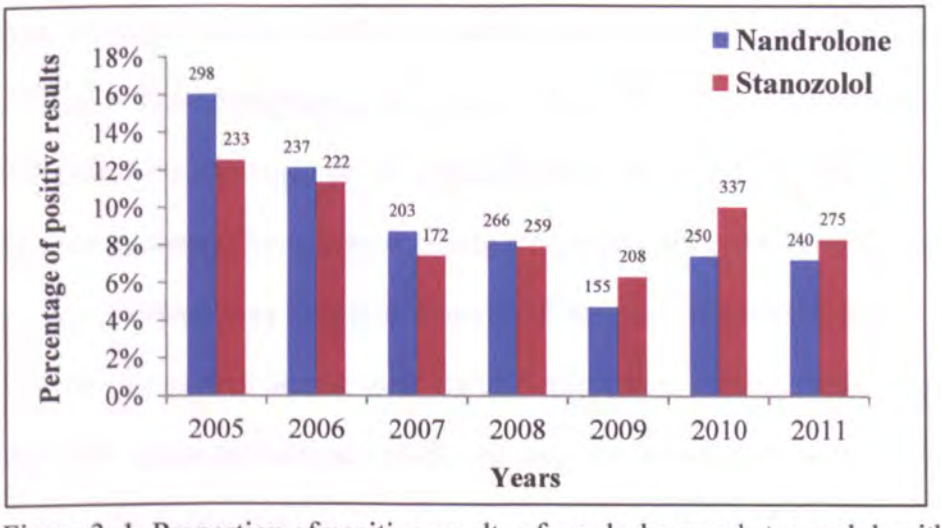

Figure 3. 1: Proportion of positive results of nandrolone and stanozolol, within the class of anabolic agents (from year 2005 to 2011). Numbers above the bars represent the number of positive results of nandrolone and stanozolol reported per year [7-13]

Doping with AASs, particularly nandrolone and stanozolol is an increasing problem for the WADA's anti-doping effort and hence there is a constant need to develop new strategies for the detection, reduction, prevention and intervention of doping with AASs. Social science research studies on doping predominantly rely on self-reports [170, 174-182, 198]. However, self-reported methodology is known to be constrained by the potentially distorting effect of socially desirable responding. Owing to the fear of consequences, most users can deny or under-report illicit drug consumption. Thus, self-reported information can lead to erroneous assumptions about doping in the society. Owing to this, the validity of self-report methodology is often challenged and it is recommended that the self-reported information should be verified using appropriate biochemical analysis. Previously reported validity studies of self-reported drug use behaviour have employed chemical analysis for the detection of mainly social drugs in urine, blood, saliva or hair [177-182].

Though the standard method for detecting doping with AASs is urinalysis [60, 71], it is vulnerable to confounding doping results. For instance, once in the body, stanozolol gets rapidly metabolised and the metabolites are generally detected in urine until ca. 6 days (depending on the dose) [199]. AASs are so called 'training drug' which are taken for a prolonged period, typically in cycles, during preparation, in order to obtain the desired performance-enhancing effects [49, 200]. Owing to the inability of urinalysis in determining long term histories of drug use, it generally fails to identify individuals who partake in long term use of AASs during training periods and deliberately stop their intake to result in a sufficient drug free period prior to providing their urine samples for testing [99].

Genetic and metabolic variations are known to influence urinalysis based doping tests (as discussed in section 1.8.1) [40, 45, 99, 100, 130, 131]. Urinalysis is vulnerable

to give ambiguous doping results owing to single accidental intake of AASs or their metabolites through contaminated food or water [201]. It has been found that nutritional supplements that are generally taken by athletes are either accidentally contaminated (during manufacturing, transportation or packaging) or deliberately adulterated with AASs in an attempt to improve the effectiveness of supplement products. De Cock et al. carried out a study and found that urinalysis reported positive results for healthy volunteers who were administered with a nutritional supplement that was found to contain 19-nor-4-androstene-3, 17-dione as a non labelled ingredient [100]. Urinalysis confirmed the presence of 19-norandrosterone (nandrolone metabolite) at a concentration above 2 ng/mL. According to the WADA, urinary concentrations of nandrolone or its metabolite exceeding 2 ng/mL indicates offence [60]. Similarly, Colker *et al.* reported that short term administration of 19-nor-4-androstene-3, 17-dione can result in a positive drug test result after urinalysis [202].

These findings highlights the drawbacks of urinalysis in determining doping with AASs and also indicates that if urinalysis is employed to verify self-reported drug use information, the results are susceptible to be misleading. On the other hand, it is well known that hair analysis (compared to urine, blood or saliva testing) has the unique potential for determining the retrospective history of drug use (depending on the length of hair analysed). Hair can be collected under close supervision without embarrassment and is not vulnerable to evasive maneuvers such as temporary abstention, excessive fluid intake, and substitution or adulteration of specimens. Hair analysis is also not subject to evidential false positives, such as those caused by single accidental intake of contaminated food or drinks [203]. Furthermore, the samples are easy to store and are free of biohazards. Hence, it is suggested that hair analysis, either alone or in combination with

urinalysis [132-134], can be an effective approach for detecting doping and thus for validating athletes' self-reported doping attitude and drug use information.

Thus, to support social science studies on doping [170, 175] the aim of this work was to develop sensitive, specific and reproducible methods for the detection and quantification of two of the most widely used synthetic AASs, nandrolone and stanozolol, in human hair using ELISA and LC-MS/MS. The main advantages of ELISA in large scale sample screening operation involve speed, low cost and simplicity. Hence, the hair samples can be initially screened by ELISA. All positive samples can then be confirmed using the robust, quantitative technique of LC-MS/MS.

These methods were employed for the first time for verifying self-reported doping informations by analysing the participants' hair samples for the presence of two of the widely used AASs [170, 175]. However, the work presented in this chapter mainly focuses on the analytical findings.

3.2. MATERIALS AND METHODS

3.2.1. Chemicals and reagents

Reference standards of nandrolone, stanozolol and stanozolol D3 (IS) were obtained from LGC standards (Teddington, UK). Sodium hydrogen phosphate heptahydrate, sodium phosphate monobasic dihydrate, sodium hydroxide, hydrochloric acid, HPLC grade deionised water, dichloromethane, pentane, formic acid and acetonitrile were obtained from Sigma Aldrich (Poole, UK). ELISA kits for nandrolone and stanozolol were obtained from Neogen Corporation (Lexington KY 40511 USA). Each kit comprised of a specific enzyme immunoassay (EIA) buffer, wash buffer concentrate,

drug-enzyme conjugate, 3, 3', 5, 5' tetramethylbenzidine (TMB) substrate, stop solution and antibody coated plate containing 96 wells.

3.2.2. Preparation of standard solutions

The reference standards of nandrolone (1 mg/mL in acetonitrile) and stanozolol (1 mg/mL in 1, 2-dimethoxyethane) were used as stock solutions. These stock solutions were diluted with acetonitrile to obtain two working solutions at concentrations of 1000 ng/mL and 10 ng/mL, for each drug. The working solutions of nandrolone and stanozolol at a concentration of 1000 ng/mL were used for optimising the mass spectrometric parameters. The working solutions of nandrolone and stanozolol, at a concentration of 10 ng/mL were used to optimise the chromatographic parameters. Samples for calibration curve were prepared from the stock solutions by diluting with acetonitrile. QC samples were prepared similarly, but from separate stock solutions. A stock solution of stanozolol D3 (0.1 mg/mL in 1, 2-dimethoxyethane) was diluted with acetonitrile to obtain working solutions of 1000 and 10 ng/mL. The former working solution was used for optimising the mass spectrometric parameters and the latter was used to optimise the chromatographic conditions for the IS. Stock solutions were stored at -20^{0}C in amber, silanised, glass vials and all diluted solutions were stored at 4^{0}C in amber, silanised, glass vials.

3.2.3. Hair specimens

Hair samples analysed in this study were obtained from 180 European subjects (108 males, 72 females, 62% athletes) as part of social science studies [170, 175]. Hundred and fifteen partipitants were from Hungary (mainly Budapest) and the remaining 65 were from the UK. The participants were asked to answer a questionnaire linked to doping and also to provide their hair samples. Further details on hair sample collection is provided in section 2.2. The age of the participants ranged between 18 and 53 years. A positive hair control sample for stanozolol was obtained from a male body builder who reported use of stanozolol for the past 30 years by oral and intramuscular route. Blank hair samples were obtained from healthy, non-athlete volunteers.

3.2.4. Analysis

Hair samples were initially decontaminated by rinsing them with dichloromethane (section 2.4.1). After decontamination, hair samples were cut (using scissors) into *ca.* 1 mm segments which were then used to carry out both ELISA screening as well as LC-MS/MS confirmatory analysis.

3.2.4.1. Enzyme linked immunosorbent assay (ELISA)

For ELISA screening, 50 mg of hair segments were incubated with 1 mL of 1 M sodium hydroxide at 95 ^{0}C for 15 minutes. The homogenate was neutralised with hydrochloric acid and then diluted with EIA buffer (1:1 v/v) prior to screening. The ELISA screening system consisted of a Cary 50 MPR microplate reader (Varian, UK). The

manufacturer's protocol was followed for analysis [204]. The ELISA kit employed operated on the basis of competition between the drug in the hair matrix and the drug-enzyme conjugate for a limited number of antibody binding sites on the microplate. Initially, the samples were added to the wells followed by addition of the drug-enzyme conjugate. After incubation, the wells were washed using the wash buffer supplied with the kit. Washing was essential to remove any unbound drug from each well. The presence of bound drug-enzyme conjugate was recognised using TMB. The enzymatic reaction was stopped after 30 minutes using a stop solution. Figure 3.2 represents a schematic diagram of the principle of ELISA [204]. The extent of colour development is inversely proportional to the amount of AAS present in the hair. The absorbance of each well was measured at a wavelength of 450 nm.

A calibration curve and QCs were prepared in EIA buffer to ensure the kit was working properly. Controls were made by spiking blank hair preparations with known concentrations of selected drug. Hair samples were first screened by ELISA and all positive samples were then analysed on LC-MS/MS for confirmation (quantification).

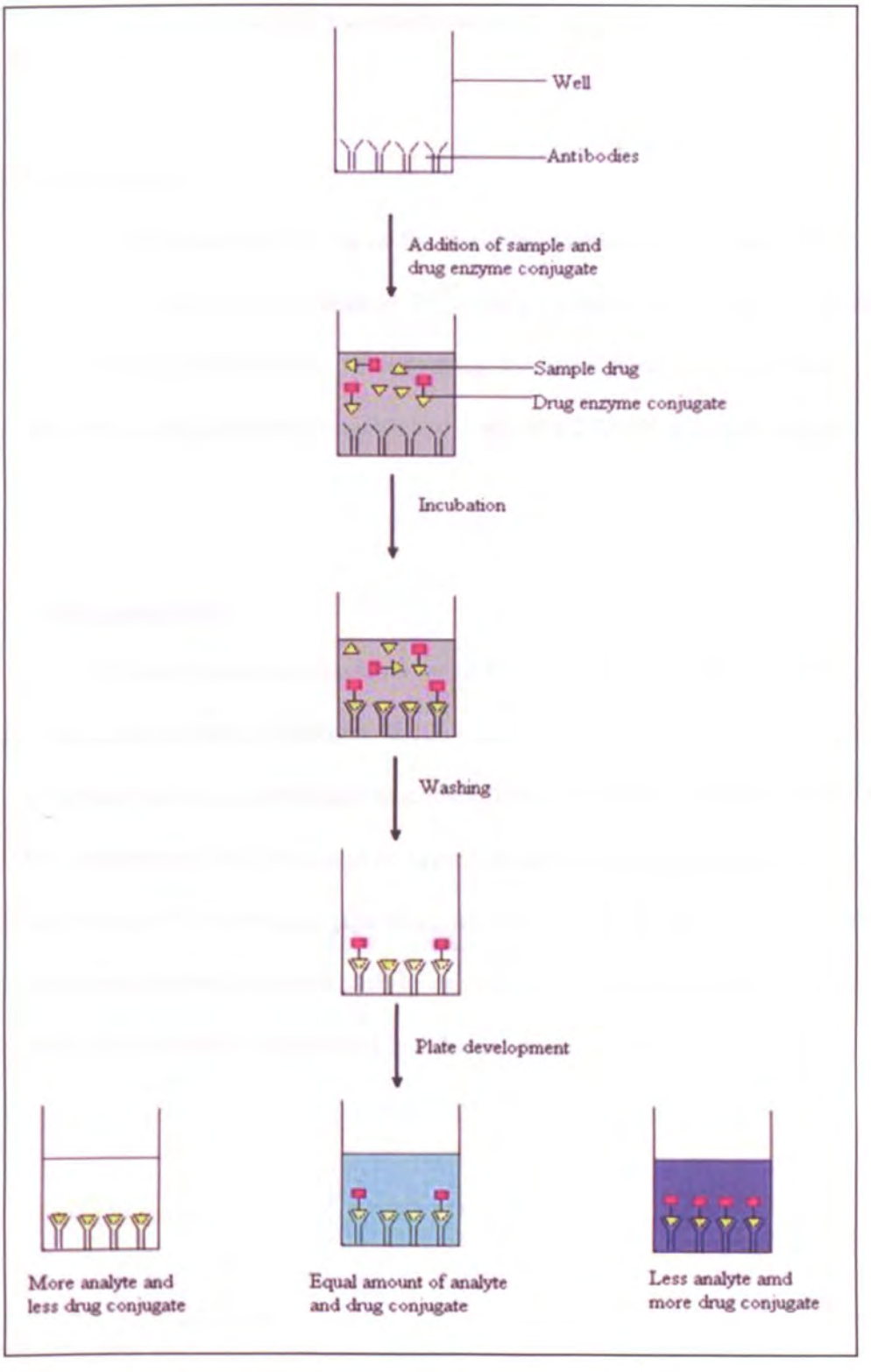

Figure 3. 2: Schematic diagram showing ELISA principle [204]

3.2.4.2. Confirmation of ELISA results using LC-MS/MS

Alkali digestion

For confirmation, 20 mg of the decontaminated hair segments were incubated with 1 mL 1 M sodium hydroxide at 95 ^{0}C for 15 minutes in the presence of stanozolol D3, which was used as an IS. After cooling, the homogenate was neutralised with 1 M hydrochloric acid followed by addition of 2 mL of 0.2 M phosphate buffer (pH 7.0).

Sample purification

The homogenate was purified by LLE using 3.5 mL pentane. After vortex mixing and centrifugation (4 minutes at 1257 x g) at room temperature, the pentane layer was transferred into a fresh glass tube after filtering through a syringe driven, 0.45 μm PTFE membrane filter. The organic layer was then dried by heating at 50 ^{0}C under a gentle stream of nitrogen gas. The extracted residue was reconstituted with 100 μL acetonitrile and filtered through a syringe driven, 0.2 μm PTFE membrane filter. A 4 μL aliquot of the solution was injected into the LC-MS/MS system.

LC-MS/MS analysis

A Thermoscientific LC-MS/MS system was used in this study (details in section 2.5).

Liquid-chromatography conditions

The two mobile phase solvents used were acetonitrile (solvent A) and water with 0.1 % formic acid (solvent B). The gradient flow composition is shown in Table 3.1. The total flow rate through the column was 100 μL/min. An SB-C18 column (2.1 x 50 mm, 1.8 μm) was used for analysis. To prevent the column from blocking, a 0.2 μm in-line filter was installed prior to the column. The column oven temperature was maintained at 60 ^{0}C. The autosampler was maintained at 4 ^{0}C. Acetonitrile was used as a solvent for syringe wash and needle wash after each injection. The autosampler was programmed to perform syringe and needle wash after each injection. The LC was interfaced with the MS/MS system without a flow split.

Table 3. 1: LC mobile phase gradient composition

LC run time (min)	Solvent A (%)	Solvent B (%)
	Acetonitrile	Water (0.1 % formic acid)
0	50	50
10	80	20
11	100	0
12	50	50
15	50	50

Mass spectrometric conditions

The mass spectrometer was operated in the positive electrospray ionisation mode at a spray voltage of 4500 V and capillary temperature of 350 ^{0}C. The protonated molecules, $[M+H]^{+}$, of nandrolone (m/z 275.2), stanozolol (m/z 329.2), and stanozolol D3 (m/z 332.2) generated, act as precursor ions for collision induced dissociation (CID) for MS/MS analysis. Selective reaction monitoring (SRM) was used to monitor the precursor ions and diagnostic product ions for the confirmation of analytes. The most abundant SRM ion transitions for each analyte were acquired using the collision energies given in Table 3.2.

Table 3. 2: Retention times, SRM transitions and conditions of nandrolone, stanozolol and stanozolol D3

Analytes	Retention time (min)	Transition (m/z)	Collision energy (eV)
Nandrolone	3.53	275.2 → 109.2	27
Stanozolol	4.74	329.2 → 81.1	50
Stanozolol D3 (IS)	4.79	332.2 → 81.2	42

3.2.5. Method validation

The performance of the assays were validated according to the FDA guidelines for the following set of parameters: LLOQ, LLOD, selectivity, accuracy, intra-day precision, inter-day precision, linearity and extraction recovery for each analyte [197]. Blank hair samples obtained from healthy, non-athlete volunteers were initially analysed to confirm that they are drug free. These drug-free hair samples were used as negative controls for developing and validating the method. To validate the method, cali-

bration samples were prepared by spiking 20 mg drug-free hair specimens with known amounts of working standard solutions of nandrolone, stanozolol and IS. QC samples were prepared similarly at three concentration levels equally distributed over the linear range. Samples for calibration curve and QCs were treated in a way similar to unknowns. A calibration curve for each target analyte was constructed by plotting the analyte to IS ratio against the known concentrations of analyte in each sample. The analyte to IS ratio for each analyte was obtained by dividing the peak area of analyte by the peak area of the IS. Seven calibration points (5, 10, 20, 50, 100, 200 and 400 pg/mg) were used for plotting each calibration curve for nandrolone, whereas for stanozolol, 9 calibration points per curve (1, 2.5, 5, 10, 20, 50, 100, 200 and 400 pg/mg) were used. Three sets of spiked samples were prepared, each on a different day. The linearity of the calibration curve for each target analyte was investigated by using linear regression analysis.

Accuracy, intra-day precision and inter-day precision of the method for nandrolone and stanozolol were determined at three concentration levels equally distributed over the linear range. For determining the accuracy of the method, six replicates per concentration levels were analysed and the mean calculated values obtained were compared with the respective known concentrations. Intra-day precision was determined by analysing six replicates per concentration levels, on the same day. Inter-day precision was evaluated by measuring six replicates per concentration levels, on three consecutive days. Inter-day and intra-day precision was estimated from the calculation of RSD. LLOQ for nandrolone and stanozolol was defined as the lowest concentration which could be reliably quantified with a S/N of at least 10, precision not exceeding 20% of the RSD and accuracy not deviating more than ± 20% of the actual value. LLOD for nandrolone and stanozolol was defined as the lowest concentration which could be reli-

ably differentiated from the background noise by giving a peak response equivalent to 3 times to that of the background noise (i.e. S/N $\geq$3).

The extraction recoveries were determined at three concentration levels by spiking the blank hair samples with known concentrations of analytes and IS. The mixtures were then subjected to alkali digestion, LLE followed by analysis. The ratios of peak areas of analytes to IS were determined and compared with the ratios of peak areas of analyte to IS of standard solutions prepared in acetonitrile at same final concentrations. The components of the matrix may interact, either chemically or physically, with the analytes. These interactions may suppress or enhance the response of the analytes. To determine such matrix effects, negative control hair samples were extracted (without analytes and IS). The extracted residue was spiked with known amount of analytes and IS. The peak areas of analytes and IS obtained after analysis were then compared with the peak areas obtained after analysing standard solutions of analytes and IS at same theoretical concentration.

The method selectivity was confirmed by the absence of interfering peaks at the retention times of analytes and IS in extracted blank hair samples. Stability of the analytes in the hair matrix was assessed by storing the extracted hair samples (spiked with analytes and IS) at 4^0C and room temperature at three time points: 24 hours, 7 days and 14 days, away from light. These samples were analysed daily for up to 14 days to determine a period after which the sensitivity of the analytes begins to vary, potentially due to matrix components and storage conditions.

3.3. RESULTS AND DISCUSSION

3.3.1. Method development

The ELISA kits were successfully employed for the qualitative analysis of AASs. The results for calibration curves and QC samples were within the range specified by the kit manufacturer. This ensured that the kits were working properly. Negative control and positive control samples were prepared in hair. Any measurements higher than the positive control were suspected positive and were confirmed using LC-MS/MS. Quantitative analysis using LC-MS/MS was based on the retention times of analytes and the relative abundance of their respective product ions.

Optimisation of chromatographic and mass spectrometric conditions

A combination of acetonitrile and water as mobile phase solvent A and B respectively was examined. Different gradient and isocratic mobile phase compositions were investigated. Addition of formic acid (0.001% v/v, 0.01% v/v and 0.1% v/v) to solvent A and/or solvent B did not influence sensitivity drastically. However, maximum sensitivity with good peak shape were obtained when acetonitrile was used as solvent A and water with 0.1% formic acid was used as solvent B under the conditions shown in Table 3.1. Though addition of formic acid (0.1% v/v) to water did not influence sensitivity, it was used to prevent algae growth in water. Different mobile phase flow rates ranging from 100 uL/min to 250 uL/min were investigated. Flow rates higher than 200 uL/min led to negligible decrease in sensitivity. No drastic changes in the sensitivity

were observed when using flow rates between 100 μL/min to 200 uL/min. Hence, to save organic solvents, a flow rate of 100 μL/min was used.

The intensity of each compound (nandrolone, stanozolol and stanozolol D3) was optimised by varying the mass spectrometric conditions, such as capillary temperature, spray voltage, auxiliary gas pressure, sheath gas pressure, tube lens voltage and skimmer offset voltage. For this, a 1000 ng/mL solution of each compound was infused directly into the mass spectrometer in presence of mobile phase (100 μL/min). While infusing the compounds, different mass spectrometric conditions were investigated and the conditions which gave highest intensity for all the compounds were employed. The optimised parameters are summarised in Table 3.3.

Table 3. 3: Optimisation of mass spectrometric parameters for the analysis of nandrolone, stanozolol and stanozolol D3

Parameters	Range investigated	Optimised conditions
Capillary temperature (^{0}C)	300 to 400	350
Spray voltage (V)	3500 to 5000	4500
Auxiliary gas pressure (psi)	5 to 55	5
Sheath gas pressure (psi)	10 to 60	10
Tube lens voltage (V)	-10 to 240	105
Skimmer offset voltage (V)	0 to 50	14
Collision pressure (mTorr)	0 to 3	1.5
Collision energy (eV)	0 to 70	Please see Table 3.2

Figure 3.3 represents the product ion spectrum (mass range m/z 50-350) of the protonated molecules of nandrolone and stanozolol. The most abundant product ions for nandrolone and stanozolol were at m/z 109.20 and 81.10 respectively. The possible fragmentation pathway is also depicted in Figure 3.3 (with the help of red line) . Operating the mass spectrometer in MRM mode enhanced the method selectivity, sensitivity and specificity.

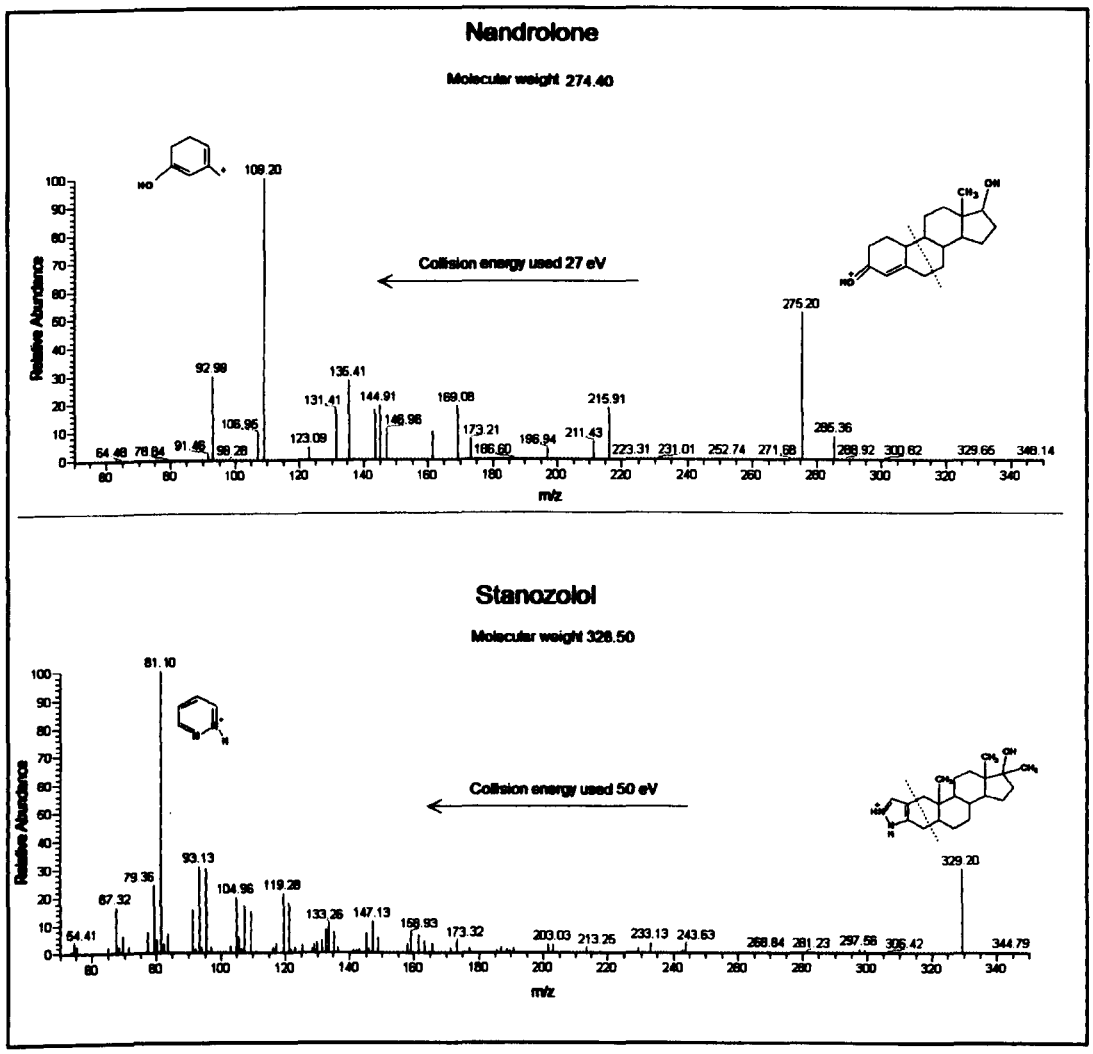

Figure 3. 3: Positive product ion spectrum (mass range m/z 50-350) of nandrolone and stanozolol

Generally, drugs are extracted from hair by solubilisation or digestion of the hair itself. Alkali and enzymatic digestion lead to complete dissolution of hair, which is generally known to give good recoveries of drugs entrapped in the hair matrix [136, 137, 146, 205]. Some drugs like mephedrone, heroin, cocaine and benzodiazepines are known to degrade under alkali conditions [184]. For such drugs, enzymatic digestion or solvent extraction are known to work well. Enzymatic digestion is a softer technique and is proposed to be a universal extraction procedure for all substances. However, enzymatic digestion can be time consuming and expensive, compared to other methods. Hence, it may be used for the extraction of those drugs that at are susceptible to degradation owing to the harsh conditions or for those drugs that may give poor recoveries by direct solvent extraction. Acidic hydrolysis and direct extraction using organic solvents like methanol have also been employed for drug extraction. Such techniques are capable of extracting the entrapped drugs without hair dissolution, which may result in poor recoveries [206, 207].

In this study alkali digestion was employed for the extraction of nandrolone and stanozolol to ensure good recoveries. However, owing to complete dissolution of hair, components of hair matrix in the solution may interfere with the analysis of drugs. Thus, an effective sample purification step is essential for good results. Extraction efficacies of hexane and pentane were investigated. When LLE was performed using hexane as the organic solvent, the extraction recoveries of stanozolol and nandrolone at LLOQ concentration levels were found to be 66.2% and 31.0% respectively. However, extraction recoveries of stanozolol and nandrolone at LLOQ concentration levels with pentane were slightly better than hexane and were found to be 79.8% and 41.7% respectively. Thus, LLE using pentane was an effective sample purification step as presented in Table 3.5.

During the early stages of method development, centrifugation was carried out using 15 mL polyethylene teraphthalate (PET) centrifuge tubes. However, the pentane layer was observed to get turbid, indicating potential dissolution of the plastic tube. Also, after the evaporation of pentane layer, a sticky residue was left which may block the analytical column. Hence, glass centrifuge tubes with PTFE lined screw caps were used throughout the project to prevent such potential risks of column blockage and contamination of mass spectrometer. In addition, the reconstituted extract was filtered through a syringe driven 0.2 μm PTFE filter membrane before injecting into the LC-MS/MS system and an inline column filter (0.2 μm) was installed prior to the column.

Sporadically, during LLE, vortex mixing pentane and neutralised hair homogenate led to formation of gel like substance. Centrifugation at room temperature did not get rid of the gel at all times. In such cases, the separation of pentane layer was made difficult leading to sample loss. To avoid this, such samples were kept in an ultrasound bath for up to 20 seconds at room temperature to disintegrate the gel like mass followed by centrifugation. Thus, the separation of pentane layer was made facile and sample loss was prevented. Stanozolol D3 was used as an IS to compensate for any: i) loss of analytes during sample preparation, ii) any matrix effects and iii) variations in the instrument response from injection to injection. However, under the chromatographic conditions employed in the method, there were no detectable interferences by extractable endogenous materials present in hair that affected the analysis of nandrolone and stanozolol.

Owing to the efficient hair decontamination step employed, no external interferences were observed with hair analyses. The presence of external contaminants on the hair surface was investigated by collecting the washings of ten random hair samples and pooling them. The mixture was dried, reconstituted with 100 μL of acetonitrile, filtered

through 0.2 μm PTFE membrane and analysed. There were no detectable interference peaks at the retention times of nandrolone, stanozolol and stanozolol D3. Overall, decontamination, alkali digestion of hair followed by purification using LLE combined with injection of 4 μL aliquot through the column were analytical prerequisites for proficient identification of target AASs in the hair matrix.

To prevent carry over effects from previous injections, blank acetonitrile was injected periodically in between samples. In addition, a washing method was developed to ensure that the LC-MS/MS system, mainly the mass spectrometer and column, is not contaminated due to large number of back to back injections. In this method, 4 μL acetonitrile (blank) was injected in to the LC-MS/MS system. The mobile phase composed of 80% acetonitrile and 20% water (with 0.1% formic acid) at a flow rate of 100 μL/min. The total run time was 20 minutes during which the ESI source was operated in positive ion mode for 10 minutes and in negative ion mode for the remaining 10 minutes. The mass spectrometer was operated in full scan mode (mass range m/z 50 to 1000). After the washing run, the column was equilibrated with 50 % acetonitrile and 50 % water (0.1% formic acid) for 10 minutes before injecting the next sample. The washing method was run after every 4 samples.

3.3.2. Method validation

The assay for nandrolone was linear in the range 5 to 400 pg/mg with determination coefficient (r^2) values higher than 0.9967. The assay for stanozolol was linear in the range 1 to 400 pg/mg with r^2 values greater than 0.9984. Figure 3.4 represents calibration curves, regression equations and r^2 values obtained during method validation.

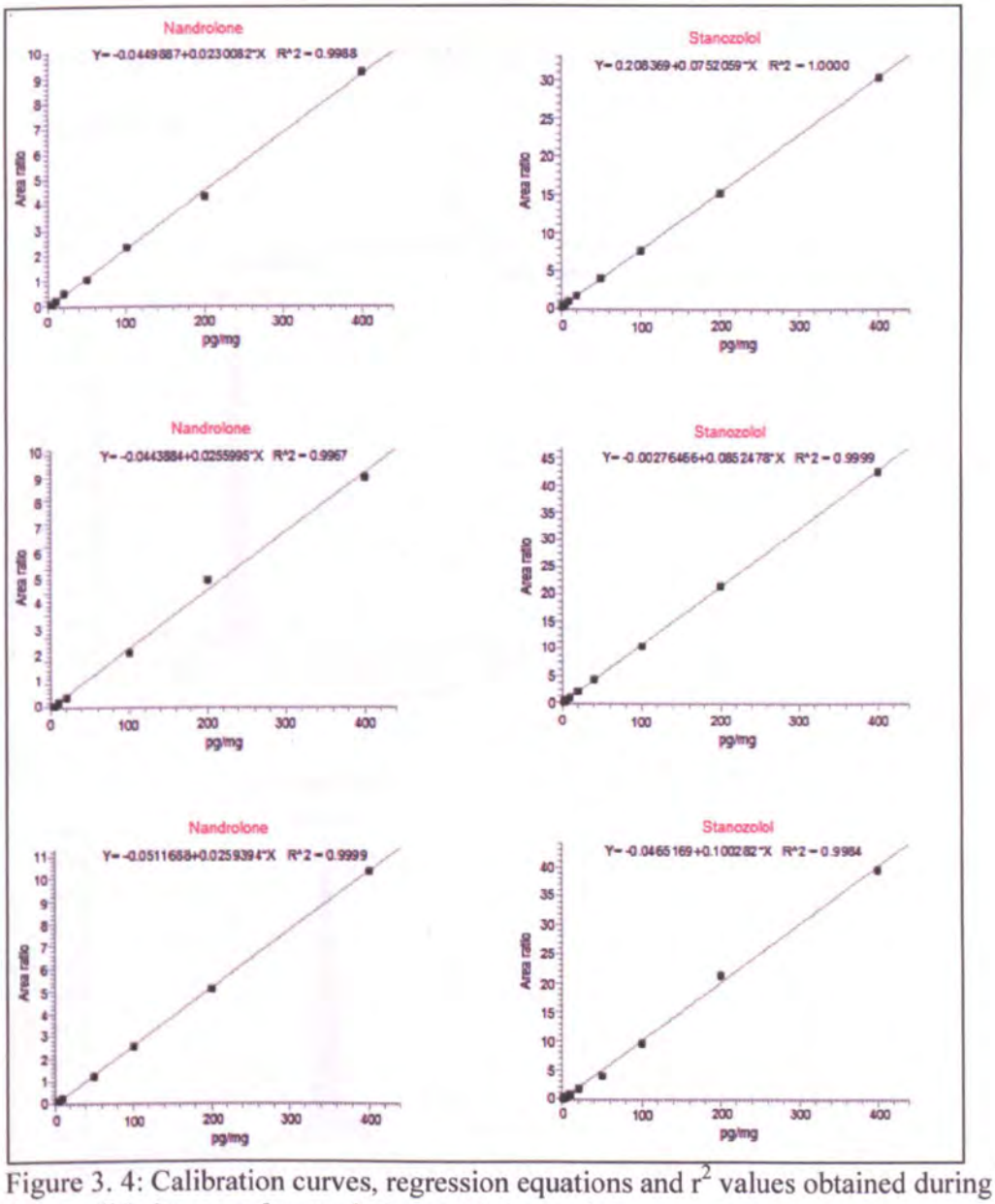

Figure 3. 4: Calibration curves, regression equations and r^2 values obtained during the three validation runs for nandrolone and stanozolol

The assays were capable of detecting (LLOD) 3.0 pg nandrolone and 0.5 pg stanozolol per mg of hair. The average S/N values (N=6) of nandrolone and stanozolol at LLOD concentration levels were 7 and 8 respectively. The LLOQ values for nandrolone and stanozolol were 5 pg/mg and 1 pg/mg respectively. The average S/N values (N=6) of nandrolone and stanozolol at LLOQ concentration levels were 12 and 17 respectively. Figure 3.5 represents chromatograms of nandrolone and stanozolol at LLOQ concentration level, in human hair matrix, thus demonstrating that the method is selective and there are no interferences at the retention times of analytes, which may affect analysis.

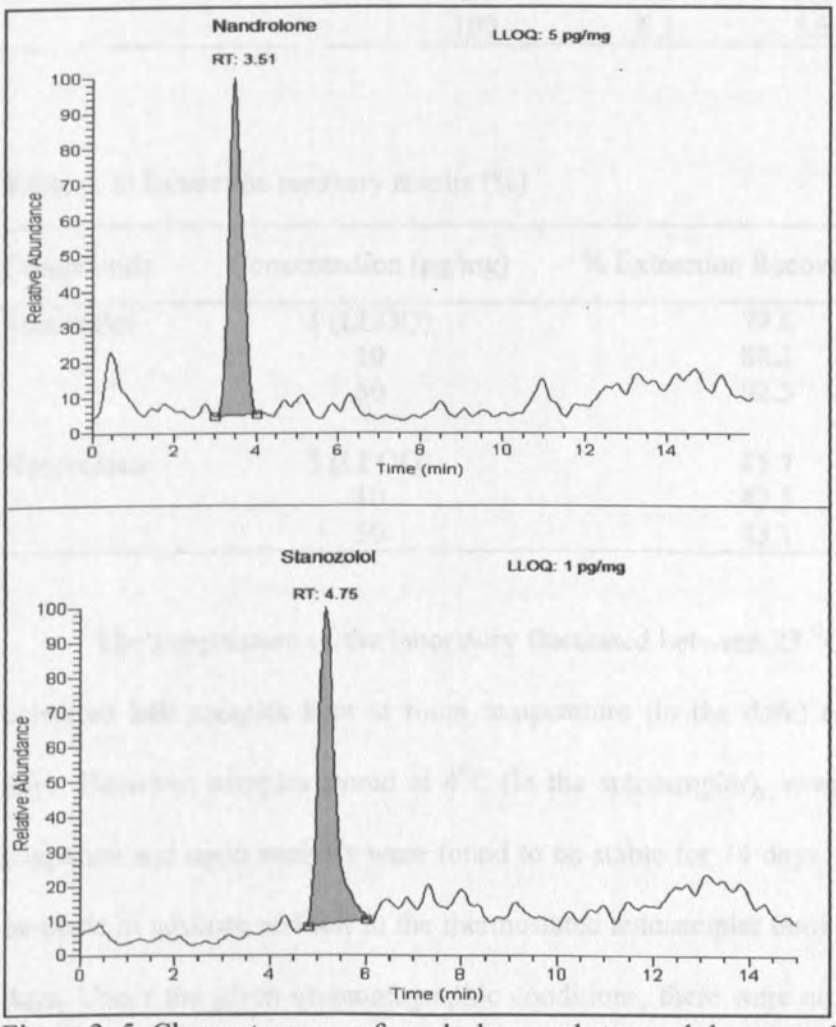

Figure 3. 5: Chromatograms of nandrolone and stanozolol extracted from blank hair spiked at LLOQ concentrations (5 pg/mg nandrolone and 1 pg/mg stanozolol)

The accuracy, inter-day precision and intra-day precision of the analytical method were within the limits set by the FDA guidelines and are summarised in Table 3.4. The extraction recoveries are summarised in Table 3.5.

Table 3. 4: Summary of assay validation results

Compounds	Linear range (pg/mg)	Concentration (pg/mg)	Precision RSD (%)		Accuracy (%)
			Intra-day N=6+6+6	Inter-day N=18+18+18	
Stanozolol	1-400	2.5	6.3	9.6	103.9
		20	11.4	6.7	92.9
		100	7.6	7.3	92.2
Nandrolone	5-400	10	9.1	8.8	99.4
		20	8.6	7.4	97.5
		100	6.3	5.5	106.7

Table 3. 5: Extraction recovery results (%)

Compounds	Concentration (pg/mg)	% Extraction Recovery (N=6)
Stanozolol	1 (LLOQ)	79.8
	10	88.2
	50	82.5
Nandrolone	5 (LLOQ)	41.7
	10	42.5
	50	43.1

The temperature of the laboratory fluctuated between 23 ^{0}C to 30 ^{0}C and hence extracted hair samples kept at room temperature (in the dark) evaporated before 14 days. However, samples stored at 4^{0}C (in the autosampler), away from light, did not evaporate and upon analysis were found to be stable for 14 days. Thus, samples could be made in advance and left in the thermostated autosampler until analysis for up to 14 days. Under the given chromatographic conditions, there were no matrix interferences affecting the analysis.

3.3.3. Application to real samples

The analytical method comprising of ELISA screening and LC-MS/MS confirmation was applied to the analyses of 180 hair samples. These hair samples were obtained from individuals who participated in social science studies [170, 175] based on survey questionnaires. Amongst the 180 hair samples screened by ELISA, 16 were found to be positive for stanozolol and 3 for nandrolone. The ELISA results are detailed in the appendix (Table 3.1A and Table 3.2A). In order to avoid any false positive results due to cross reactivity in ELISA, these 19 samples were analysed on LC-ESI-MS/MS for confirmation.

Typical chromatogram and CID spectrum of nandrolone and stanozolol obtained by analysing hair samples of athletes screened positive by ELISA are depicted in Figure 3.6 and 3.7 respectively. Twelve athletes were successfully confirmed positive for stanozolol and 1 for nandrolone. An athlete who admitted use of stanozolol for the past 30 years by oral and intramuscular routes was considered as a positive control. However, since stanozolol and nandrolone are exogenous AASs, any detectable amounts may suggest doping. Table 3.6 shows the quantity of AASs detected in hair. The results suggest that ELISA screening led to six false positive results. Hence, it is essential to confirm the ELISA results using a more reliable technique like LC-MS/MS. Also, it is arguable that ELISA may lead to false negative results, this questions the reliability of ELISA for screening purposes as well.

Results indicated that most of the athletes who abused stanozolol were females. This could be owing to the suggestions that stanozolol binds with lesser affinity to androgenic receptors and is associated with lesser androgenic side effects, compared to other commonly abused AASs like testosterone and nandrolone [38].

Table 3. 6: Hair analysis results

	Sex	Age	Stanozolol		Nandrolone	
			ELISA screening	LC-MS/MS pg/mg	ELISA screening	LC-MS/MS pg/mg
Control *	M	53	Positive	47.4	Negative	NA
Sample 23	F	21	Positive	BDL	Negative	NA
Sample 30	F	25	Positive	BQL	Negative	NA
Sample 32	F	20	Positive	86.3	Negative	NA
Sample 35	F	19	Positive	9.8	Negative	NA
Sample 39	F	22	Positive	10.0	Negative	NA
Sample 41	F	20	Positive	56.1	Negative	NA
Sample 50	M	23	Positive	BDL	Positive	BDL
Sample 53	F	18	Positive	BDL	Negative	NA
Sample 56	M	19	Positive	11.2	Negative	NA
Sample 69	F	22	Positive	63.3	Negative	NA
Sample 72	F	20	Positive	26.9	Negative	NA
Sample 78	F	20	Positive	12.7	Negative	NA
Sample 79	M	21	Positive	33.0	Negative	NA
Sample 80	F	18	Negative	NA	Positive	14.0
Sample 82	M	22	Positive	5.0	Negative	NA
Sample 94	F	22	Positive	BDL	Negative	NA
Sample 134	F	18	Positive	40.2	Negative	NA
Sample 159	M	32	Negative	NA	Positive	BDL

*Male bodybuilder who admitted use of stanozolol for the past 30 years by intramuscular and oral route. NA means not analysed (where ELISA showed negative results). BQL means below quantification level (1 pg/mg and 5 pg/mg for stanozolol and nandrolone respectively). BDL means below detection level (0.5 pg/mg and 3 pg/mg for stanozolol and nandrolone respectively). Results for ELISA screening is presented in the appendix (Table 3.1A and Table 3.2A).

The results indicate that the newly developed LC-MS/MS methods are efficient in detecting nandrolone and stanozolol in hair even at very low levels when only circa 20 mg hair was processed (Figure 3.6 and 3.7). Less hair required for analysis makes the method more convenient for drug testing. Min Shen et al. quantified nandrolone and stanozolol in guinea pig hair at a concentration as low as 10 pg/mg using LC-MS/MS [146]. These newly developed methods for human hair analysis now provide extended

LLOQs and LLODs and hence can be employed more efficiently for doping testing using *ca.* 20 mg human hair. These LC-MS/MS methods for the detection of nandrolone and stanozolol in human hair are novel and reported for the first time in the literature [4].

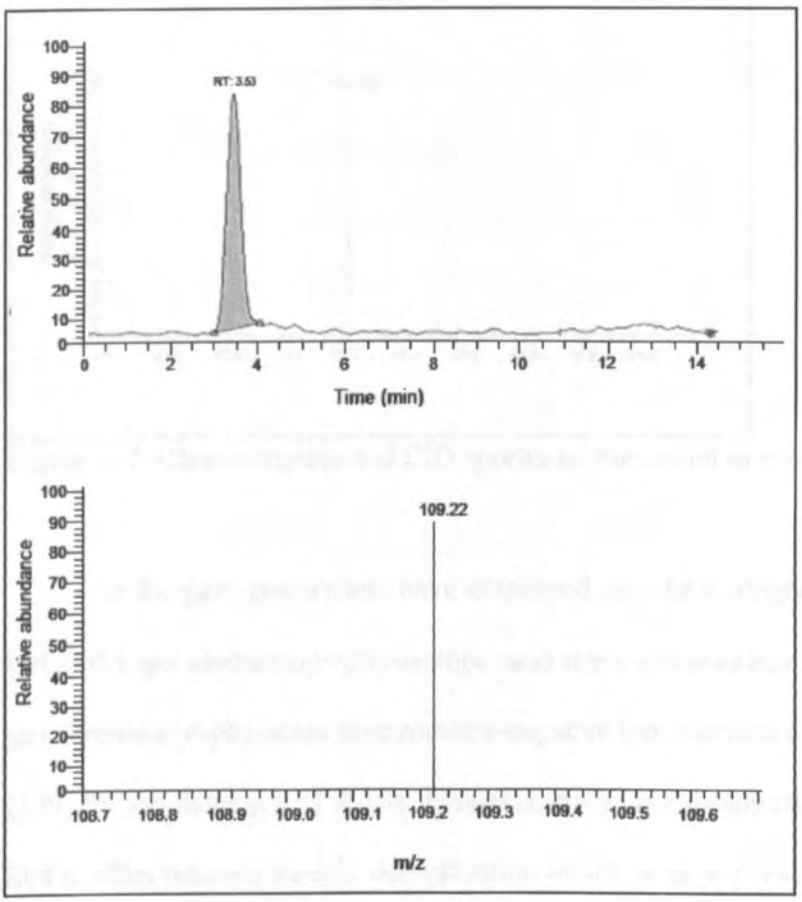

Figure 3. 6: Chromatogram and CID spectra of nandrolone in hair (14.0 pg/mg)

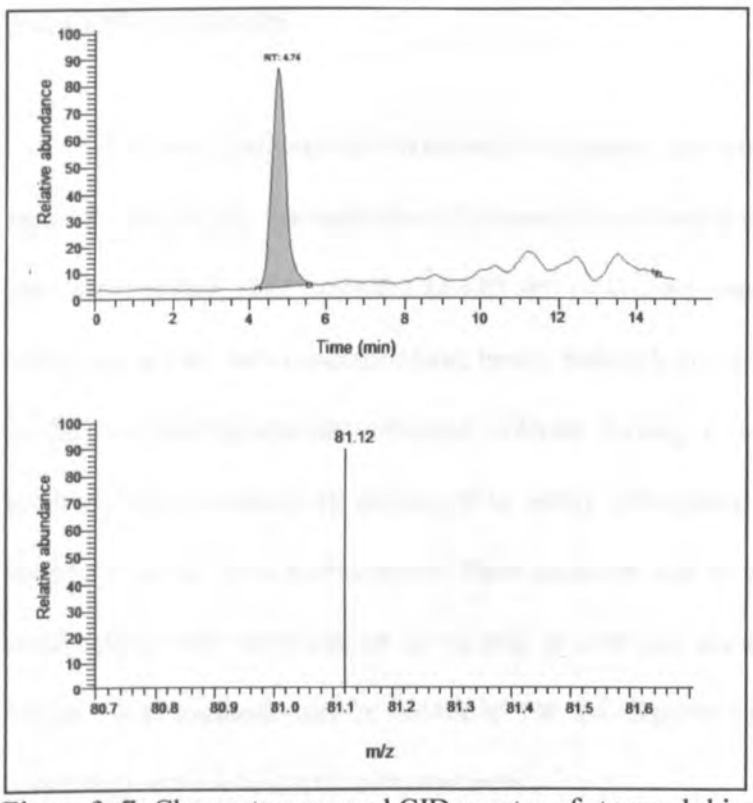

Figure 3. 7: Chromatogram and CID spectra of stanozolol in hair (5.0 pg/mg)

In the past, researchers have employed gas chromatography-mass spectrometry (GC-MS), gas chromatography-isotope ratio mass spectrometry (GC-IRMS) [208, 209], gas chromatography-mass spectrometry-negative ion chemical ionisation (GC-MS-NCI) [139] for the detection of AASs. However, the major disadvantage of using GC-MS is that it often requires sample derivatisation which is an expensive and laborious procedure. Generally, the derivatives are unstable and susceptible to thermal decomposition during analysis, thus affecting the reproducibility of the method. In contrast, LC-MS/MS generally does not require any additional derivatisation step. Thus, LC-MS/MS can be considered as a more economical and feasible approach for analysing AASs.

3.4. CONCLUSIONS

The new methods that have been developed are sensitive, specific, reliable and reproducible for the determination of stanozolol and nandrolone when *ca.* 20 mg human hair is processed. The extended LLOD and LLOQ are capable of detecting these steroids even at very low concentrations, hence reducing the amount of hair required to 14-15 hairs affording sample collection without leaving a noticeable bald patch. These methods were successfully employed to verify participants' self-reported drug use information [170, 175]. Furthermore, these methods can be employed either alone or in combination with urinalysis or blood tests to reinforce the current anti-doping regimes. These novel methods can be extended for the detection and quantification of other commonly used AASs such as testosterone.

CHAPTER 4

DETECTION OF STANOZOLOL AND 3'-HYDROXYSTANOZOLOL IN ENVIRONMENTAL WATERS USING LC-MS/MS

4.1. INTRODUCTION

Growing use of over-the-counter and prescription pharmaceutical drugs has led to growing concerns for the safety of drinking water [210]. It has been reported that high concentrations of pharmaceutical drugs reach waste water treatment plants, which is mainly through human excretions, pharmaceutical manufacturing discharges and improper disposals [211]. Pharmaceutical compounds cannot be eliminated completely in the waste water treatment plants and thus such pharmaceutical substances can reach ground and surface waters. Even modern sewage treatment works are not constructed to specifically eliminate pharmaceuticals from potable water supplies. For the past few years, a vast range of pharmaceutical drugs like bronchodilators, oral contraceptives, steroids, antidepressants, beta-blockers, antibiotics, anti-inflammatory and analgesics have been reported to be present in sewage, surface, drinking and ground waters [212-217]. This suggests that pharmaceutical products are widespread contaminants, which may impose serious concerns to human health and the environment [218].

Stanozolol is one of the most commonly misused exogenous AAS in sport and in veterinary practice, where it is used for growth promoting purposes [68, 110-114]. In humans, 3'-hydroxystanozolol is reported to be one of the major metabolites of stanozolol, which is mainly excreted in urine in the form of glucuronide conjugate. Unlike testosterone (endogenously produced hormone), the synthetic stanozolol and / or its main metabolite should only appear in environmental waters if the former is used for veterinary purposes, taken under medical supervision or illegally by athletes for performance enhancement or if either one or both of these compounds are accidently discharged into environmental waters [123].

Stanozolol has been detected (qualitatively) in the past in sludge samples collected from Huiyang and Meihu waste water treatment plants [115]. Chang *et al.* have also reported the presence of stanozolol in Beijing influent waste water at a concentration of *ca.* 0.54 pg/mL [120]. Recently, Tölgyesi *et al.* have reported the presence of cortisol, dexamethasone, flumethasone, prednisolone and epitestosterone in the River Danube, but their selection of analytes did not include stanozolol [118].

In the previous study discussed in chapter 3, stanozolol was detected in the hair samples collected from subjects living in Budapest (Appendix, Table 3.1A) [4, 170]. More than 1.8 million people living in Budapest receive water from the River Danube, which is the second largest river in Europe. In the past (before July 2010), only 30-40% of the waste water was being treated before flowing into the River Danube [118]. Hence, this study was initiated to investigate whether people in Budapest are accidentally exposed to AASs through contaminated drinking water. Thus, the primary aim of this study was to develop a method for the detection of stanozolol in aqueous matrices using LC-MS/MS. The secondary aim was to develop a method for the detection of 3'-hydroxystanozolol (one of the major urinary metabolite in humans) in aqueous matrices.

4.2. MATERIALS AND METHODS

4.2.1. Chemicals and reagents

Reference standards for stanozolol, 3'-hydroxystanozolol and stanozolol D3 were obtained from LGC standards (Teddington, UK). HPLC grade acetonitrile, water, pentane, chloroform, ethylacetate and formic acid were obtained from Sigma Aldrich (Poole, UK).

4.2.2. Preparation of standard solutions

Solutions for stanozolol, 3'-hydroxystanozolol and stanozolol D3 (IS) were prepared in way similar to that discussed in previous chapter. The reference standards of stanozolol (1 mg/mL in 1, 2-dimethoxyethane) and 3'-hydroxystanozolol (1 mg/mL in methanol) were used as stock solutions. The stock solutions of stanozolol and 3'-hydroxystanozolol were diluted with acetonitrile and methanol respectively, to obtain working solutions at concentrations of 1000 ng/mL and 10 ng/mL for each analyte. The working solutions of stanozolol and 3'-hydroxystanozolol at a concentration of 1000 ng/mL were used for optimising the mass spectrometric parameters. The working solutions of stanozolol and 3'-hydroxystanozolol at a concentration of 10 ng/mL were used to optimise the chromatographic conditions. The stock solutions of stanozolol and 3'-hydroxystanozolol were diluted with acetonitrile and methanol respectively, to prepare samples for calibration curve. QC samples were prepared similarly, but from separate stock solutions. These calibration curve and QC samples were used for spiking negative control water samples for method development and validation. A reference standard of stanozolol D3 (0.1 mg/mL in 1, 2-dimethoxyethane) was used as a stock solution and was diluted with acetonitrile to obtain working solutions of 1000 and 10 ng/mL. The

former working solution was used for optimising the mass spectrometric parameters and the latter was used to optimise the chromatographic conditions and for spiking water samples. Although the mass spectrometric parameters for stanozolol and stanozolol D3 were optimised in the previous study, they were re-optimised for this study as the LC-MS/MS underwent its annual maintenance before the start of this study. Stock solutions were stored at -20^{0}C in amber, silanised, glass vials and all diluted solutions were stored at 4^{0}C in amber, silanised, glass vials.

4.2.3. Water sample collection

Environmental water samples were obtained from Budapest (Hungary) and collected from the River Danube and an urban tap in clean, amber bottles. Water samples were collected periodically from December 2009 to November 2010. Samples from Lake Balaton and spring water (Rózsika forrás, Solymár, near Budapest) were also collected for comparison. Some commonly-consumed, bottled non-carbonated, natural mineral water samples (Evian, Badoit, Perrier, Volvic, Visegradi, Aquasol, Natur Aqua, Theodora, Imola, Dr Szalay, S Pellegrino, Isklar, Highland spring, Drench and Buxton) were purchased from local supermarkets. The majority of the natural mineral waters (bottled) that were analysed, are recognised by the European Union [219]. All water samples were stored at -20^{0}C and protected from light until analysis.

4.2.4. Sample preparation

Stanozolol was extracted from water samples by performing LLE using pentane. Suspended particles were not filtered from the water samples. Thus, the drug adsorbed on them could also be extracted efficiently. A 5 mL aliquot of each water sample was spiked with stanozolol D3 (IS, 50 μL of 10 ng/mL) followed by the addition of 3 mL pentane. The contents were vortex mixed vigorously for 10 to 20 seconds in a 10 mL glass centrifuge followed by centrifugation at 3500 g max at ambient temperature for 5 minutes. The pentane layer was separated and collected in a silanised glass tube. To ensure good recovery, the extraction procedure was performed twice. Both organic fractions were pooled and filtered using a 0.45 μm PTFE filter. The filtered organic fraction was then dried by heating at 45 ^{0}C. A gentle stream of nitrogen gas was used to assist evaporation. The dried residue was then reconstituted with 50 μL acetonitrile. A 5 μL aliquot of the reconstituted solution was injected into the LC-MS/MS system for analysis.

The second analyte, 3'-hydroxystanozolol was extracted in a similar way, but by using a mixture of pentane, chloroform and ethylacetate (total 3 mL) in the ratio 3:2:1 v/v/v. The dried residue was reconstituted with 50 μL methanol, followed by injecting 5 μL aliquot into the LC-MS/MS system.

4.2.5. LC-MS/MS analysis

A Thermoscientific LC-MS/MS system was used in this study (details in section 2.5).

LC conditions:

An Agilent Zorbax SB-C18 column (2.1 mm x 50 mm, 1.8 μm) was used for analysis. The mobile phase composed of water with 0.1% formic acid as solvent A and acetonitrile with 0.1% formic acid as solvent B. The total flow rate through the column was 100 μL/minute. The column was maintained at 60 ^{0}C. The gradient flow composition is shown in Table 4.1. Acetonitrile was used as a solvent for syringe and needle wash. The autosampler was maintained at 4^{0}C and was programmed to wash the syringe and the needle after each injection.

Table 4. 1: LC mobile phase gradient composition

LC run time (min)	Solvent A (%) Acetonitrile (0.1% formic acid)	Solvent A (%) Water (0.1% formic acid)
0	50	50
4	100	0
6.5	100	0
7	50	50
10	50	50

Mass spectrometric conditions:

The mass spectrometer was equipped with an electrospray interface, which was operated in positive ion mode. The capillary temperature was maintained at 350 ^{0}C. An ion spray voltage of 4000 V was essential for optimum ionisation of stanozolol, 3'-hydroxystanozolol and stanozolol D3 (IS). The protonated molecules, $[M+H]^{+}$, of stanozolol (*m/z* 329.2), 3'-hydroxystanozolol (*m/z* 345.2) and stanozolol D3 (*m/z* 332.2), were used as precursor ions for CID for MS/MS analysis. SRM was used to monitor the precursor ions and diagnostic product ions for unambiguous quantification of stanozolol and 3'-hydroxystanozolol. The collision energies and SRM, *m/z* transitions for stanozolol, 3'-hydroxystanozolol and IS are shown in Table 4.2.

Table 4. 2: Retention times, SRM transitions and collision energies of stanozolol, 3'-hydroxystanozolol and stanozolol D3

Analytes	Retention time (min)	Transition (m/z)	Collision energy (eV)
Stanozolol	3.58	329.2 → 81.2	42
		329.2 → 121.2	50
3'-hydroxystanozolol	3.08	345.2 → 97.2	40
		345.2 → 121.2	36
Stanozolol D3	3.56	332.2 → 81.2	42

4.2.6. Method validation

The performance of the method was validated according to the FDA guidelines for the following parameters: LLOD, LLOQ, linearity, accuracy, selectivity, inter-day precision, intra-day precision, linearity and extraction recoveries [197]. Drug-free water (HPLC grade) was used for method development and validation.

Samples for calibration curves and QCs were prepared by spiking 5 mL HPLC grade water with known concentrations of stanozolol, 3'-hydroxystanozolol and IS followed by LLE and LC-MS/MS analysis. QC samples were prepared similarly at three concentration levels equally distributed over the linear range. A calibration curve was constructed by plotting the analyte to IS ratio versus the known concentration of analyte in each sample. The analyte to IS ratio was calculated by dividing the area of analyte peak by the area of the IS peak. Eight calibration points (0.5, 1, 2, 4, 8, 32, 100 and 200 pg/mL) were used for plotting each calibration curve for stanozolol, whereas for 3'-hydroxystanozolol, 7 calibration points per curve (1, 2.5, 5, 10, 20, 50, and 100 pg/mL) were used. Three sets of spiked samples were prepared, each on a different day. Linear regression analysis using the least squares method was employed to evaluate the calibration curve of both target analytes as a function of their concentrations in water.

The accuracy and intra-day precision of the method was assessed by analysing QC samples, at 3 different concentration levels (for each analyte) equally distributed over the linear range, in six replicates each. The inter-day precision was determined by repeating this for three consecutive days. For determining the accuracy of the method, the mean calculated values at each concentration level were compared with the respective known concentrations. The precision of the assay was estimated by calculating the RSD at each concentration level. The LLOQ was defined as the lowest concentration of

analyte which could be reliably quantified with acceptable precision and accuracy stated by the FDA guidelines. LLOQ was determined as the concentration of stanozolol and 3'-hydroxystanozolol corresponding to a signal-to-noise ratio (S/N) $\geq$ 10. To determine the LLOD, a number of serial 1:2 dilutions were made from the low standard (LLOQ). LLOD was defined as the concentration which gave a response equivalent to three times the background noise.

The extraction recovery for both analytes were determined by comparing the analyte to IS peak area ratios obtained after extracting negative control HPLC grade water fortified with stanozolol and 3'-hydroxystanozolol at a final concentration of 2 pg/mL and 5 pg/mL respectively (in presence of IS) with the un-extracted standard working solutions at the same concentrations. To evaluate the selectivity of method, blank HPLC grade water extracted and analysed to confirm the absence of any detectable ghost peak that may elute at the retention similar to analytes or IS. The matrix effects were assessed by initially extracting blank water samples (HPLC grade, tap water and river water) as described in section 4.2.4. After extraction, each sample was spiked with known concentrations of stanozolol, 3'-hydroxystanozolol and stanozolol D3 followed by analysis. The resulting peak responses of stanozolol, 3'-hydroxystanozolol and stanozolol D3 were compared with those obtained by analysing neat standard solution of analytes and IS at the same final concentrations. The stability of stanozolol, 3'-hydroxystanozolol and IS in extracted water samples was investigated. For this, blank water samples (HPLC grade, river water and tap water) were extracted and then spiked with stanozolol, 3'-hydroxystanozolol and stanozolol D3. The samples were stored at 4^0C and room temperature at three time points: 24 hours, 7 days and 14 days, away from light. The samples were analysed daily for 14 days to determine the temperature and time period for which samples remain stable without variations in results.

4.3. RESULTS AND DISCUSSION

4.3.1. Method development

Stanozolol and 3'-hydroxystanozolol were unambiguously analysed on the basis of their retention times and SRM transitions (Figure 4.1). For stanozolol, the two ion transitions monitored were 329.2 >121.2 and 329.2 >81.2, whereas for 3'-hydroxystanozolol the two ion transitions monitored were 345.2 >121.2 and 345.2 >97.2.

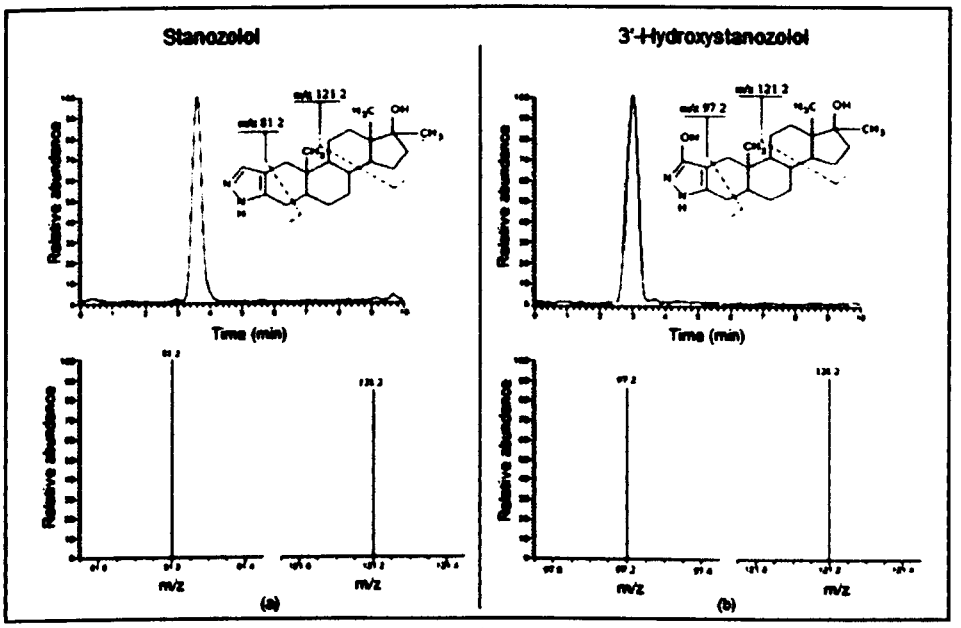

Figure 4. 1: Typical chromatogram and mass spectrum of (a) stanozolol and (b) 3'-hydroxystanozolol

Optimisation of chromatographic and mass spectrometric conditions

Optimisation of chromatographic conditions was similar to that discussed in chapter 3. A combination of acetonitrile and water as mobile phase solvent A and B respectively was examined. Different gradient and isocratic mobile phase compositions were investigated. Addition of formic acid (0.001% v/v, 0.01% v/v and 0.1% v/v) to solvent A and/or solvent B did not influence sensitivity drastically. However, maximum sensitivity with good peak shape for all compouds were obtained when acetonitrile with 0.1% formic acid was used as solvent A and water with 0.1% formic acid was used as solvent B under the conditions shown in Table 4.1. Though addition of formic acid (0.1% v/v) to acetonitrile and water did not influence sensitivity, it was used to prevent algae growth in water and in the LC system. Different mobile phase flow rates ranging from 100 μL/min to 250 μL/min were investigated. Flow rates higher than 200 μL/min led to negligible decrease in sensitivity. No drastic changes in the sensitivity were observed when using flow rates between 100 μL/min to 200 μL/min. Hence, to save organic solvents, a flow rate of 100 μL/min was used.

In the previous study (chapter 3), only one product ion for stanozolol, with m/z 81.2, showed high intensity, whereas the product ion m/z 121.2 was not distinguishable from the background noise and hence not used. However, in this study both the product ions were detectable. Before the beginning of this study, the LC-MS/MS underwent its annual maintenance, which improved its sensitivity and thus two abundant product ions were detected for stanozolol after reoptimising the mass spectrometric parameters.

The intensity of each compound (stanozolol, 3'-hydroxystanozolol and stanozolol D3) was optimised by varying the mass spectrometric conditions, such as capillary temperature, spray voltage, auxiliary gas pressure, sheath gas pressure, tube lens voltage and skimmer offset voltage. For this, a 1000 ng/mL solution of each compound was infused directly into the mass spectrometer in presence of mobile phase (100 μL/min). While infusing the compounds, different mass spectrometric conditions were investigated and the conditions which gave highest intensity for all the compounds were employed. The optimised parameters are summarised in Table 4.3.

Table 4. 3: Optimisation of mass spectrometric parameters for the analysis of nandrolone, stanozolol and stanozolol D3

Parameters	Range investigated	Optimised conditions
Capillary temperature (^{0}C)	300 to 400	350
Spray voltage (V)	3500 to 5000	4000
Auxiliary gas pressure (psi)	5 to 55	5
Sheath gas pressure (psi)	10 to 60	10
Tube lens voltage (V)	-10 to 240	105
Skimmer offset voltage (V)	0 to 50	14
Collision pressure (mTorr)	0 to 3	1.5
Collision energy (eV)	0 to 70	Please see Table 4.2

During the early stage of method development, water samples were filtered through syringe driven filter (0.45 μm) with a mixed ethylcellulose (MEC) membrane. Samples were then subjected to LLE followed by analysis. It was observed that filtration led to appearance of ghost peaks near the retention time of analytes, which could lead to false results. Also, filtration discards the suspended particles on which target drugs could be adsorbed. Hence, the filtration step (using an MEC membrane) prior to LLE was abandoned. Instead, samples were filtered after LLE using PTFE membrane filter, prior to injecting into LC-MS/MS system.

The efficiencies of pentane and hexane for the extraction of stanozolol were investigated. For stanozolol (2 pg/mL in HPLC water), optimum extraction recovery was obtained using pentane and was found to be 95.5%. With hexane, the extraction recovery of stanozolol was slightly less and was found to be 88.2%. However, the extraction recoveries of 3'-hydroxystanozolol (5 pg/mL in HPLC water) when hexane or pentane was employed were found to be less than 20.7%. This could be owing to the hydroxyl group of the 3'-hydroxystanozolol. Hence, attempts were made to use more polar solvents for the extraction of 3'-hydroxystanozolol. For this, different combinations of pentane, chloroform and ethylacetate were investigated. A mixture of pentane, chloroform and ethylacetate in the ratio 3:2:1 v/v/v facilitated maximum recovery of 3'-hydroxystanozolol as summarised in Table 4.5.

High performance liquid chromatography (HPLC) coupled to fluorescence detector and GC-MS have been commonly employed to analyse steroids [118]. However, HPLC coupled to fluorescence detector and GC-MS generally require laborious sample preparation steps like derivatisation which makes the method more time consuming and expensive [118, 220]. Hence, use of LC-MS/MS for analysing steroids is a feasible approach as the sample preparation step involved is facile, economical and does not re-

quire any additional derivatisation step. Compared to previous methods for detecting steroids in environmental waters, the major advantage of this method is that only 5 mL of water sample (opposed to up to 1000 mL) is required for analysis [115, 118-120]. Another advantage includes the use of LLE for purification of water samples, which is less time consuming and more economical in comparison to the solid phase extraction (SPE) processes employed in previous studies.

4.3.2. Method validation

The method validation results obtained were in accordance with the FDA guidelines [197]. The regression coefficient (r^2) values obtained were greater than 0.9960 for both analytes, thus indicating that the assay showed excellent linearity within the quantification range of 0.5 to 200 pg/mL water for stanozolol and 1 to 100 pg/mL water for 3'-hydroxystanozolol. Figure 4.2 represents calibration curves, regression equations and r^2 values obtained during method validation.

The LLOD for stanozolol and 3'-hydroxystanozolol were found to be 0.25 pg/mL and 0.5 pg/mL respectively. The average S/N values (N=6) of stanozolol and 3'-hydroxystanozolol at LLOD concentration levels were 6 and 5 respectively. The LLOQ for stanozolol and 3'-hydroxystanozolol were found to be 0.5 pg/mL and 1 pg/mL respectively. The average S/N values (N=6) of stanozolol and 3'-hydroxystanozolol at LLOQ concentration levels were 12 and 11 respectively. Figure 4.3 represents chromatograms of stanozolol and 3'-hydroxystanozolol at LLOQ concentration level, in aqueous matrix.

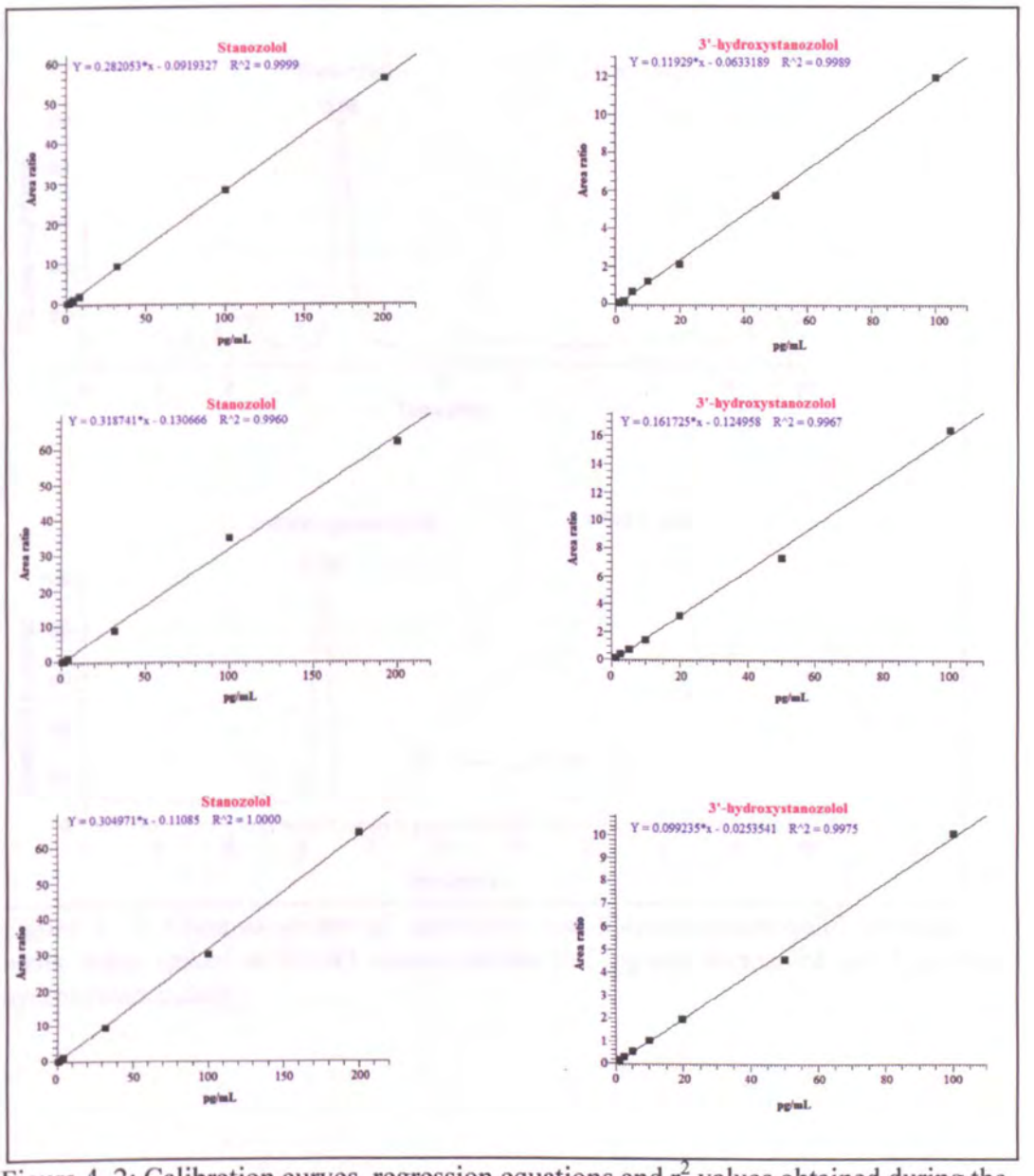

Figure 4. 2: Calibration curves, regression equations and r^2 values obtained during the three validation runs for stanozolol and 3'-hydroxystanozolol

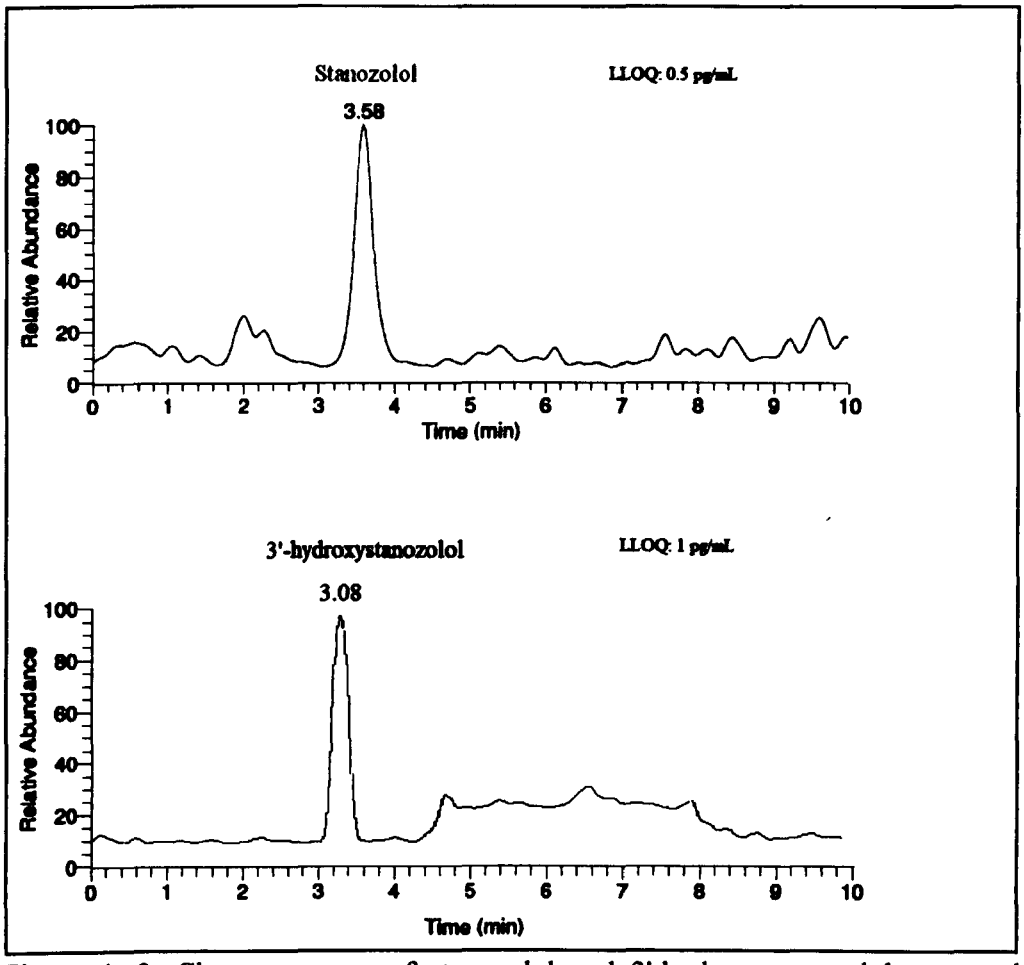

Figure 4. 3: Chromatograms of stanozolol and 3'-hydroxystanozolol extracted from blank water spiked at LLOQ concentrations (0.5 pg/mL stanozolol and 1 pg/mL 3'-hydroxystanozolol)

The analytical characteristics of this method including accuracy, inter-day precision, intra-day precision and linear range are summarised in Table 4.4. The RSD was used to assess method precision and it indicated good reproducibility.

Table 4. 4: Summary of assay validation results

Analytes	Linear range (pg/mL)	Concentration (pg/mL)	Precision RSD (%)		Accuracy (%)
			Intraday	Interday	
Stanozolol	0.5 to 200	2	8.5	7.4	91.7
		16	3.7	8.8	100
		100	6.1	7.4	106.6
3'-hydroxystanozolol	1 to 100	2.5	4.28	7.36	97.36
		5	3.61	5.24	92.80
		10	3.04	4.33	96.84

The extraction recoveries (with IS correction) for stanozolol and 3'-hydroxystanozolol in three water types; namely HPLC grade water, River Danube water and tap water were in the range 93.8% to 95.7% (Table 4.5). This indicated that the method is capable of detecting stanozolol and 3'-hydroxystanozolol in different types of aqueous matrix.

Table 4. 5: Extraction recovery results (%)

Matrix	Stanozolol (2 pg/mL)	3'-hydroxystanozolol (5 pg/mL)
HPLC water (N=6)	95.5	95.7
Tap water (N=6)	94.5	94.8
River water (N=6)	94.2	93.8

Matrix interference was observed with river water leading to a reduction in peak areas of stanozolol, 3'-hydroxystanozolol and stanozolol D3 by 22.3%, 23.9% and 18.4%, respectively. Comparatively, tap water and HPLC water showed lesser matrix effects. The reduction in peak areas was possibly attributed to ion suppression in the ESI source. However, after IS correction, the matrix effects in all three types of water samples were comparable and in the range 93.3-97.3% as shown in Table 4.6. Thus, stanozolol D3 was used as an IS to: i) compensate for matrix effects, ii) correct any loss of analyte during sample preparation, iii) compensate for any variations in the instrument response from injection to injection.

Table 4. 6: Matrix effect results for stanozolol, 3'-hydroxystanozolol (3'-HS) and stanozolol D3 in HPLC water, tap water and river water

Matrix	ME^1 %			ME^2 %	
	Stanozolol	3'-HS	IS	Stanozolol	3'-HS
HPLC water (N=6)	95.9	95.5	98.7	97.3	96.8
Tap water (N=6)	88.8	89.7	92.3	96.2	97.2
River water (N=6)	77.7	76.1	81.6	95.4	93.3

ME1 is matrix effect expressed as the ratio of mean peak area of analyte spiked post extraction to the mean peak area of the same analyte standard multiplied by 100. A value less than 100 indicates ion suppression. ME2 is matrix effect corrected with IS.

Extracted water samples were found to be stable when stored at 4^0C (in the autosampler), in the dark, and did not evaporate before 14 days. Thus, samples could be made in advance and left in the thermostated autosampler until analysis for up to 14 days. The analytical prerequisites for efficient detection of stanozolol and 3'-hydroxystanozolol at low levels in aqueous matrix were, purification of water samples using LLE in presence of a deuterated IS, stanozolol D3, followed by injecting only 5

μL aliquot through the column combined with the optimised LC-MS/MS conditions employed for analysis.

4.3.3. Application to real samples

The newly developed methods were applied to the analysis of various water samples obtained from the River Danube, urban tap water (Budapest), Balaton lake, spring water and 15 bottled drinking water. Water samples were collected periodically from December 2009 to November 2010. The pH values of all the river and tap water samples collected were found to be in the neutral range (6.5-7.5). The pH values of bottled water analysed are summarised in Table 4.7.

Table 4. 7: Bottled natural mineral water analysed

Bottled water	Origin	pH		EU recognised
		Labelled values	Laboratory values	
Evian	France	7.2	7.1	Yes
Badoit	France	6.0	6.2	Yes
Perrier	France	5.5	5.8	Yes
Volvic	France	7.0	7.0	Yes
Visegradi	Hungary	Not labelled [a]	6.9	Yes
Aquasol	Hungary	Not labelled [a]	7.2	Yes
Natur Aqua	Hungary	Not labelled [a]	7.5	Yes
Theodora	Hungary	Not labelled [a]	7.3	Yes
Imola	Hungary	Not labelled [a]	7.4	Yes
Dr Szalay	Hungary	9.0	8.8	Not known
S Pellegrino	Italy	7.7	7.6	Yes
Isklar	Norway	Not labelled [a]	7.5	Not known
Highland spring	Scotland	7.7	7.7	Not known
Drench	UK	7.4	7.4	Not known
Buxton	UK	7.4	7.2	Yes

'a' indicates pH not labelled but was found to be in neutral range

In three out of six samples from the River Danube, collected since December '09, stanozolol was detected with levels up to 1.82 pg/mL. In contrast, only one sample of urban tap water from Budapest city was found to contain stanozolol at a concentration of 1.19 pg/mL. Stanozolol was not detected in any of the fifteen bottled waters investigated. The results for stanozolol analysis in different water samples are shown in Table 4.8. To investigate the potential sources of stanozolol in River Danube and tap water, the method was extended for the detection of 3'-hydroxystanozolol. It was found that 3'-hydroxystanozolol was not detected in any of the water samples analysed in this study. Further investigation needs to be carried out for determining the source of stanozolol and reasons for gradual decrease in its concentration.

Table 4. 8: Determination of stanozolol in environmental and domestic water samples

Environmental water sample (N=3)	Average concentration pg/mL					
	31st December 2009	18th April 2010	21th July 2010	01st September 2010	24th October 2010	05th November 2010
River Danube	1.82 ± 0.19	0.71 ± 0.06	0.54 ± 0.03	BDL	BDL	BDL
Budapest Tap	1.19 ± 0.03	0.31 (BQL)	BLD	BDL	BDL	BDL
Lake Balaton	-	BDL	-	-	-	-
Spring 'Rózsika'	-	BDL	-	-	-	-

* BDL means below detection level (0.25 pg/mL), BQL means below quantification level (0.5 pg/mL)

The possible sources of stanozolol entering the river are unknown, but may be from human or animal consumption and excretion of un-metabolised drug or due to a single accidental discharge of the parent compound. It should be noted that stanozolol was only found once in tap water and that this level does not present a threat to health based on recommended intake levels. The National Health Service (NHS) recommends a minimum water intake of 1.2 litres every day [221]. Hence, individuals who were drinking stanozolol contaminated urban tap water (1.19 pg/mL) had involuntarily consumed approximately 1.43 ng stanozolol per day. Since the effective doses of stanozolol for men and women are 50-100 mgs/day and 2.5-10 mgs/day respectively [222], such low levels detected in drinking water may not cause significant harm to the general public, especially as they were found only at one time point.

Water samples from river and tap were collected periodically until November 2010 and stanozolol concentrations were found to be reducing over time as shown in Table 4.8. The possible reasons for a gradual reduction in concentration could be due to: i) variations in rates of contamination, ii) dilution of river water due to rise in water levels (Table 4.9, Figure 4.4), iii) degradation of the steroid in the river water due to other constituents in the river or photolysis etc or deposition in the sediment.

The average water level of River Danube from 31st December 2009 to 5th November 2010 is presented in Table 4.9. The water level data were obtained from Ovisz [223]. The water level is measured hourly at various points on the river along its course while crossing Hungary (1646.500 fk, approximately 400 km). Data shown are from the closest measuring point to the sample collection site. Maximum water temperature is around 22°C. The fluctuation in water level is shown in Figure 4.4. The water analysis results indicate that stanozolol was found to be present in the River Danube and Budapest tap water in the month of December 2009, when the water level in river was found

to be low. Furthermore, in July 2010 a biological sewage treatmened plant was opened in Budapest in order to treat most of the water supplied to the city (in contrast to only 30-40% water being processed in the past). Thus, the decrease in the levels of stanozolol compared to those we previously observed could be potentially owing to this major environmental protection investment.

Table 4. 9: River Danube water level, volume and temperature

Date	Water level (daily average in cm)	Water flow rate (m^3min^{-1})	Temperature (°C)
31st December 2009	249.47 ± 1.97		
18th April 2010	325.83 ± 5.45		
21th July 2010	310.80 ± 6.67	2650	22.0
1st September 2010	349.86 ± 9.15	3070	17.5
24th October 2010	190.13 ± 1.39	1690	10.0
5th November 2010	192.00 ± 0.98	1690	8.6

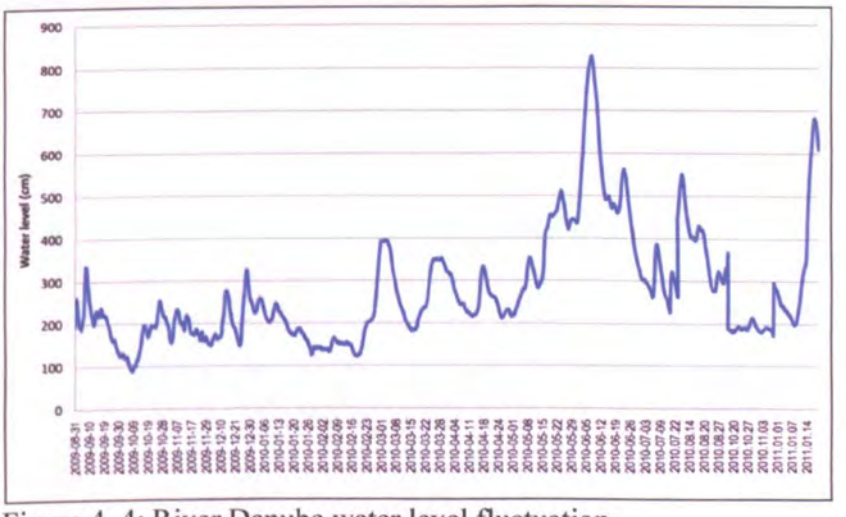

Figure 4. 4: River Danube water level fluctuation

4.4. CONCLUSIONS

Rapid, highly sensitive and reproducible methods have been developed to detect stanozolol and 3'-hydroxystanozolol in different types of water samples. These assays are capable of detecting stanozolol and 3'-hydroxystanozolol at a concentration as low as 0.25 pg/mL water and 0.5 pg/mL water, respectively when only 5 mL water is processed. The performance of this method gives acceptable relative recoveries for both compounds in river and tap water samples. The method can be extended to detect other chemicals and pharmaceutical drugs which may be hazardous to human health and environment. The major urinary metabolite of stanozolol, 3'-hydroxystanozolol was not detected in any of the samples analysed. Since 95% of stanozolol metabolites are excreted in urine in the form of glucuronide conjugates, further research is required by carrying out a deglucuronidation step and re-analysing the samples, provided the samples are available from a reliable source and from the same time point.

CHAPTER 5

DETECTION OF TESTOSTERONE AND EPITESTOSTERONE IN HUMAN AND RAT HAIR USING LC-MS/MS

5.1. INTRODUCTION

Testosterone (T) is an endogenously-produced hormone which is capable of increasing lean body mass, strength and aggressiveness [4, 59, 99, 224]. Owing to its ability to improve overall performance in sports, use of testosterone is banned by majority of sports organisations. According to 'The 2013 Prohibition List' of the World Anti Doping Agency (WADA), testosterone belongs to the class S1.1 b and its use in and out-of-competition is prohibited [6]. Despite the restrictions, testosterone is one of the most frequently used PED. Figure 5.1 presents the proportion of positive results of testosterone, within the class of anabolic agents reported by WADA accredited laboratories from year 2005 to 2011[7-13].

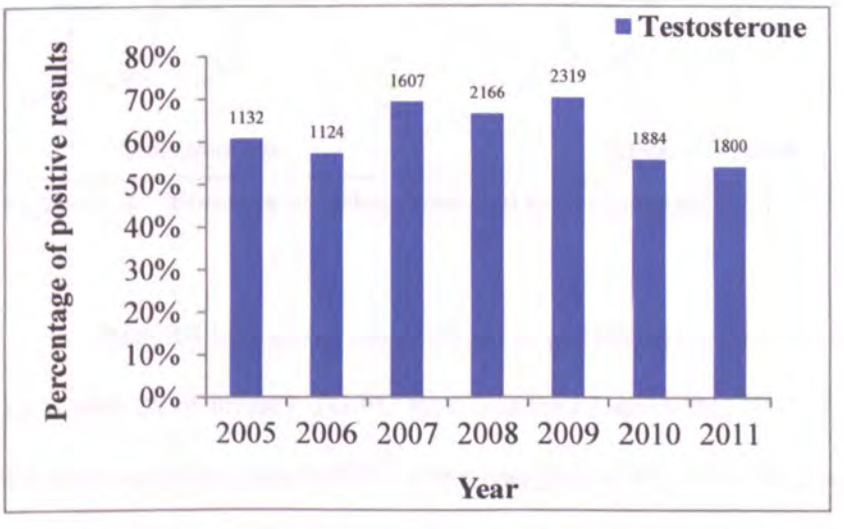

Figure 5. 1: Proportion of positive results of testosterone, within the class of anabolic agents (year 2005 to 2011). Numbers above the bars represent the number of positive results of testosterone reported per year [7-13]

Since testosterone is produced endogenously, detecting doping is often challenging. For identifying doping with testosterone, it is essential to distinguish between supra-physiologically administered testosterone and endogenously produced testosterone. This is achieved by determining the urinary ratio of testosterone glucuronide (TG) to epitestosterone glucuronide (EG) [225]. TG and EG are the main glucuronidated metabolites of testosterone and epitestosterone respectively, and are excreted in urine [55]. Epitestosterone is an endogenously-produced biologically inactive epimer of testosterone (not a metabolite of testosterone) [56]. Testosterone and epitestosterone have similar chemical structure but they differ in the configuration of hydroxyl-bearing carbon at C-17 position, as depicted in Figure 5.2. According to the WADA, if the TG/EG ratio exceeds the population based threshold value of 4 [57, 58, 61], then doping with testosterone is suspected and further investigation may be required.

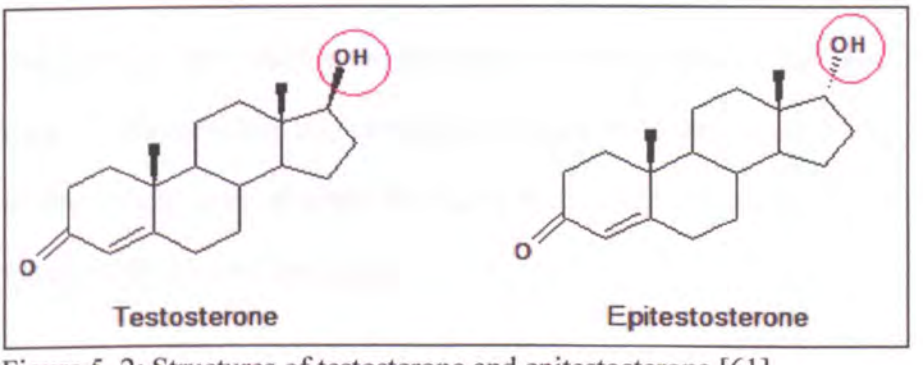

Figure 5. 2: Structures of testosterone and epitestosterone [61]

Inequitable doping results owing to genetic variations, is reported to be the major drawback of urinary TG/EG test. It is well known that UGT2B17 and UGT2B7 are the main enzymes responsible for the catalysis of the phase II glucuronidation reaction of testosterone and epitestosterone respectively. A number of studies have been carried out which indicate that there are inter-ethnic and inter-individual variations in the preva-

lence of deletion polymorphism in the gene coding of the UGT2B17 enzyme, and the excretion rate of testosterone-glucuronide is dependent on the genotype of UGT2B17 enzyme [48, 49, 52, 57]. It has been observed that individuals with *del/del* genotype excrete less than normal amounts of testosterone-glucuronide in urine than those individuals carrying *ins/del* or *ins/ins* genotypes. As the major enzyme involved in the glucuronidation of epitestosterone is UGT2B7, deletion mutations in the UGT2B17 gene may not significantly affect the excretion rate of epitestosterone-glucuronide [62].

In a study carried out by Sjoqvist *et al.*, it was observed that the occurrence of UGT2B17 deletion genotype was seven times more frequent in Koreans than in the Swedish population [49]. They also investigated the association between deletion polymorphism and urinary levels of testosterone and epitestosterone. They observed that the excretion of testosterone was 16 times higher in Swedish people compared to the Koreans [49]. However, the excretion rate of epitestosterone was not dependent on UGT2B17 genotype. Jakobsson-Schulze *et al.* reported that, when testosterone was administered to individuals deficient in the UGT2B17 gene (*del/del* genotype), their urinary TG/EG ratio [48] did not reach the population based ratio of 4. Thus, false negative doping results were obtained. Owing to such genetic variations, the accuracy of urine based TG/EG test is challenged.

T and E are also excreted in urine as sulfate conjugates, but this is a minor elimination pathway compared to glucuronidation. The urinary fraction of testosterone sulphate conjugate is reported to be only 4% of that of glucuronidated testosterone [226]. It can be argued that in order to compensate for inefficient glucuronidation activity, AASs could be excreted mainly as sulphate conjugates. However, it has also been reported that low-mode excretors do not produce more than normal levels of testosterone sulfate in order to compensate for impaired glucuronidation activity [51, 226]. Alternatively, gen-

otype based threshold levels can be employed to improve the sensitivity of the TG/EG test which may not be facile for a larger population.

It has also been reported that commonly used drugs NSAIDS like ibuprofen and diclofenac competitively inhibit the testosterone glucuronidation activity of UGT2B17 and other UGTs, *in vitro*. Thus, the urinary TG/EG ratio may also get affected by the use of drugs that act as substrates for UGTs contributing to testosterone glucuronidation. However, further research needs to be carried out to determine if similar inhibitory effects are observed *in vivo*. These findings indicate that urinalysis based TG/EG ratio is susceptible to false doing results owing to genetic and metabolic variations. Hence, analysis of other biological samples (other than urine) can provide vital information related to doping.

For the past few decades, hair analysis has been investigated for anti-doping purposes as it is reported to be an effective method (compared to urinalysis) for retrospective detection of steroids in their unconjugated form [4, 99, 140, 143]. Impaired glucuronidation of testosterone due to genetic and metabolic variations may not restrict the detection of unconjugated testosterone in hair. Thus, hair analysis can provide valuable information on individuals' drug use. The concentration of testosterone in the hair of testosterone users has been found to be higher than the natural concentration [99]. It can be assumed that the T/E ratio in hair of a testosterone user will be higher than the natural ratio and this can provide vital information for controlling doping. Thus, hair analysis can compliment urinalysis by providing the levels of testosterone, epitestosterone and thus T/E ratios to identify drug users, especially over a longer time-frame.

GC-MS based methods are available in the literature for the determination of endogenous steroids including T and E in human hair [99, 190, 194, 227]. However, GC-MS methods often require a complicated, time consuming and expensive sample derivatisation step for analysing steroids. Generally, the derivatives are unstable and are susceptible to thermal decomposition during analysis, thus affecting the method reproducibility [61]. In contrast, LC-MS is a feasible approach for analysing steroids as the sample preparation step involved is facile, economical and does not require any additional derivatisation step.

The work described in this chapter includes the first time application of LC-MS/MS for the detection of testosterone and epitestosterone in human and rat hair. The primary aim of this study was to develop a method which is capable of detecting testosterone and epitestosterone in hair using LC-MS/MS and thus determine the natural levels of T and E in human hair. The secondary aims of this study included: i) the determination of T/E ratio in human hair and ii) investigation of the relationship between hair colour and the concentration of testosterone and epitestosterone in hair, using Long Evans rats.

5.2. MATERIALS AND METHODS

5.2.1. Chemicals and reagents

Sodium hydrogen phosphate heptahydrate, sodium phosphate monobasic dihydrate, sodium hydroxide, hydrochloric acid, melanin, reference standard of epitestosterone (purity ≥98), LC-MS grade water (ultra chromsolv) and acetonitrile (chromsolv), HPLC grade dichloromethane pentane and reference standard of testosterone were purchased from Sigma Aldrich (Poole, UK). Reference standard of stanozolol D3 was purchased from LGC standards (Teddington, UK).

5.2.2. Preparation of standard solutions

Stock solutions of T and E were prepared in acetonitrile at a concentration of 1 mg/mL. These were mixed and diluted to obtain both analytes combined at working solutions of 1000 ng/mL and 10 ng/mL for each analyte. The former working solution was used to optimise mass spectrometric parameters and the latter solution was used to optimise chromatographic parameters for T and E. Samples for standard curve were prepared from the stock solutions by diluting with acetonitrile. QC samples were prepared similarly, but from separately prepared stock solutions. A stock solution of stanozolol D3 (0.1 mg/mL, IS) was diluted with acetonitrile to obtain working solutions of 1000 and 10 ng/mL. These were used to optimise mass spectrometric and chromatographic parameters, respectively, for stanozolol D3 (IS). All stock solutions were stored at -20 ^{0}C in amber, silanised, glass vials. All diluted solutions were stored at 4^{0}C in amber, silanised, glass vials.

5.2.3. Hair specimens

Hair samples analysed in this study were available from 75 subjects (49 males, 26 females). The age of subjects ranged from 18 to 55 years. Further details on hair sample collection is provided in section 2.2. Hair samples were also obtained from 11 Long Evans rats (details provided in section 2.3). Each animal produced pigmented and non-pigmented hair. Hair samples were collected by shaving the pigmented and non-pigmented stripes of animals. All the hair samples were stored individually in labelled, sealable, envelopes until analysis.

5.2.4. Sample preparation

5.2.4.1. Decontamination

An important step in hair sample preparation involves decontamination using dichloromethane (section 2.4.1).

5.2.4.2. Alkaline digestion

After decontamination, hair samples were allowed to air dry, followed by cutting them into *ca.* 1 mm segments using scissors. Fifty milligrams of these hair segments supplemented with stanozolol D3 as IS were incubated with 1 mL of 1M sodium hydroxide at 95 ^{0}C for 10 minutes. The homogenate was then neutralised with 1 M hydrochloric acid, followed by addition of 2 mL of 0.2 M phosphate buffer (pH 7.0).

5.2.4.3. Liquid-liquid extraction

The neutralised homogenate was then subjected to LLE using 3.5 mL pentane. After vortex mixing for 20 seconds, the mixture was centrifuged at 4000 x g for 20 minutes at 4 ^{0}C. The pentane layer was transferred into a clean, silanised, glass vial and dried down at 40 ^{0}C using a gentle stream of nitrogen gas. The dried residue was reconstituted with 100 μL acetonitrile. The reconstituted solution was filtered through a 0.2 micron PTFE membrane filter, prior to injecting into the LC-MS/MS system.

5.2.5. LC-MS/MS analysis

An Agilent LC-MS/MS system was used for analysis (details in section 2.5).

LC conditions

The LC separation was achieved on an SB C-18 column (2.1 mm, 150 mm, 1.8 μm) maintained at 45 ^{0}C. To prevent the analytical column from blocking, a 0.2 micron inline filter was installed prior to the column. Acetonitrile (solvent A) and water (solvent B) were used as mobile phase solvents at a flow rate of 200 μL/min through the column. The gradient flow composition is shown in Table 5.1. Acetonitrile was used for washing the needle after each injection.

Table 5. 1: LC mobile phase gradient conditions for chromatographic separation of analytes

LC run time (minutes)	Acetonitrile (%)	Water (%)
0	47	53
5	47	53
10	100	0
11	100	0
12	47	53
25	47	53

Mass spectrometric conditions

The mass spectrometer was operated in positive ion mode. The protonated molecules, $[M+H]^+$, of testosterone (*m/z* 289.2), epitestosterone (*m/z* 289.2) and stanozolol D3 (*m/z* 332.2), were used as precursor ions for CID for MS/MS analysis. The precursor ions and the diagnostic product ions of T, E and IS were monitored in multiple reaction monitoring (MRM) mode. For the optimum ionisation of analytes, the following ESI parameters were applied: capillary voltage, 4000 V; drying gas temperature, 325 ^{0}C; drying gas flow rate, 10 L/min and nebulizing gas pressure, 35 psi. The electron multiplier voltage was set at 1560 V. The dwell time for each transition was set at 200 ms. The MRM transitions, fragmentor voltages and the collision energies of each analyte are given in Table 5.2. The mass spectrometric parameters were optimised using the Masshunter optimiser software (version B.03.01). For optimisation, a 2 μL aliquot of 1000 ng/mL solution of each compound was injected through the column in presence of mobile phase. The conditions which gave highest sensitivity for each compound were automatically saved in the method.

Table 5. 2: Retention times, MRM transitions, collision energies and fragmentor voltages of testosterone, epitestosterone and stanozolol D3 (IS)

Analytes	Retention time (min)	MRM transition	Collision energy (eV)	Fragmentor voltage (V)
Testosterone	8.0	289.2 → 109.2	26	100
		289.2 → 97.2	22	100
Epitestosterone	9.5	289.2 → 109.2	25	45
		289.2 → 97.2	21	45
Stanozolol D3	13.1	332.2 → 81.2	50	100

5.2.6. Method validation

The performance of the analytical method was validated according to the FDA guidelines [197] for the following set of parameters: selectivity, accuracy, precision, LLOQ, LLOD, linearity and extraction recoveries.

Since, melanin is one of the main constituents of hair which influences the incorporation of drugs; it was used as a negative control representative matrix for method development and validation [59, 183, 194]. The mean concentration of melanin in 50 mg of human hair is 0.5 mg [194]. Hence, calibration curve samples were prepared by spiking 0.5 mg melanin with known concentrations of T, E and IS [194]. QC samples were prepared similarly at three concentration levels, equally distributed over the linear range. Samples for calibration and QC were treated in a way similar to unknowns. An eight point calibration curve for testosterone (0.25, 0.5, 1, 5, 10, 20, 40, 100 pg/mg) and epitestosterone (0.5, 1, 2.5, 5, 10, 20, 40, 100 pg/mg) was constructed by plotting the ratio of analyte to IS against the known concentration of analyte in each sample. Analyte to IS ratio was calculated by dividing the peak area of analyte by the peak area of

IS. Data analysis and method performance were evaluated using the Masshunter work-station software (quantitative analysis, version B.04.00).

The selectivity of the method was confirmed by the absence of any detectable interfering peaks at the retention times of T, E and IS in extracted melanin samples. For both, T and E, the two ion transitions monitored were 289.2 >109.2 and 289.2 > 97.2. Quantification of T and E was performed based on the m/z 109.2 product ion, whereas the m/z 97.2 product ion was used for confirmation. For each analyte, the ratio of intensities of two product ions were determined and compared between the samples and reference standards to enhance method specificity.

Accuracy, intra-day precision and inter-day precision were determined at three different concentration levels (low, medium and high), equally distributed over the linear range (Table 5.3). Accuracy was evaluated by comparing the mean calculated values (6 replicates per concentration level) with the respective nominal concentration values. Intra-day precision was determined by measuring 6 replicates per concentration level, on the same day. Inter-day precision was assessed by analysing 6 replicates per concentration level, on three consecutive days. LLOD was defined as the lowest concentration of the analyte which gave a peak response with a signal to noise ratio (S/N) of at least three. LLOQ was defined as the lowest concentration of the analyte: (i) which could be reliably quantified with a precision not exceeding 20% of the RSD and accuracy not deviating more than ±20% of the actual value, and (ii) which gave a peak response with a S/N of at least 10. Both LLOQ and LLOD were determined empirically using consecutive 1:2 dilutions of spiked samples. The linearity of the method was investigated by using linear regression analysis.

For the extraction recovery, melanin was fortified with analytes (at three concentration levels, Table 5.4) and IS and then extracted as described in section 5.2.4.3. The analyte to IS peak area ratios obtained after extraction were then compared with analyte to IS peak area ratios of standard solutions prepared in acetonitrile at same final concentrations. To determine matrix effects, blank melanin and pooled hair samples were extracted and spiked with known concentrations of analytes and the IS. The resulting peak areas of T, E and IS were then compared with the peak areas of standard solutions of T, E and IS at the same theoretical concentrations. However, to assess the matrix effects associated with real hair samples, un-spiked pooled human hair samples were also analysed to determine the peak responses of endogenously present T and E (to make the appropriate corrections). Matrix effects associated with rat hair (pigmented as well as non-pigmented) was also determined similarly. Stability of analytes in the presence of matrix was assessed by storing extracted hair samples (spiked with T, E and IS) at either room temperature or 4 ^{0}C at three time points; 24 hours, 7 days and 14 days.

5.2.7. Statistical analysis

Group differences in T and E levels were tested using t-test and ANOVA. The level of significance was set at 0.05. All tests were confirmed by a nonparametric method (Mann-Whitney). All statistical analyses were performed using SPSS 19.0.

5.3. RESULTS AND DISCUSSION

5.3.1. Method development

Both testosterone and epitestosterone were detected and quantified on the basis of their retention times and MRM transitions. Both, testosterone and epitestosterone have similar chemical structures, but they differ in the configuration of the hydroxyl-bearing carbon at C-17 position. Since, both epimers have similar MRM transitions, co-elution of testosterone and epitestosterone (physiologically inactive), may represent inflated levels of testosterone in hair. Hence, to avoid such inaccurate results, baseline separation of testosterone from epitestosterone peaks was essential. Separation was tested on an Agilent C-18 column (2.1 mm, 1.8 μm) of three different lengths, 50 mm, 100 mm and 150 mm. The maximal baseline separation was achieved on a 150 mm long column (Figure 5.3), when acetonitrile and water were used as solvents A and B respectively, at a flow rate of 200 μL/min under the conditions shown in Table 5.1. The resolution factor obtained was around 3.4, which allowed accurate integration of peaks and subsequent quantification. Normally, for accurate integration of peaks, a resolution factor higher than 1.5 is required [228]. Different grades of mobile phase solvents were tried to get a low background baseline and hence to improve the sensitivity. LC-MS grade water (ultra chromasolv) and acetonitrile (chromasolv) gave lower baselines compared to other grades of solvents.

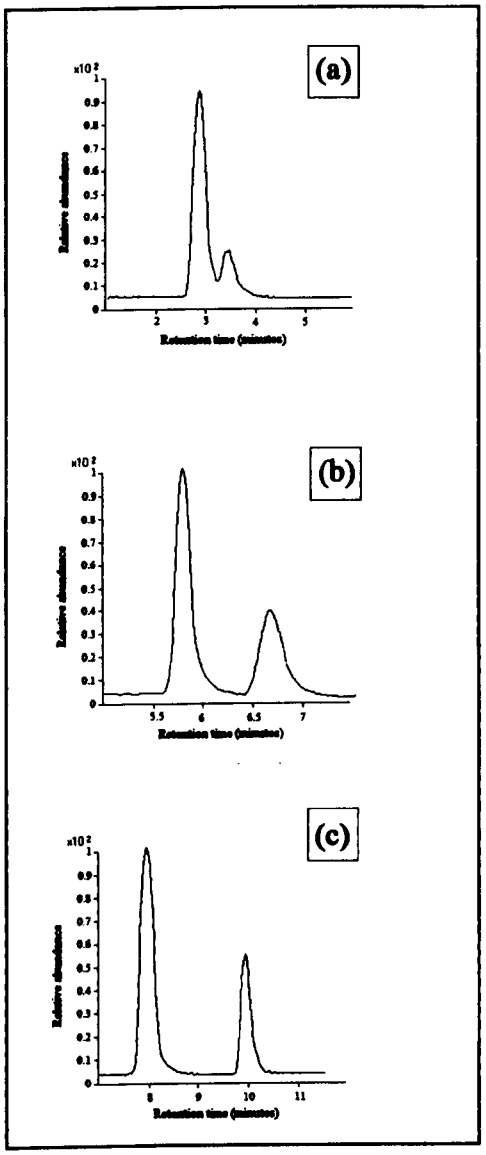

Figure 5. 3: Chromatographic separation of T and E using (a) 50 mm column, (b) 100 mm column and (c) 150 mm column

Addition of formic acid (0.001 to 0.1%) to solvent A and/or B resulted in a decrease in signal intensity (up to 50 %). Different mobile phase flow rates ranging from 150 μL/min to 250 μL/min were investigated. This range was selected for optimal column efficiency. No drastic changes in the relative abundance and peak resolution were observed within this flow rate range. However, flow rates higher than 200 μL/min led to a minor decrease in sensitivity, possibly attributed to discharge as a result of solvent accumulation in the ionisation source. A flow rate of 200 μL/min gave best results, with

optimal baseline separation, peak resolution and relative abundance of analytes and IS. A chromatogram and a mass spectrum of testosterone (4.26 pg/mg), epitestosterone (3.76 pg/mg) and IS extracted from hair are also represented in Figure 5.4. The structures and predicted fragmentation pathways for testosterone, epitestosterone and IS are depicted in Figure 5.4.

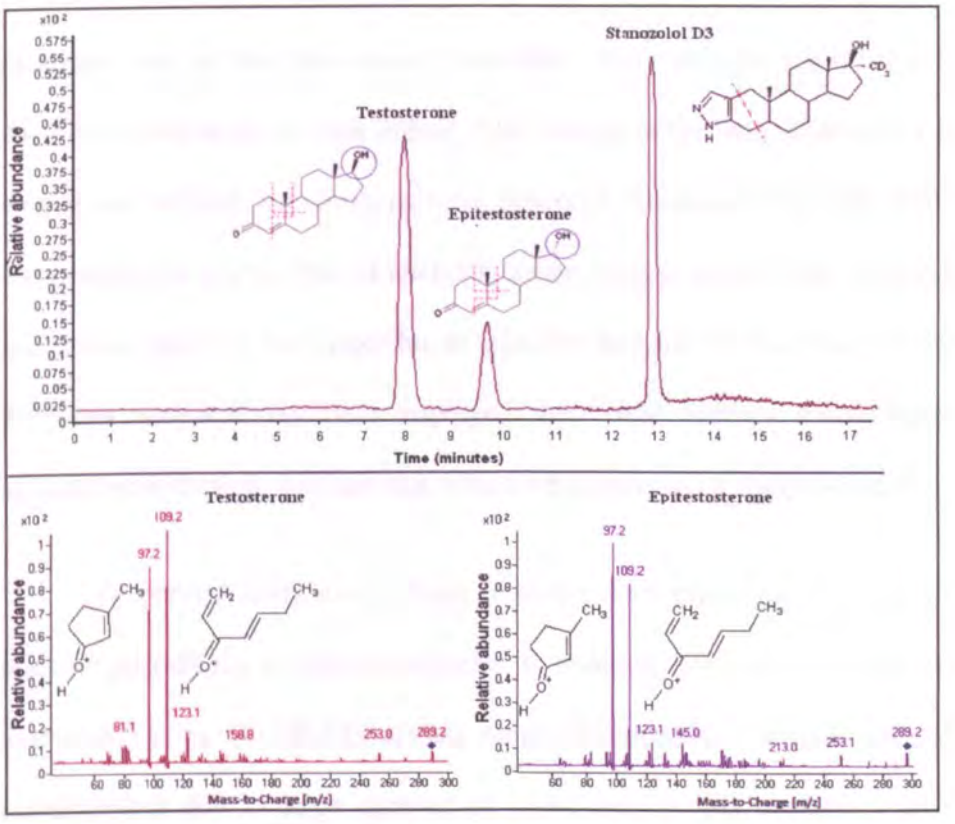

Figure 5. 4: Chromatogram and mass spectrum of testosterone (4.26 pg/mg), epitestosterone (3.76 pg/mg) and stanozolol D3 extracted from human hair [59]

Alkaline digestion was employed for complete dissolution of hair, so that the majority of the drugs incorporated in hair could be extracted. LLE using pentane was a sufficient purification step to reduce the unwanted components of hair matrix that may affect the analysis. As reported in the previous chapter, vortex mixing neutralised hair homogenate with pentane, occasionally led to the formation of a gel like substance. This caused difficulty in separating the pentane layer, leading to sample loss. To prevent

such sample loses, it was essential to separate the gel from the pentane layer. This was achieved by carrying out centrifugation at 4000 x g for 20 minutes at 4 ^{0}C. Thus, in contrast to the method discussed in previous chapter where centrifugation was carried out at room temperature at a comparatively low speed (1257 x g), this method ensured better separation of the two layers in gelled samples.

Overall, the analytical procedure was capable of detecting testosterone and epitestosterone in hair without any detectable interference by any other extractable endogenous compounds present in hair. Also, owing to the hair decontamination step employed, no external interferences were observed. Stanozolol D3 was used as an IS to compensate for any: i) loss of analytes during sample preparation, ii) variations in the instrument response from injection to injection and iii) matrix effects. However, under the given analytical conditions employed, there were no major matrix interferences associated with melanin and hair that affected the analysis of analytes and IS.

To prevent carry over effects from previous injections, blank acetonitrile was injected periodically in between samples. In addition, a washing method was developed to ensure that the LC-MS/MS system, mainly the mass spectrometer and column, is not contaminated due to large number of injections. In this method, 4 μL acetonitrile (blank) was injected in to the LC-MS/MS system. The mobile phase composed of 80% acetonitrile and 20% water at a flow rate of 200 μL/min. The total run time was 20 minutes during which the ESI source was operated in positive ion mode for 10 minutes and in negative ion mode for the remaining 10 minutes. The mass spectrometer was operated in full scan mode (mass range m/z 50 to 1000). After the washing run, the column was equilibrated with 47 % acetonitrile and 53 % water for 15 minutes before injecting the next sample. The washing method was run after every 2 samples.

The major advantage of this method compared to previously published methods [194] is that no sample derivatisation is required, which makes the method more rapid, reproducible, facile, economical and less laborious. During analysis, samples (extracted hair) were found to be stable for up to 14 days when stored at 4 ^{0}C (in the autosampler) in the dark.

5.3.2. Method validation

The validation results demonstrate that the analytical method is capable of detecting (LLOD) 0.1 pg testosterone and 0.25 pg epitestosterone per mg of hair. The average S/N values (N=6) of testosterone and epitestosterone at LLOD concentration levels were found to be 6 and 5 respectively. The LLOQ values for T and E were 0.25 pg/mg and 0.5 pg/mg respectively. The average S/N values (N=6) of testosterone and epitestosterone at LLOQ concentration levels were found to be 13 and 10 respectively. Figure 5.5 represents a chromatogram of T and E extracted from a melanin calibrant spiked at LLOQ concentrations.

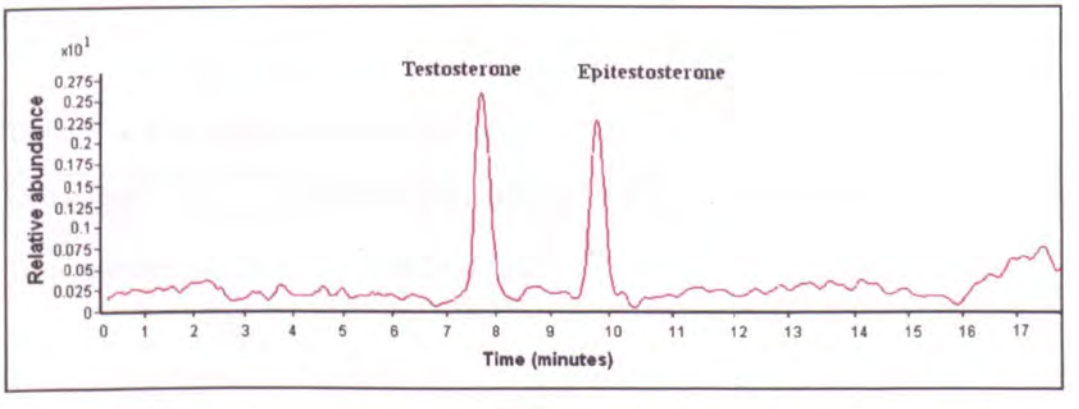

Figure 5. 5: Chromatogram of testosterone and epitestosterone extracted from a melanin calibrant spiked at LLOQ concentrations (0.25 pg/mg T and 0.5 pg/mg E)

The assay showed good linearity within the quantification range of 0.25-100 pg/mg for T and 0.5-100 pg/mg for E, with regression coefficient (r^2) values higher than 0.9987. Figure 5.6 represents calibration curves, regression equations and r^2 values obtained during method validation. Excellent peak shape and peak resolution were achieved for T, E and IS. The accuracy, inter-day precision and intra-day precision of the assay were within the limits set by FDA guidelines and are summarised in Table 5.3. The extraction recovery results are summarised in Table 5.4.

Table 5. 3: Summary of assay validation results

Compounds	Concentration (pg/mg)	Level	Precision RSD (%)		Accuracy (%)
			Intra-day	Inter-day	
Testosterone	0.5	Low	3.75	6.88	101.93
	5	Medium	3.24	3.68	97.50
	10	High	2.90	4.45	104.70
Epitestosterone	1	Low	4.28	7.36	97.36
	5	Medium	3.61	5.24	92.80
	10	High	3.04	4.33	96.84

Table 5. 4: Extraction recovery results (%)

Compounds	Concentration (pg/mg, N=6)	Extraction recovery (%)
Testosterone	0.25 (LLOQ)	94.16
	2.5	98.41
	10	97.62
Epitestosterone	0.5 (LLOQ)	90.39
	2.5	92.00
	10	94.69

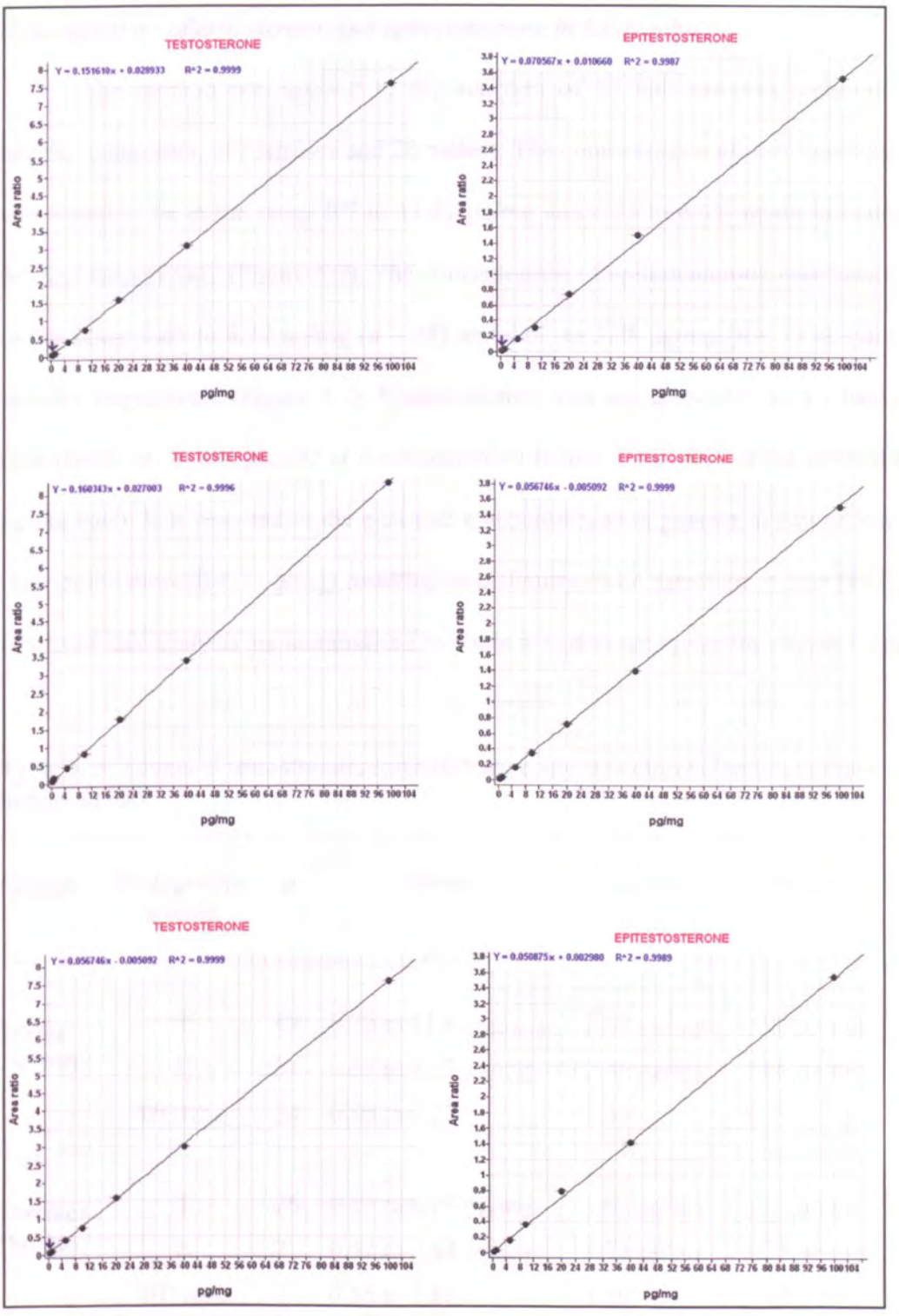

Figure 5. 6: Calibration curves, regression equations and r^2 values obtained during the three validation runs for testosterone and epitestosterone

5.3.3. Application to real samples

Determination of testosterone and epitestosterone in human hair

The method was applied to the analyses of 75 hair samples collected from healthy volunteers, (49 females and 26 males). The concentration of testosterone in hair was found to be in the range 0.7 to 11.81 pg/mg and 0.33 to 6.05 pg/mg in males and females respectively (Figure 5.7). The concentration of epitestosterone was found to be in the range 0.63 to 8.27 pg/mg (n = 34) and 0.52 to 3.88 pg/mg (n = 7) in males and females respectively (Figure 5.7). Epitestosterone was not detectable in the hair of 34 individuals i.e. it was present at a concentration below the LLOD of the method (0.25 pg/mg hair). It is reported in the past that epitestosterone is present in hair at low concentration levels (0-8.7 pg/mg) resulting in difficulty in its detection in hair [194]. The results of hair analysis are summarised in Table 5.5 (also see appendix, Figure 5.1A).

Table 5. 5: Levels of testosterone, epitestosterone and testosterone/epitestosterone ratio in human hair

Gender	Endogenous steroid	*n*	Range	Mean	Median	Standard error
Males (N=49)	T	49	0.70 to 11.81 pg/mg	2.67 pg/mg	1.98 pg/mg	0.29
	E	34	0.63 to 8.27 pg/mg	2.46 pg/mg	1.86 pg/mg	0.34
	T/E ratio	34	0.50 to 3.27	1.33	1.11	0.13
Females (N=26)	T	26	0.33 to 6.05 pg/mg	1.62 pg/mg	1.03 pg/mg	0.27
	E	7	0.52 to 3.88 pg/mg	1.73 pg/mg	1.23 pg/mg	0.45
	T/E ratio	7	0.56 to 1.81	1.29	1.30	0.17

N is the total number of hair samples analysed, n is the number of hair samples out of N in which endogenous steroid(s) were successfully detected

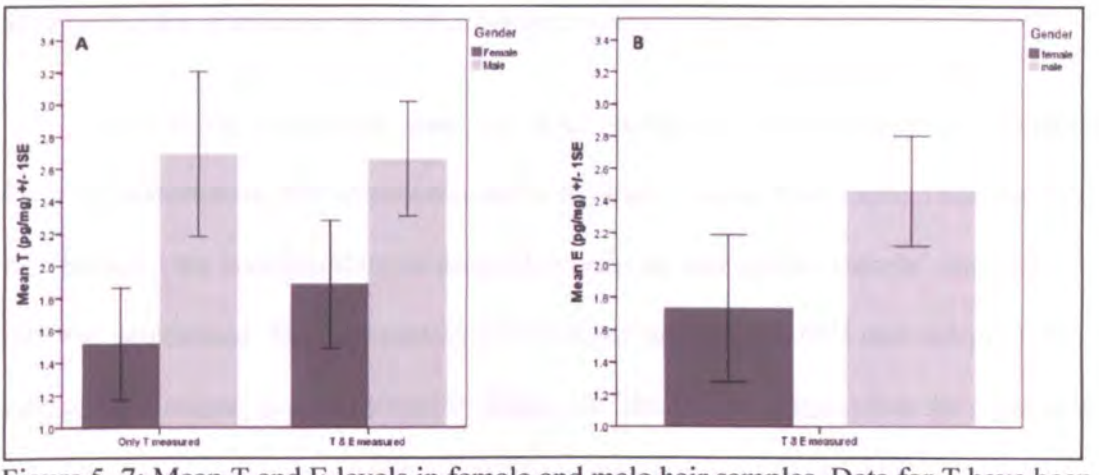

Figure 5. 7: Mean T and E levels in female and male hair samples. Data for T have been segregated depending on whether E was also detectable: (A) mean T levels; (B) mean E levels [59]

The concentrations of testosterone and epitestosterone found in this study were comparable to the physiological levels of testosterone and epitestosterone in hair reported previously using other techniques such as GC-MS [194]. The testosterone level in males was observed to be significantly higher [$t(73) = 2.378$, $p = 0.020$] than in females [59]. However, there was no statistically significant difference in the epitestosterone levels [$t(39) = 0.928$, $p = 0.359$]. The ratio of T/E in hair was also determined and was found to be in the range of 0.5 to 3.27 in males and 0.56 to 1.81 in females. The average ratio in males and females was found to be 1.33 ± 0.75 and 1.2 ± 0.45, but no statistically significant difference was observed [$t(39) = 0.117$, $p = 0.908$]. All tests were confirmed by a nonparametric method (Mann-Whitney) [59]. The measured testosterone levels were lower among those where epitestosterone was not detected (2.04 pg/mg vs. 2.53 pg/mg), particularly in females (1.52 pg/mg vs. 1.89 pg/mg), but the differences were not significant [$F(1,73) = 1.278$, $p = 0.262$ and $F(1,71) = 0.162$, $p = 0.689$, respectively] [59].

Determination of testosterone and epitestosterone in rat hair

This newly developed assay was also applied to the determination of natural levels of testosterone and epitestosterone in the hair of Long Evans rats. Thus, the relation between the concentration of drugs (testosterone and epitestosterone) and hair colour was determined. The concentration of testosterone in pigmented and non-pigmented hair of each animal is summarised in Table 5.6. The results suggest that the concentration of testosterone in pigmented hair is higher than in non-pigmented hair.

Table 5. 6: Concentration of testosterone in pigmented and non-pigmented hair of rats

Animal	Concentration of testosterone (pg/mg)		Ratio of pigmented to non-pigmented
	Pigmented hair	Non-pigmented hair	
1	6.33	4.8	1.32
2	8.74	1.81	4.83
3	6.14	1.51	4.07
4	7.84	1.84	4.26
5	5.88	1.58	3.72
6	4.84	1.28	3.78
7	5.61	1.49	3.77
8	3.66	1.11	3.30
9	3.06	1.41	2.17
10	4.81	1.14	4.22
11	2.86	0.84	3.40
Mean ± SD	5.43 ± 1.85	1.71 ± 1.07	3.53 ± 1.00

The mean ratio of concentration of testosterone in pigmented hair to that in non-pigmented hair was found to be 3.53 ± 1.00. In line with previous methods based on GC-MS, we found that testosterone, which is a neutral steroid (or a less basic molecule than stanozolol) gets preferentially accumulated into pigmented hair [163, 164]. Since testosterone was also detectable in non-pigmented hair, it indicates that although melanin is an important factor for the incorporation of drugs in hair, hair protein (keratin) or other hair components may also account for a significant part of drug accumulation in hair. This is in line with previously reported findings [157].

Epitestosterone was not detectable in the pigmented or the non-pigmented hair of rats. This could be owing to the reason that it is reported to present in hair at low concentration ranges (than testosterone) thus making its detection difficult [194]. In addition, the sensitivity of the epitestosterone assay was less than testosterone. It has been observed that there is a linear correlation in the levels of T and E in human hair [59]. It can be expected that the relationship between the concentration of epitestosterone in hair and hair colour will be similar to testosterone. Hence, the T/E ratio in human hair could be independent of hair colour. However, further research is required to determine population based natural levels of testosterone and epitestosterone in blonde hairs of healthy individuals.

5.4. CONCLUSIONS

A novel LC-MS/MS based method for the detection of T and E in human and rat hair has been developed and validated. This method is sensitive, specific, reliable, rapid, facile and reproducible for detecting 0.1 pg/mg T and 0.25 pg/mg E when *ca.* 50 mg hair was processed. The chromatographic conditions of the method offered maximal baseline separation of T and E peaks that was essential to prevent inaccurate T results. The validated method was successfully employed for the analysis of human and rat hair samples. This work reports for the first time the natural ratio of unconjugated testosterone to unconjugated epitestosterone (T/E) in human hair. The results indicate that this comprehensive method can be used to determine the levels of T, E and also the T/E ratio in human hair and can compliment urinalysis or blood test in doping testing. This method can be employed to verify self-reported drug use information in future. Also, this method was successfuly applied to determine the levels of testosterone in pigmented and non-pigmented hair of rats. Since, epitestosterone was difficult to detect in hair, further research is required to improve the sensitivity of the epitestosterone assay.

CHAPTER 6

INVESTIGATION OF INHIBITORY EFFECT OF DICLOFENAC ON THE URINARY EXCRETION OF STANOZOLOL AND 3'-HYDROXYSTANOZOLOL IN RATS

6.1. INTRODUCTION

It is well known that the major elimination and deactivation pathway of AASs and their phase I metabolites is through glucuronide conjugation (phase II metabolism) followed by excretion in urine [43, 44, 46, 47]. Generally, the glucuronidation reaction of AAS is catalysed by the enzyme UGT2B17. However, inter-individual and inter-ethnic variations in the prevalence of deletion polymorphism in the gene coding of the UGT2B17 enzyme have been reported, which eventually influence the urinary excretion of AAS and potentially lead to false doping results [48, 96].

Furthermore, UGTs are not only involved in the metabolism of AASs, but they also contribute to the metabolism of various compounds including NSAIDs [43]. Drugs like ibuprofen and diclofenac belong to the category of NSAIDs and are commonly used by athletes for the treatment of pain and inflammation [124-129]. It has been reported that both the drugs competitively inhibit the testosterone glucuronidation activity of UGT2B17 and other UGTs, *in vitro* [45]. Common dietary substances such as red wine [130], white tea and green tea [131] have also shown similar inhibitory effects in *in vitro* studies. Although the inhibitory effect is yet to be examined and reported *in vivo*, these *in vitro* results indicate that concomitant use of such over-the-counter medica-

tion or common dietary products with AAS may lead to impaired urinary excretion of AAS and their metabolites.

Considering that such genetic and metabolic variations may limit the efficacy of urinalysis in testing doping, it can be suggested that urinalysis, if used as a stand-alone test, is susceptible to confounding doping results [43, 44, 46, 47, 99, 199, 200]. The current anti-doping regime can be reinforced by employing additional biological samples like blood and hair analysed along with urine. Since impaired glucuronidation leads to reduction in the urinary excretion rate of AAS, it can be assumed that the levels of unconjugated AAS and their phase I metabolites in the systemic circulation will be elevated and thus higher levels of AAS and their phase I metabolites will be available to get incorporated into hair and other body tissues [96]. Hair analysis has been used in the past for detecting drug use as it predominantly favours the direct detection of parent AAS and determines a retrospective history of drug use. Thus, hair analysis and blood analysis can provide complementary information to urinalysis to prevent false doping results.

However, to investigate this option further, *in vivo* studies are required to establish a relationship between the drug levels detected in hair, urine and blood. This study was designed to investigate the influence of diclofenac on the urinary excretion of stanozolol and 3'-hydroxystanozolol, *in vivo*, using rat models. Also, the potential application of hair analysis when the urinary excretion is impaired was investigated. To support this study, new and sensitive LC-MS/MS based methods were developed, which are capable of determining the concentrations of stanozolol and 3'-hydroxystanozolol in pigmented hair, urine and blood sera samples of rats [185].

6.2. MATERIALS AND METHODS

6.2.1. Chemicals and reagents

Reference standards for stanozolol, 3'-hydroxystanozolol, 3'-hydroxystanozolol glucuronide, stanozolol D3 and 3'-hydroxystanozolol D3 were purchased from LGC standards (Teddington, UK). Sodium hydrogen phosphate heptahydrate, sodium phosphate monobasic dihydrate, sodium hydroxide, formic acid, hydrochloric acid, LC-MS grade water, acetonitrile, methanol, HPLC grade dichloromethane, pentane, chloroform and ethylacetate were purchased from Sigma Aldrich (Poole, UK). β-glucuronidase from *E. Coli* was purchased from Roche Diagnostics (Burgess Hill, UK). For the animal experiment, stanozolol, ketamine (2.5%), xylazine (Rompun, 2%) and diclofenac were purchased from Desma (Madrid, Spain), Kőbányai Gyógyszerárugyár (Budapest, Hungary), Haver-Lockhart laboratories (Kansas, US) and Sigma Aldrich (Deisenhofen, Germany) respectively.

6.2.2. Preparation of standard solutions

The reference standards of stanozolol (1 mg/mL in 1, 2-dimethoxyethane) and 3'-hydroxystanozolol (1 mg/mL in methanol) were used as stock solutions. These stock solutions were mixed and diluted with methanol to obtain both analytes combined at working solutions of 1000 ng/mL and 10 ng/mL for each drug. The working solutions of stanozolol and 3'-hydroxystanozolol at a concentration of 1000 ng/mL were used for optimising the mass spectrometric parameters. The working solutions of stanozolol and 3'-hydroxystanozolol at a concentration of 10 ng/mL were used to optimise the chroma-

tographic parameters. Samples for calibration curve were prepared from the stock solutions by diluting with methanol. QC samples were prepared similarly, but from separate stock solutions. These calibration curve and QC samples prepared in methanol were used for spiking negative control samples for method development and validation. Reference standards of stanozolol D3 (0.1 mg/mL in 1, 2-dimethoxyethane) and 3'-hydroxystanozolol D3 (0.1 mg/mL in methanol) were used as stock solutions. These were mixed and diluted with methanol to obtain both ISs combined at working solutions of 1000 ng/mL and 10 ng/mL for each compound. The working solutions of stanozolol D3 and 3'-hydroxystanozolol D3 at a concentration of 1000 ng/mL were used for optimising the mass spectrometric parameters. The working solutions of stanozolol D3 and 3'-hydroxystanozolol D3 at a concentration of 10 ng/mL were used to optimise the chromatographic parameters. Stock solutions were stored at -20^{0}C in amber, silanised, glass vials and all diluted solutions were stored at 4^{0}C in amber, silanised, glass vials.

6.2.3. Animals

As mentioned in section 2.3, twenty four male, brown Norway rats were purchased from Charles River laboratories (Sulzfeld, Germany). Each animal weighed around 280-340 g and were approximately 5 months old. All the rats were kept in an animal house located in Semmelweis University, Budapest, Hungary. Animals were housed in groups of three individuals in standard laboratory cages. They were kept in a constant room temperature environment with an alternating 12h lightdark cycle. Food and water were available *ad-libitum*.

6.2.4. Administration of diclofenac and stanozolol

Twenty four brown Norway rats (males) were divided into four groups of six animals each (Groups: G1, G2, G3 and G4). Diclofenac was dissolved in normal saline and administered subcutaneously, daily, to each animal belonging to group G2, G3 and G4, six days a week at doses detailed in Table 6.1. Two days after the beginning of diclofenac treatment, animals of all four groups received stanozolol (in saline) intraperitoneally [146], at a dose of 5 mg/kg/day, six days a week. The diclofenac treatment lasted for a period of six weeks, whereas the stanozolol treatment lasted for 3 weeks (Figure 6.1). Stanozolol and diclofenac were injected at the same time on every treatment day.

Table 6. 1: Dose of stanozolol and diclofenac administered to individual animals

Group	Individual animals	Diclofenac
G1	No. 1-6	0 mg/kg
G2	No. 7-12	1 mg/kg
G3	No. 13-18	5 mg/kg
G4	No. 19-24	25 mg/kg

Group G2 received diclofenac at a daily dosage of 1 mg/kg and the recommended daily dose of diclofenac in adults is 75 to 150 mg daily [229]. Thus, depending on the body weight of adult individuals, dosage of diclofenac received by the G2 group approximately resembles the typical daily dose of diclofenac in adults. The dose of stanozolol selected was in line with previous steroid studies using rat models and considered equivalent to those abused by humans on a milligram per kilogram of body weight basis [230-233].

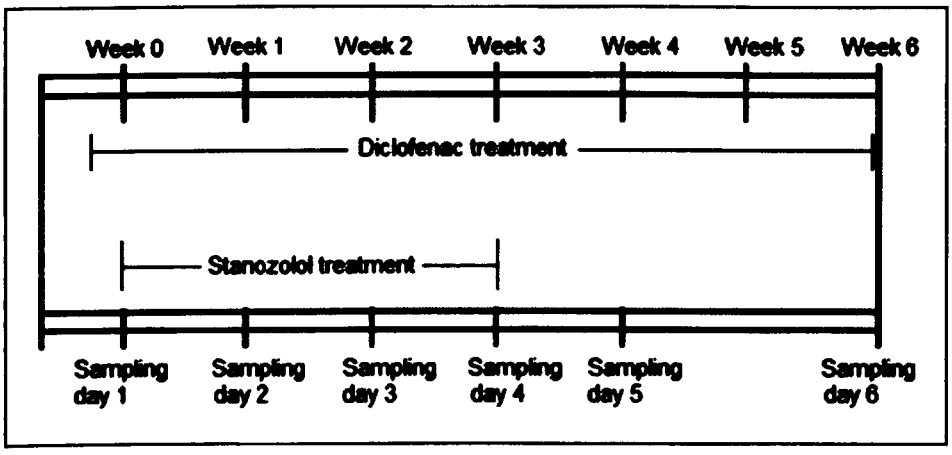

Figure 6. 1: Drug treatment and sample collection period

6.2.5. Sample collection

The hair segments, blood and two urine samples were collected once every week for six weeks with one exception at the fifth week, when no sample was collected. In group G4, which received 25 mg/kg diclofenac, 5 out of the six individuals died after 10 days of treatment. Hence, the experiment was continued without further samples from that group. Hair samples were collected by shaving an area on the back of animals using and electric shaver. Exactly the same dorsal surface was sampled each time to avoid any diluting effect of the hair grown before the stanazolol treatment period. The growth rate of rat hair was tested prior to the treatment regime by shaving the back of the experimental animals and the sampling protocol was adjusted accordingly. Urine was collected by gently pressing the abdomen. The first urine sample was collected shortly after the anaesthesia and the second urine sample was collected after one hour of stanozolol and diclofenac injections. A blood sample was taken from the tail vein one hour after stanozolol and diclofenac injection.

Blood samples were left to clot for 45 to 60 minutes and then centrifuged (at 1000 x g for 10 minutes at room temperature) to harvest serum. Before collecting blood and urine samples, the animals were anaesthetised with a mixture of ketamine and xylazine. The schedule of drug treatment and sample collection is depicted in Figure 6.2.

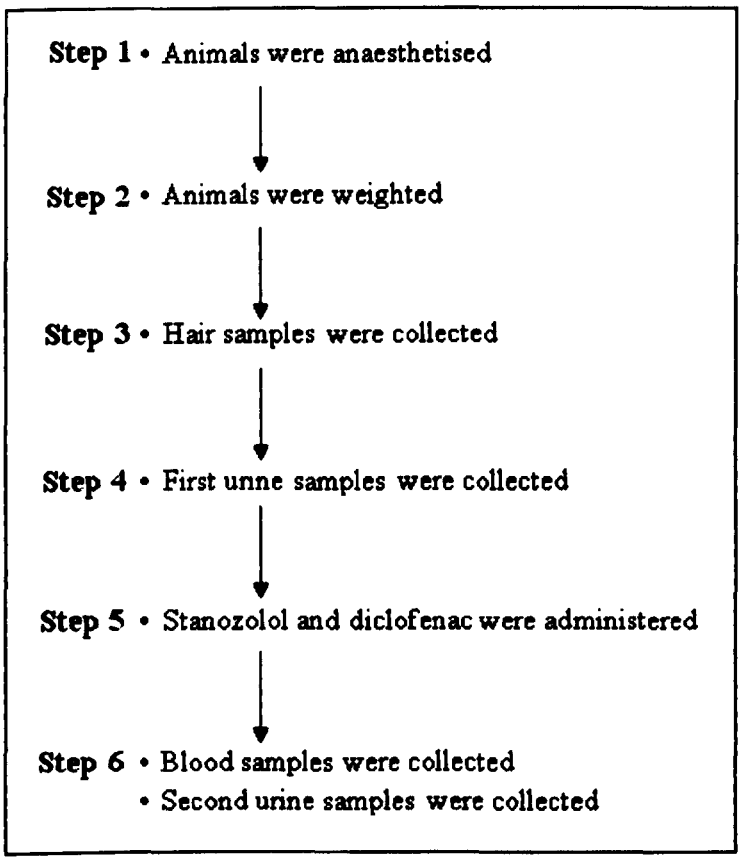

Figure 6. 2: Drug treatment and sample collection schedule

Two weeks before the experiment, the entire dorsal surface of the animal was shaved to the skin with an electric shaver and drugfree control hair was collected and preserved. Drugfree blood and urine samples were also collected before the experiment was initiated. Serum and urine samples obtained were stored at 80 ^{0}C. Hair samples were stored in sealed, clean envelopes at room temperature. The administration of drugs

and sample collection was conducted under the institutional license of Semmelweis University, Budapest, Hungary in accordance with the EC Council directives on laboratory animals (86/609/EEC). Samples were analysed in Kingston University.

6.2.6. Sample preparation

6.2.6.1. Hair samples

Decontamination

Hair samples were initially decontaminated as discussed in section 2.4.1

Alkali digestion

After decontamination, hair samples were allowed to air dry and then pulverised using a ball mill. As described in section 2.4.1, fifty milligrams of decontaminated hair powder was incubated with 1 mL 1 M sodium hydroxide at 95 ^{0}C for 10-15 minutes in the presence of deuterated ISs stanozolol D3 and 3'-hydroxystanozolol D3. After cooling, the homogenate was neutralised with 1 M hydrochloric acid, followed by addition of 2 mL of 0.2 M phosphate buffer (pH 7.0).

6.2.6.2. Serum and urine samples

Serum and urine samples were thawed and vortex mixed. A 100 μL aliquot of each was used for analysis.

6.2.6.3. Enzymatic hydrolysis of glucuronide conjugates

The enzyme β-glucuronidase was used for the enzymatic hydrolysis of glucuronide conjugates to determine the total concentration (glucuronide conjugated + unconjugated) of stanozolol and 3'-hydroxystanozolol in each matrix (hair, urine and serum). For this step, and, in a similar manner to the hair samples, the serum and urine samples were also neutralised by mixing with 1 mL of 0.2 M phosphate buffer (pH 7.0). The neutralised solutions of hair, serum and urine were hydrolysed by incubation with 50 μL of β-glucuronidase at 50 ^{0}C for two hours in the presence of ISs. After cooling, the samples were purified by performing LLE.

6.2.6.4. Liquid-liquid extraction

LLE was carried out by using a mixture of pentane, chloroform and ethylacetate (4 mL in total) in the ratio 3:2:1 v/v/v. The mixture was vortex mixed for 20 seconds and then centrifuged at 4000 x g for 20 minutes at 4 ^{0}C. The organic layer was transferred into a clean, silanised, glass vial and evaporated at 40 ^{0}C using a gentle stream of nitrogen gas. The dried residue was reconstituted with 100 μL methanol. The reconstituted solution was filtered through a 0.2 micron PTFE membrane filter, prior to injecting (3 μL) into the LC-MS/MS system.

6.2.7. LC-MS/MS analysis

The analysis of stanozolol and its metabolite 3'-hydroxystanozolol in the three matrices was carried out using an Agilent LCMS/MS system (details in section 2.5).

LC conditions

The analytical column used was a SB C18 column (2.1 mm, 50 mm, 1.8 μm), kept in a column oven at 45 ^{0}C. A 0.2 micron inline filter was installed prior to the column to prevent the analytical column from blocking. Mobile phase solvents comprised of water with 0.001% v/v formic acid as solvent A and 50:50 mixture of acetonitrile and methanol as solvent B. The flow rate of mobile phase through the column was 300 μL/min. The gradient flow composition is shown in Table 6.2.

Table 6. 2: LC mobile phase gradient conditions

LC run time (minutes)	Solvent A Water (0.001% formic acid)	Solvent B Acetonitrile : methanol (50:50)
0	60	40
1	60	40
2	15	85
5	0	100
6	0	100
7	60	40
15	60	40

Mass spectrometric conditions

The mass spectrometer was equipped with an electrospray ionisation (ESI) source, which was operated in positive ion mode. The protonated molecules, $[M+H]^+$, of stanozolol (*m/z* 329.5), 3'-hydroxystanozolol (*m/z* 345.5), stanozolol D3 (*m/z* 332.5) and 3'-hydroxystanozolol D3 (*m/z* 348.5) were used as precursor ions for CID for MSMS analysis. The mass spectrometer was operated in MRM mode to monitor the precursor ions and the diagnostic product ions of each analyte and IS. The MRM transitions, collision energies and retention times of each analyte and ISs are detailed in Table 6.3.

Table 6. 3: Retention times, MRM transitions and collision energies of stanozolol, 3'-hydroxystanozolol, stanozolol D3 and 3'-hydroxystanozolol D3

Compounds	Retention time (min)	MRM transitions	Collision energy (eV)
Stanozolol	6.0	329.5 → 81.1	50
		329.5 → 121.1	46
3'-Hydroxystanozolol	5.6	345.5 → 97.1	50
		345.5 → 121.1	42
Stanozolol D3	6.1	332.2 → 81.2	50
3'-Hydroxystanozolol D3	5.8	348.5 → 97.1	50

For the optimum ionisation of analytes, the following mass spectrometric conditions were applied: capillary voltage, 4000 V; drying gas temperature, 325 ^{0}C; drying gas flow rate, 10 L/min; nebulising gas pressure, 35 psi and fragmentor voltage of 125 V. The electron multiplier voltage was set at 1660 V. The dwell time for each transition was set at 200 ms. The mass spectrometric parameters were optimised automatically

using the Masshunter optimiser software (version B.03.01). For optimisation, a 2 μL aliquot of 1000 ng/mL solution of each compound was injected through the column in presence of mobile phase. The conditions which gave highest sensitivity for each compound were automatically saved in the method.

6.2.8. Method validation

The validation of the analytical methods was performed according to the FDA guidelines [197], by determining accuracy, precision, LLOQ, LLOD, linearity, selectivity, and extraction recoveries. Drug-free rat hair, urine and sera samples were used for method development and validation. Samples for calibration curves were prepared by spiking known amounts of stanozolol, 3'-hydroxystanozolol and ISs (stanozolol D3 and 3'-hydroxystanozolol D3) to drug free hair, urine and serum. QC samples were prepared similarly at three concentration levels (for each matrix) equally distributed over the linear range. Calibration curves were prepared for each matrix by plotting the analyte to IS ratio against the known concentrations of analyte in each sample. The analyte to IS ratio for each analyte was obtained by dividing the peak area of analyte by the peak area of the IS. Samples for calibration curves and QCs were treated in a way similar to unknowns. The linearity of the method was investigated by using linear regression analysis. Data analysis and method performance were evaluated using the Masshunter workstation software (quantitative analysis, version B.04.00).

The selectivity of the method was determined by analysing the drug free samples of hair, urine and serum in replicates and confirming the absence of any detectable peaks at the retention times of stanozolol, 3'-hydroxystanozolol and ISs. The accuracy of each assay was determined by analysing QC samples at three concentration levels in

replicates (N=6, per concentration level) and comparing the mean calculated values with the respective nominal concentration values. Intraday precision was determined by measuring 6 replicates per concentration level, on the same day. Interday precision was assessed by analysing 6 replicates per concentration level, on three consecutive days. Intraday and interday precision of the method was characterised in terms of RSD. The limits of acceptable variability were set at 15% for all the concentrations, except at LLOQ, for which 20% was accepted. LLOD was defined as the lowest concentration of the analyte which gave a peak response equivalent to three times the background noise [i.e. signal to noise ratio (S/N) $\geq$ 3]. LLOQ was defined as the lowest amount of analyte which gave a peak response with a S/N $\geq$ 10 and which could be measured with adequate precision and accuracy (RSD less than 20% and an inaccuracy ±20%). Both LLOQ and LLOD were determined empirically using consecutive 1:2 dilutions of spiked samples. Linearity of the method was investigated by using linear regression analysis.

The extraction recovery for each analyte was determined at three concentration levels by replicate analysis (N=6) of blank matrices (urine, serum and hair) spiked with known concentrations of analytes and ISs and then extracted as described in section 6.2.5.4. The analyte to IS peak area ratios obtained after extraction were then compared with analyte to IS peak area ratios of standard solutions prepared in methanol at the same final concentrations. To determine matrix effects, blank hair, urine and sera samples were extracted as described in section 6.2.5.4. In order to consider only the matrix effect and not losses during the extraction procedure, the blank extracts were spiked with known concentrations of analytes and ISs after the extraction step, followed by analysis. The resulting peak areas of stanozolol, 3'-hydroxystanozolol and ISs were then

compared with the peak areas of standard solutions of stanozolol, 3'-hydroxystanozolol and ISs at the same theoretical concentrations.

6.2.9. Statistical analysis

Group differences in stanozolol and 3'-hydroxystanozolol levels were tested using one way ANOVA followed by Tukey post hoc test when required (only when there is a significant difference). The level of significance was set at 0.05. To determine the magnitude of the difference between groups, the effect size was also analysed and was expressed as partial eta-squared: 0.01 is considered a small effect, 0.06 is considered a medium effect, and 0.14 is considered a large effect [234]. All statistical analyses were performed using SPSS 19.0.

6.3. RESULTS AND DISCUSSION

6.3.1 Method development

Both stanozolol and 3'-hydroxystanozolol were detected and quantified on the basis of their retention time and MRM transitions (Table 6.3). The most abundant product ions that were monitored for stanozolol were m/z 81.1 and 121.1, whereas for 3'-hydroxystanozolol, the most abundant product ions that were monitored were m/z 97.1 and 121.1. Figure 6.3 represents the product ions mass spectra (full scan) of stanozolol and 3'-hydroxystanozolol. Operating the mass spectrometer in MRM mode enhanced the method selectivity, sensitivity and specificity. Stanozolol D3 and 3'-hydroxystanozolol D3 were used as ISs for stanozolol and 3'-hydroxystanozolol respectively. ISs were used to compensate for any: i) ionisation suppression, ii) variations in

the instrument response from injection to injection and iii) loss of analytes during sample preparation.

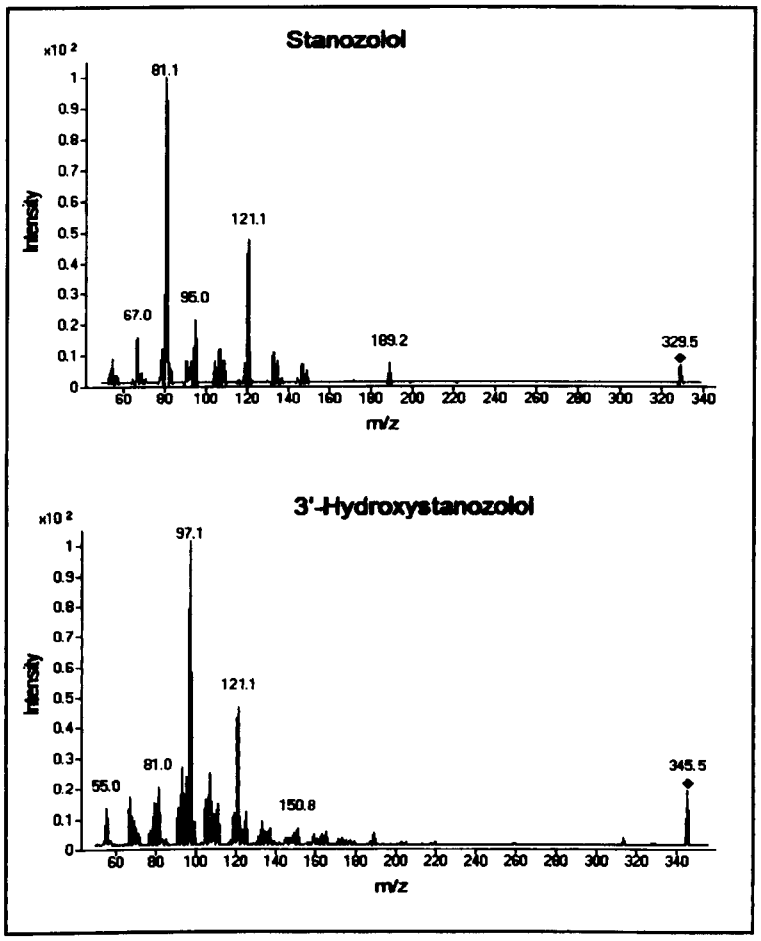

Figure 6. 3: Product ion mass spectra (full scan) of stanozolol and 3'-hydroxystanozolol

Different grades of mobile phase solvents were tried to get a low background baseline and hence to improve the sensitivity. LCMS grade water (ultra chromasolv), acetonitrile (chromasolv) and methanol (chromasolv) gave lower baselines compared to other grades of solvents and hence were used for this study. The use of different mobile phase solvents was investigated. For instance, use of water as solvent A in combination with methanol or acetonitrile or a mixture of methanol and acetontrile (50:50) as solvent B was examined. Different gradient and isocratic mobile phase compositions were investigated. Addition of formic acid (0.001% v/v, 0.01% v/v and 0.1% v/v) to solvent A

and/or solvent B was also investigated. Optimum sensitivity and excellent peak shapes for all analytes and ISs were obtained when water with formic acid (0.001% v/v) was used as solvent A and a mixture of acetonitrile and methanol (50:50) was used as solvent B under the gradient conditions shown in Table 6.2. It was observed that when formic acid was added to solvent A and/or solvent B at concentrations ≥ 0.01% v/v, there was a drastic reduction in the sensitivity of all analytes and ISs (up to 50%). However, when formic acid was added only to water (solvent A) at a concentration of 0.001% v/v, there was no effect on the sensitivity and peak shapes.

For hair analysis, alkali digestion was employed for the extraction of drugs from hair matrix. Alkali digestion ensures complete dissolution of the hair matrix and hence it is generally known to give good recoveries of drugs entrapped in the hair matrix. However, a potential drawback of complete dissolution of hair is that the components of hair matrix in solution may interfere with the analysis. Thus, to reduce the unwanted matrices that may affect the analysis, sample purification was carried out using LLE. The extraction efficiencies of different solvents like pentane, hexane, chloroform, ethylacetate and ethanol, and their combinations were investigated. It was found that a mixture of pentane, chloroform and ethylacetate in the ratio 3:2:1 v/v/v facilitated maximum recovery of analytes and ISs. Also, owing to the hair decontamination step employed (using dichloromethane), no external interferences were observed. The LLE step employed was also efficient for the extraction of stanozolol, 3'-hydroxystanozolol and ISs from the urine and sera samples. Under the analytical conditions employed there were no matrix interferences that affected the analysis of stanozolol and 3'-hydroxystanozolol in hair, urine and serum. The enzymatic hydrolysis of glucuronide conjugates of stanozolol and 3'-hydroxystanozolol was carried out to ensure that the total concentration (glucuronide conjugated plus unconjugated) of stanozolol and 3'hydroxystanozolol was determined in each matrix. The deglucuronidation step was

optimised by varying the incubation temperature (35 to 60 ^{0}C) and incubation time (30 minutes to 12 hours). For this, known amounts of 3'-hydroxystanozolol glucuronide were spiked to neutralised solutions of drug free hair, urine and serum. Samples were incubated with 50 μL of β-glucuronidase at various temperatures (35, 40, 45, 50, 55 and 60 ^{0}C) and time points (0.5, 1, 2, 4, 6 and 12 hours). After incubation, these samples were subjected to LLE followed by analysis, as described above. Optimum recovery of 3'-hydroxystanozolol, post deglucuronidation of 3'-hydroxystanozolol glucuronide was obtained when the solutions were incubated at 50 ^{0}C for 2 hours.

6.3.2. Method validation

The validation results are within the limits set by the FDA guidelines. The methods were selective and specific for unambiguous determination of stanozolol and 3'-hydroxystanozolol in all three matrices. Excellent peak shape was achieved for stanozolol, 3'-hydroxystanozolol, stanozolol D3 and 3'-hydroxystanozolol D3. Figure 6.4 represents the chromatograms of stanozolol and 3'-hydroxystanozolol extracted from hair, urine and serum at LLOQ concentration levels. Typical calibration curves of stanozolol and 3'-hydroxystanozolol in all three matrices are provided in Figure 6.5.

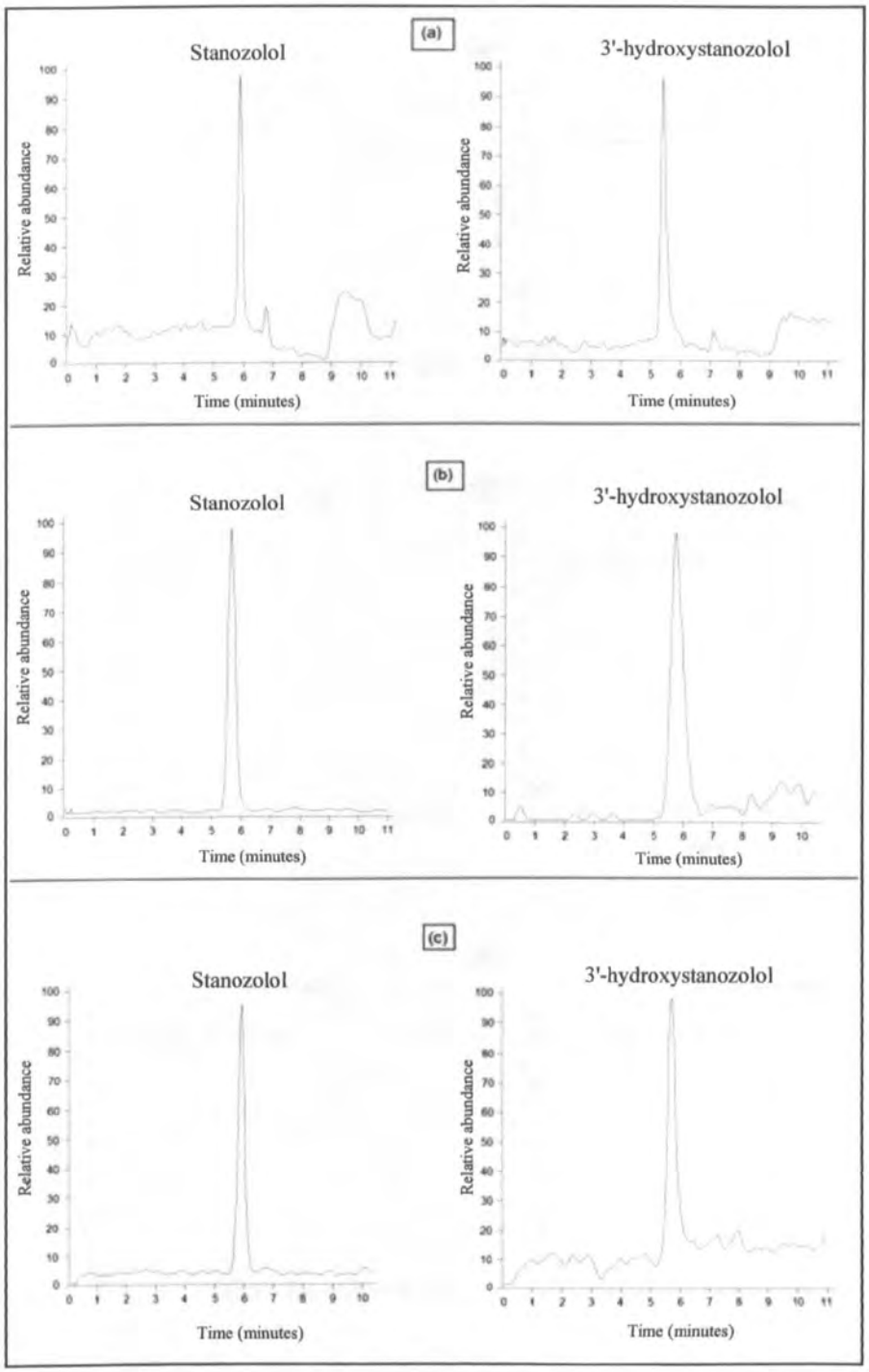

Figure 6. 4: Chromatograms of stanozolol and 3'-hydroxystanozolol extracted from (a) hair, (b) urine and (c) serum at LLOQ concentration levels

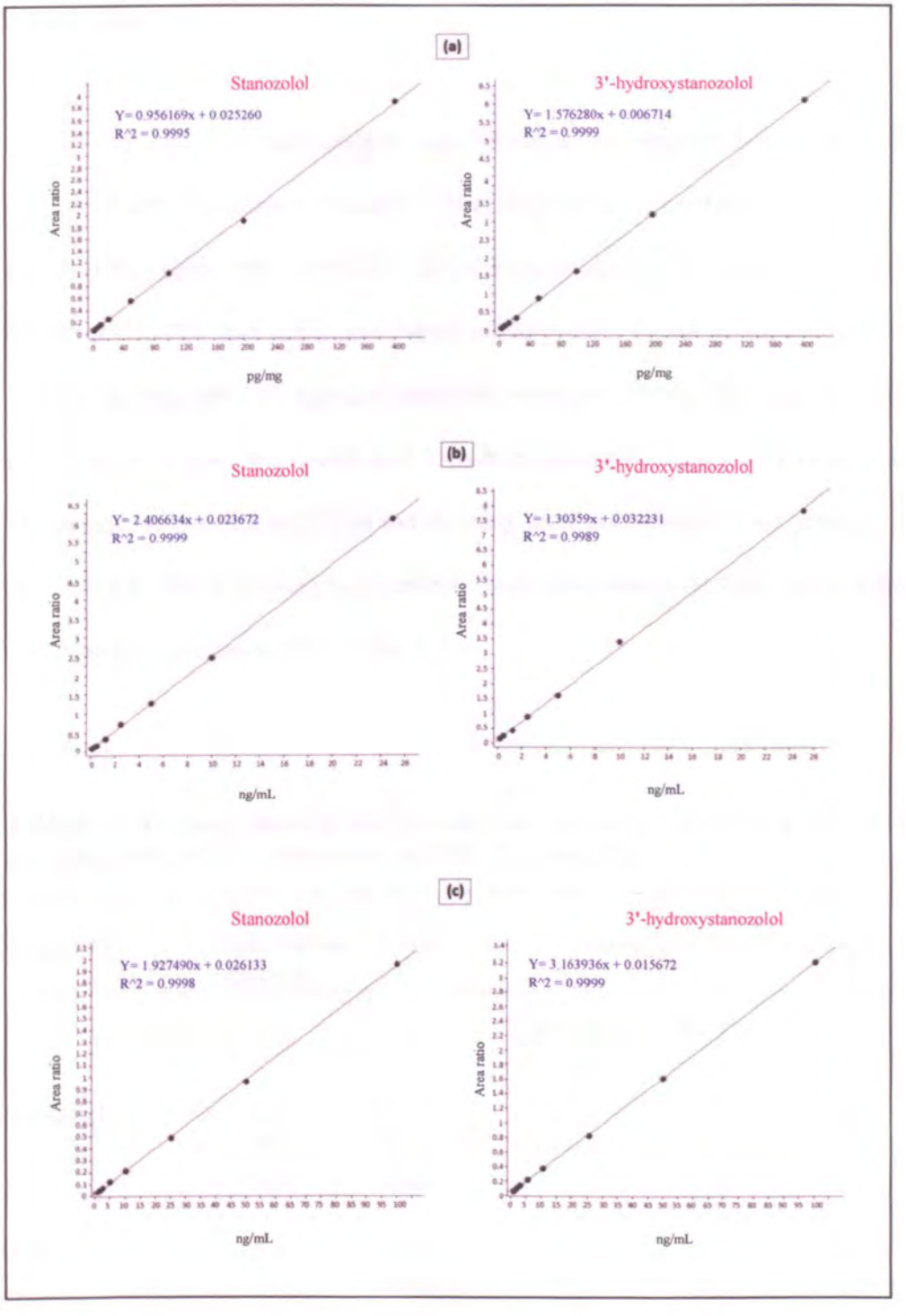

Figure 6. 5: Typical calibration curves of stanozolol and 3'-hydroxystanozolol in (a) hair, (b) urine and (c) serum

6.3.2.1. Hair

The assay for hair analysis was linear in the range 0.5-400 pg/mg for both stanozolol and 3'-hydroxystanozolol. The determination coefficient (r^2) values were found to be higher than 0.9986 for all calibration curves. The method was capable of detecting (LLOD) stanozolol and 3'-hydroxystanozolol in hair at concentrations as low as 0.125 pg/mg and 0.25 pg/mg respectively when *ca.* 50 mg hair was processed. The LLOQ level of both stanozolol and 3'-hydroxystanozolol was found to be 0.5 pg/mg. The accuracy, intraday precision and interday precision results of the assay are detailed in Table 6.4. The extraction recoveries of both compounds (at three concentration levels) from hair are presented in Table 6.5.

Table 6. 4: Accuracy, intraday precision and interday precision of the assay for detecting stanozolol and 3'-hydroxystanozolol (3-HS) in rat hair

Compounds	Concentration (pg/mg)	Level	Precision RSD (%)		Accuracy (%)
			Intraday	Interday	
Stanozolol	2.5	Low	3.4	2.8	105.0
	20	Medium	1.5	1.3	103.4
	100	High	1.2	2.8	100.7
3-HS	2.5	Low	1.5	4.1	97.3
	20	Medium	3.6	3.4	101.9
	100	High	3.8	5.3	100.5

Table 6. 5: Extraction recovery results of stanozolol and 3'-hydroxystanozolol from hair

Compounds	Concentration (pg/mg)	% Extraction Recovery (N=6)
Stanozolol	0.5 (LLOQ)	100.84
	2.5	103.53
	10	105.39
3'-Hydroxystanozolol	0.5 (LLOQ)	88.51
	2.5	107.99
	10	102.64

6.3.2.2. Urine

The assay for urinalysis was linear in the range 0.125-25 ng/mL for stanozolol and 0.25-25 ng/mL for 3'-hydroxystanozolol. The determination coefficient (r^2) values were found to be higher than 0.9959 for all runs. The method was capable of detecting (LLOD) stanozolol and 3'-hydroxystanozolol at concentrations as low as 0.063 ng/mL and 0.125 ng/mL urine respectively, when only 100 µL aliquot of urine was processed. The LLOQ levels of stanozolol and 3'-hydroxystanozolol were found to be 0.125 ng/mL and 0.25 ng/mL urine respectively. Table 6.6 summarises the accuracy, intraday precision and interday precision results of the assay. The extraction recoveries from urine at three concentration levels are presented in Table 6.7.

Table 6. 6: Accuracy, intraday precision and interday precision of the assay for detecting stanozolol and 3'-hydroxystanozolol (3-HS) in rat urine

Compounds	Concentration (ng/mL)	Level	Precision RSD (%)		Accuracy (%)
			Intraday	Interday	
Stanozolol	0.5	Low	10.4	6.3	90.6
	2.5	Medium	4.6	7.0	105.6
	5	High	4.1	4.0	111.4
3-HS	0.5	Low	4.9	6.2	89.7
	2.5	Medium	7.1	5.0	109.2
	5	High	6.4	5.1	104.2

Table 6. 7: Extraction recovery results of stanozolol and 3'-hydroxystanozolol from urine

Compounds	Concentration (ng/mL)	% Extraction Recovery (N=6)
Stanozolol	0.125 (LLOQ)	107.87
	2.5	111.77
	10	109.64
3'-Hydroxystanozolol	0.25 (LLOQ)	91.44
	2.5	107.92
	10	111.82

6.3.2.3. Serum

The serum assay showed good linearity within the quantification range 0.25-100 ng/mL for both stanozolol and 3'-hydroxystanozolol, with determination coefficient (r^2) values higher than 0.9981. The method was capable of detecting (LLOD) stanozolol and 3'-hydroxystanozolol in serum at concentrations as low as 0.063 ng/mL and 0.125

ng/mL respectively when only 100 μL aliquot of serum was processed. The LLOQ level of both stanozolol and 3'-hydroxystanozolol was found to be 0.25 ng/mL. The accuracy, intraday precision and interday precision results of the assay are detailed in Table 6.8. The extraction recoveries are presented in Table 6.9.

Table 6. 8: Accuracy, intraday precision and interday precision of the assay for detecting stanozolol and 3'-hydroxystanozolol in rat serum

Compounds	Concentration (ng/mL)	Level	Precision RSD (%)		Accuracy (%)
			Intraday	Interday	
Stanozolol	1.25	Low	4.4	3.0	111.0
	10	Medium	1.4	1.6	108.4
	50	High	1.7	1.4	90.4
3'-Hydroxystanozolol	1.25	Low	4.3	6.4	98.0
	10	Medium	3.5	3.9	102.3
	50	High	4.8	5.3	91.9

Table 6. 9: Extraction recovery results of stanozolol and 3'-hydroxystanozolol from serum

Compounds	Concentration (ng/mL)	% Extraction Recovery (N=6)
Stanozolol	0.25 (LLOQ)	113.24
	2.5	101.92
	10	109.42
3'-Hydroxystanozolol	0.25 (LLOQ)	95.36
	2.5	111.03
	10	105.29

6.3.3. Application of the method to real samples

The developed method was employed for determining the total (glucuronide-conjugated plus unconjugated) concentration of stanozolol and its metabolite 3'-hydroxystanozolol in rat urine (urine 1 and urine 2 samples), serum and hair samples. Samples were obtained from the stanozolol only treated group (G1 control group) and the diclofenac plus stanozolol treated groups (G2 and G3). The average concentration of 3'-hydroxystanozolol in rat urine (1), serum and hair in each group, during stanozolol treatment and post-stanozolol treatment is presented in Table 6.10. The average concentration of stanozolol in rat urine (1), serum and hair in each group, during stanozolol treatment and post-stanozolol treatment is presented in Table 6.11.

Table 6. 10: Average concentrations of 3'-hydroxystanozolol in rat urine (1), serum and hair during and after stanozolol treatment.

Average concentrations of 3'-hydroxystanozolol in urine 1, (ng/mL)		
Groups	During stanozolol treatment (week 1, week 2 and week 3)	Post-stanozolol treatment (week 4 and week 6)
G1	2.8 ± 2.4	BDL
G2	BDL	BDL
G3	BDL	BDL

Average concentrations of 3'-hydroxystanozolol in serum (ng/mL)		
Groups	During stanozolol treatment (week 1, week 2 and week 3)	Post-stanozolol treatment (week 4 and week 6)
G1	7.3 ± 2.0	0.9 ± 0.6
G2	8.7 ± 2.6	2.1 ± 1.0
G3	10.5 ± 1.8	2.1 ± 1.9

Average concentrations of 3'-hydroxystanozolol in hair (pg/mg)		
Groups	During stanozolol treatment (week 1, week 2 and week 3)	Post-stanozolol treatment (week 4 and week 6)
G1	29.5 ± 3.7	9.0 ±7.0
G2	24.6 ± 15.8	16.6 ± 15.0
G3	32.8 ± 9.4	10.8 ± 12.6

*BDL is below detection limit, which was found to be 0.125 ng/mL urine for 3'-hydroxystanozolol. G1 = 5 mg/kg stanozolol only, G2 = 5 mg/kg stanozolol + 1 mg/kg diclofenac, G3 = 5 mg/kg stanozolol + 5 mg/kg diclofenac

Table 6. 11: Average concentrations of stanozolol in rat urine (1), serum and hair during and after stanozolol treatment.

Average concentrations of stanozolol in urine 1, (ng/mL)		
Groups	During stanozolol treatment (week 1, week 2 and week 3)	Post-stanozolol treatment (week 4 and week 6)
G1	2.9 ± 2.9	0.9 ± 0.8
G2	1.2 ± 0.5	1.2 ± 1.1
G3	2.0 ± 0.9	0.8 ± 1.1
Average concentrations of stanozolol in serum (ng/mL)		
Groups	During stanozolol treatment (week 1, week 2 and week 3)	Post-stanozolol treatment (week 4 and week 6)
G1	18.3 ± 10.9	2.7 ± 1.1
G2	21.6 ± 7.5	3.8 ± 1.3
G3	23.2 ± 5.6	3.9 ± 2.5
Average concentrations of stanozolol in hair (pg/mg)		
Groups	During stanozolol treatment (week 1, week 2 and week 3)	Post-stanozolol treatment (week 4 and week 6)
G1	146.6 ± 11.7	126.7 ± 3
G2	105.2 ± 48.7	140.5 ± 53.3
G3	122.7 ± 39.4	136.3 ± 63.5

G1 = 5 mg/kg stanozolol only, G2 = 5 mg/kg stanozolol + 1 mg/kg diclofenac, G3 = 5 mg/kg stanozolol + 5 mg/kg diclofenac

6.3.3.1. Effect of diclofenac on the urinary excretion of 3'-hydroxystanozolol and stanozolol

During stanozolol treatment period (week 1, week 2 and week 3)

The concentrations of 3'-hydroxystanozolol and stanozolol in rat urine (1) during the six weeks of the study are shown in Figure 6.6 (concentrations with error bars are presented in the appendix Figure 6.1A). During the stanozolol treatment period, the urinary concentrations of 3'-hydroxystanozolol in the three groups were found to be significantly different [$F(2, 14) = 7.19, p = 0.007$, partial eta-squared = 0.507]. Also, the effect size was analysed to determine the magnitude of the difference between groups. The effect size was found to be large as indicated by the partial eta-squared value. Tukey's post hoc test revealed that the urinary concentration of 3'-hydroxystanozolol in group G1 (not treated with diclofenac) was significantly higher than the diclofenac treated groups, G2 ($p = 0.17$) and G3 ($p = 0.013$). This indicates that diclofenac reduced the overall urinary excretion of 3'-hydroxystanozolol in G2 and G3. However, there was no significant difference between groups G2 and G3 ($p = 1.000$). The metabolite, 3'-hydroxystanozolol was not detectable in any of the urine 2 samples, that were collected one hour after the administration of drugs, thus indicating that stanozolol did not get sufficiently metabolised to 3'-hydroxystanozolol (or its glucuronide conjugate) and excreted in urine, within one hour, in order to get detected by the assay. It also indicated that there was no detectable residual metabolite from previous week's treatment.

The average urinary concentrations (urine 1) of stanozolol in group G1 was slightly higher than in G2 and G3, but there was no significant difference between the groups [$F(2, 14) = 1.13, p = 0.351$]. Stanozolol was detectable in the urine 2 samples in all three groups, without any significant difference between them [$F(2, 14) = 0.440, p = 0.652$].

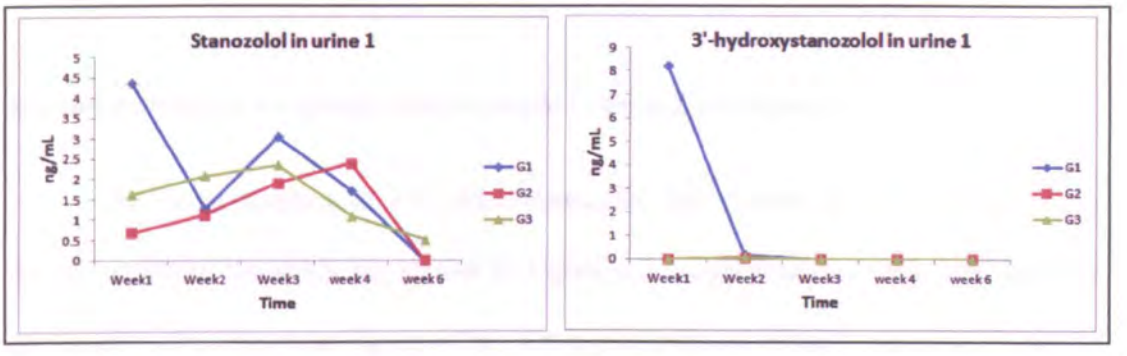

Figure 6. 6: Concentrations of stanozolol and 3'-hydroxystanozolol in urine 1 samples from groups G1, G2 and G3 during stanozolol treatment (week 1, week 2 and week 3) and poststanozolol treatment period (week 4 and week 6). G1 = 5 mg/kg stanozolol only, G2 = 5 mg/kg stanozolol + 1 mg/kg diclofenac, G3 = 5 mg/kg stanozolol + 5 mg/kg diclofenac

Post stanozolol treatment period (week 4 and week 6)

As expected, 3'-hydroxystanozolol was not detectable in urine 1 and urine 2 samples after 1 week of stopping the stanozolol treatment. Stanozolol was detectable in majority of the urine 1 and urine 2 samples for only upto one week after stopping the stanozolol treatment.

6.3.3.2. Effect of diclofenac on the serum concentration of 3'-hydroxystanozolol and stanozolol

During stanozolol treatment period (week 1, week 2 and week 3)

The concentrations of 3'-hydroxystanozolol and stanozolol in rat serum during the six weeks of the study are shown in Figure 6.7 (concentrations with error bars are presented in the appendix Figure 6.2A). Owing to reduced urinary excretion, it can be expected that the serum level of 3'-hydroxystanozolol will be significantly higher in the diclofenac plus stanozolol treated groups, than in the stanozolol only treated group. Although the average serum concentration of 3'-hydroxystanozolol in the diclofenac treated groups G2 and G3 were found to be slightly higher than in G1 (Figure 6.7), there was no significant difference [$F(2, 14) = 3.537$, $p = 0.057$]. The average serum concentrations of stanozolol in groups G1, G2 and G3 were not significantly different [$F(2, 14) = 0.543$, $p = 0.593$].

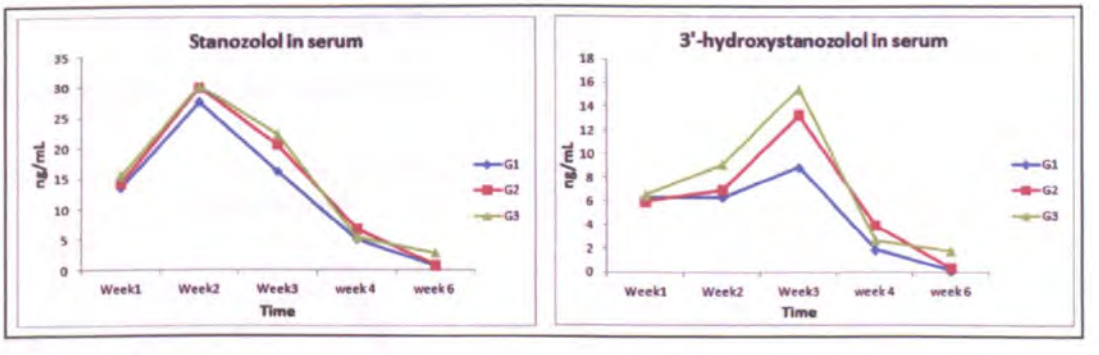

Figure 6. 7: Concentrations of stanozolol and 3'-hydroxystanozolol in serum samples from groups G1, G2 and G3 during stanozolol treatment (week 1, week 2 and week 3) and poststanozolol treatment period (week 4 and week 6). G1 = 5 mg/kg stanozolol only, G2 = 5 mg/kg stanozolol + 1 mg/kg diclofenac, G3 = 5 mg/kg stanozolol + 5 mg/kg diclofenac

Post stanozolol treatment period (week 4 and week 6)

Post stanozolol treatment, serum samples were collected for upto 3 weeks. As expected, the concentrations of both the compounds reduced over three weeks. The average serum concentrations of stanozolol and 3'-hydroxystanozolol in the three groups after stopping stanozolol treatment were also not significantly different, [F(2, 14) = 0.819, $p = 0.461$ and F(2, 14) = 1.749, $p = 0.210$; respectively].

6.3.3.3. Effect of diclofenac on the incorporation of 3'-hydroxystanozolol and stanozolol in hair

During stanozolol treatment period (week 1, week 2 and week 3)

The concentrations of 3'-hydroxystanozolol and stanozolol in rat hair during the six weeks of the study are presented in Figure 6.8 (concentrations with error bars are presented in the appendix Figure 6.3A). During the stanozolol treatment period, there was no significant difference between the groups in the concentrations of 3'-hydroxystanozolol [$F(2, 15) = 0.886$, $p = 0.433$] and stanozolol [$F(2, 15) = 1.916$, $p = 0.182$] in hair, thus suggesting that diclofenac did not inhibit the incorporation of 3'-hydroxystanozolol and stanozolol into hair.

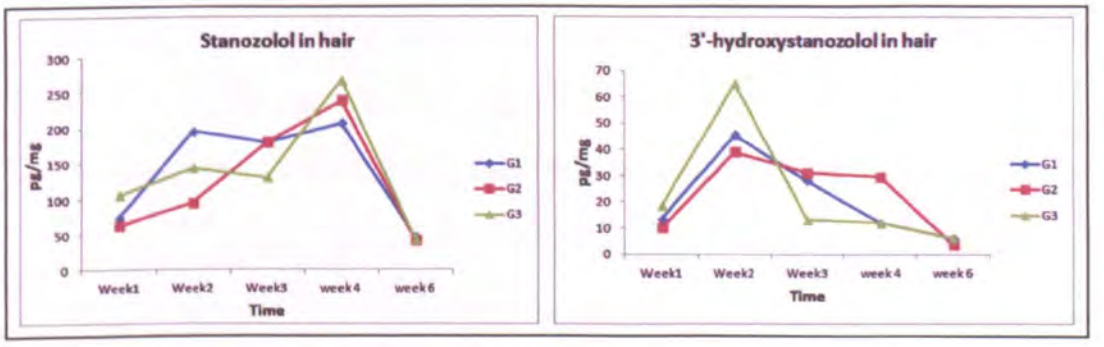

Figure 6. 8: Concentrations of stanozolol and 3'-hydroxystanozolol in hair samples from groups G1, G2 and G3 during stanozolol treatment (week 1, week 2 and week 3) and poststanozolol treatment period (week 4 and week 6). G1 = 5 mg/kg stanozolol only, G2 = 5 mg/kg stanozolol + 1 mg/kg diclofenac, G3 = 5 mg/kg stanozolol + 5 mg/kg diclofenac

Post stanozolol treatment period (week 4 and week 6)

After stopping the stanozolol treatment, hair samples were analysed for up to three weeks and as expected, even after stopping stanozolol treatment, both the compounds were detected in hair in all three groups. The concentrations of stanozolol and 3'-hydroxystanozolol in the three groups were not significantly different, [$F(2, 14) = 0.113, p = 0.894$] and [$F(2, 14) = 0.598, p = 0.563$] respectively. This indicates that diclofenac did not inhibit the incorporation of stanozolol and 3'-hydroxystanozolol in hair even after stopping the stanozolol treatment.

Overall, there was a gradual increase in the concentrations of both compounds in hair over time. In all three groups, the maximum concentration of stanozolol in hair was reached on the 4th week of the study, i.e. one week after stopping the stanozolol treatment, and then decreased gradually. However, the peak concentration of 3'-hydroxystanozolol in hair in all three groups was reached on the second week of the study, i.e. after two week of stanozolol treatment, and then decreased gradually.

The results indicate that diclofenac significantly inhibits the urinary excretion of 3'-hydroxystanozolol in rats. However, diclofenac was found to have no inhibitory effect on the incorporation of stanozolol and 3'-hydroxystanozolol in hair. Even after stopping stanozolol treatment, stanozolol was detectable in the hair of 16 animals (out of 17) for up to three weeks, whereas 3'-hydroxystanozolol was detectable in the hair of 11 animals (out of 17). In contrast, stanozolol was detectable in the urine of only 2 animals (out of 17) after three weeks of stopping stanozolol treatment, whereas 3'-hydroxystanozolol was not detectable in the urine samples of any of the animals. This highlights that the detection of both the compounds in urine can be complex, than in hair. Blood analysis can also provide important information on drug use but it is an invasive method compared to hair.

In the past, researchers have found it difficult to detect 3'-hydroxystanozolol in hair. Cirimile *et al.* [235] reported the detection of stanozolol in scalp hair of a body-builder who declared to be a regular user of stanozolol. However, 3'-hydroxystanozolol was not detectable in hair under the analytical conditions employed by them. Similarly, in another study carried out by Shen *et al.*, stanozolol was detectable in guinea pig hair after administering stanozolol at a single high dose of 60 mg/kg, whereas, 3'-hydroxystanozolol was not detectable [146]. However, the method presented in this chapter was capable of detecting stanozolol and 3'-hydroxystanozolol in rat hair after administering stanozolol for 6 days at a dose of 5.0 mg/kg/day that is considered equivalent to those levels abused by athletes. Thieme *et al* have reported a case where both stanozolol and 3'-hydroxystanozolol were detectable in the hair of a bodybuilder using gas chromatography high resolution mass spectrometry (GC-HRMS), after sample derivatisation [135]. In the past GC-MS and GC-HRMS have been frequently employed for the detection of stanozolol and or 3'-hydroxystanozolol [135, 159, 235]. The major disadvantage of such techniques is that it requires a laborious and expensive sample derivatisation step. Generally, the derivatives are unstable and susceptible to thermal decomposition during analysis, thus affecting the reproducibility of the method. In contrast, LC-MS/MS normally does not require any additional derivatisation step. Thus LC-MS/MS can be considered as a more economical and feasible approach for analysing AAS.

6.4. CONCLUSIONS

In conclusion, this study reported here for the first time that, *in vivo*, diclofenac significantly reduces the urinary excretion of 3'-hydroxystanozolol in rats. Athletes commonly use painkillers like diclofenac in order to reduce the pain associated with extensive exercise. This suggests that athletes who partake in the use of AASs along with painkillers like diclofenac are susceptible to be tested negative by urinalysis. Thus, the reliability of the conventional urinalysis based anti-doping test is challenged. On the other hand, it was found that diclofenac did not influence the concentrations of stanozolol and 3'-hydroxystanozolol in hair. In contrast to urine, hair favoured the detection of both the compounds for up to three weeks after stopping stanozolol treatment. Hence, hair analysis can be a proficient approach for testing doping when the urinary excretion of drugs is impaired due to metabolic, genetic or other factors.

The newly developed LC-MS/MS methods were accurate, reproducible and sensitive for the determination of stanozolol and its metabolite, 3'-hydroxystanozolol in rat urine, serum and hair. These methods can be extended to carry out further metabolic studies on other AASs for understanding the array of factors that may confound urinalysis results. Also, these methods can be extended for analysing human hair, urine and serum samples in combination to provide a pattern of drug use and this can be useful for testing doping with stanozolol and other commonly abused AASs. Hair can provide retrospective information on an individual's drug use and this can be used in out-of-competition testing. However, information of current drug-use, if important, can be obtained by urine and blood serum analyses. Thus, when the three tests are used in combination, useful information on an individual's drug use can be obtained and false doping results can be prevented.

CHAPTER 7

CONCLUDING REMARKS AND FUTURE WORK

To conclude, new highly sensitive, specific, reliable and reproducible LC-MS/MS methods have been developed for detecting frequently used AASs in hair and other matrices. All the methods were validated according to the FDA guidelines. Novel assays were developed for detecting nandrolone and stanozolol in human hair with only 20 mg hair. These methods were employed to analyse hair samples of subjects who participated in social science studies on doping [170, 175] and thus their self-reported drug use informations were authenticated. The methods were extended to determine the natural levels of testosterone (T), epitestosterone (E) and the T/E ratios altogether in human hair, for the first time. These assays can complement urinalysis to determine doping with nandrolone, stanozolol and testosterone when 20 to 50 mg hair is processed. These methods can be used in future to validate self-reported drug use information and thus support various social science studies on doping.

This research project also explored the influence of diclofenac on the urinary excretion patterns of stanozolol and 3'-hydroxystanozolol, *in vivo*, using a rat model and LC-MS/MS based newly developed methods. It was observed that diclofenac significantly reduced the urinary excretion of 3'-hydroxystanozolol, thus indicating that concomitant use of AASs along with diclofenac can lead to misleading doping results based on urinalysis. The results raise questions on the reliability of the urine based doping tests. Interestingly, it was also found that diclofenac did not influence the concentrations of stanozolol and 3'hydroxystanozolol in hair, *in vivo*. This indicates that hair analysis could be a proficient approach in determining doping when urinary excretion of drug is

impaired. These methods can be extended to investigate metabolism of other commonly used AASs.

This project also reports new methods for the detection of stanozolol and 3'-hydroxystanozolol in aqueous matrices when only 5 mL water was processed. These methods were successfully employed to analyse drinking and environmental water samples collected from Budapest. These methods can be extended to detect other chemicals and pharmaceutical drugs which may be hazardous to human health and environment. It is well known that there is a permanent gap between drug development and drug testing methods. Thus, the newly developed assays reported in this thesis can be further extended for detecting newly developed illicit drugs.

Future work

- To investigate the inhibitory effect of diclofenac on the urinary excretion of 3'-hydroxystanozolol in humans [185].
- To investigate the influence of diclofenac (and other NSAIDs), *in vivo*, on the urinary excretion of nandrolone, testosterone, epitestosterone and their metabolites [45].
- To investigate the inhibitory effects of common dietary substances like green tea, white tea and red wine [130, 131] and other UGT substrates on the urinary excretion of nandrolone, stanozolol, testosterone, epitestosterone and their metabolites, *in vivo*.
- To develop new methods for the detection of other widely used PEDs and new designer drugs like 4'methylethcathinone (4MEC) in hair [236].
- The sensitivity of the assay for detecting epitestosterone in hair needs to be improved.

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Hair analysis results for stanozolol (Chapter 3)

For ELISA screening, a positive control was prepared by spiking drug free hair with stanozolol to get a final concentration of 3.8 pg/mg. Drug free hair was used as a negative control. Analysis of the positive and negative controls was carried out as discussed in section 3.2.4. For the positive control, ELISA absorbance at 650 nm was found to be 0.2571, whereas for the negative control it was found to be 0.3899. Samples that gave absorbance values lower than 0.2571 were considered positive. All the positive results are given below in red (Table 3.1A). Positive results were confirmed using LC-MS/MS.

Table 3.1A: Hair analysis results for stanozolol

Sample ID	ELISA Absorbance at 650 nm	LC-MS/MS confirmation pg/mg	Nationality	Age	Sex
Sample 1	0.4329		Hungarian	30	f
Sample 2	0.3081		Hungarian	22	f
Sample 3	0.2655		Hungarian	26	f
Sample 4	0.3545		Hungarian	21	f
Sample 5	0.4302		Hungarian	24	m
Sample 6	0.3336		Hungarian	22	f
Sample 7	0.3309		Hungarian	24	f
Sample 8	0.2857		Hungarian	22	m
Sample 9	0.3151		Hungarian	18	f
Sample 10	0.3441		Hungarian	25	f
Sample 11	0.3166		Hungarian	26	m
Sample 12	0.3624		Hungarian	19	f
Sample 13	0.3834		Hungarian	22	m
Sample 14	0.3394		Hungarian	19	f
Sample 15	0.3091		Hungarian	21	f
Sample 16	0.3067		Hungarian	18	f
Sample 17	0.2882		Hungarian	20	f
Sample 18	0.3740		Hungarian	20	m
Sample 19	0.3595		Hungarian	22	m
Sample 20	0.3293		Hungarian	20	f
Sample 21	0.4281		Hungarian	23	m
Sample 22	0.2719		Hungarian	25	m
Sample 23	**0.2502**	**BDL**	**Hungarian**	**21**	**f**
Sample 24	0.3167		Hungarian	21	m
Sample 25	0.2712		Hungarian	20	f
Sample 26	0.3282		Hungarian	21	m
Sample 27	0.3333		Hungarian	18	m

Sample 28	0.2807		Hungarian	22	m
Sample 29	0.3834		Hungarian	23	m
Sample 30	**0.2269**	**BQL**	**Hungarian**	**25**	**f**
Sample 31	0.2899		Hungarian	24	f
Sample 32	**0.2422**	**86.3 pg/mg**	**Hungarian**	**20**	**f**
Sample 33	0.3411		Hungarian	21	f
Sample 34	0.3256		Hungarian	21	f
Sample 35	**0.2529**	**9.8 pg/mg**	**Hungarian**	**19**	**f**
Sample 36	0.4180		Hungarian	23	m
Sample 37	0.4577		Hungarian	22	m
Sample 38	0.3731		Hungarian	23	m
Sample 39	**0.2523**	**10.0 pg/mg**	**Hungarian**	**22**	**f**
Sample 40	0.4523		Hungarian	22	m
Sample 41	**0.2533**	**56.1 pg/mg**	**Hungarian**	**20**	**f**
Sample 42	0.3798		Hungarian	20	m
Sample 43	0.3815		Hungarian	30	f
Sample 44	0.3849		Hungarian	29	m
Sample 45	0.2877		Hungarian	23	m
Sample 46	0.3513		Hungarian	19	f
Sample 47	0.3413		Hungarian	18	m
Sample 48	0.2643		Hungarian	20	f
Sample 49	0.3269		Hungarian	28	f
Sample 50	**0.2266**	**BDL**	**Hungarian**	**23**	**m**
Sample 51	0.3182		Hungarian	19	f
Sample 52	0.2671		Hungarian	20	f
Sample 53	**0.2494**	**BDL**	**Hungarian**	**18**	**f**
Sample 54	0.2870		Hungarian	21	f
Sample 55	0.3623		Hungarian	20	m
Sample 56	**0.2506**	**11.2 pg/mg**	**Hungarian**	**19**	**m**
Sample 57	0.2915		Hungarian	22	m
Sample 58	0.2886		Hungarian	21	m
Sample 59	0.3534		Hungarian	20	f
Sample 60	0.3066		Hungarian	30	m
Sample 61	0.2921		Hungarian	20	f
Sample 62	0.3365		Hungarian	22	m
Sample 63	0.3557		Hungarian	22	m
Sample 64	0.3423		Hungarian	20	f
Sample 65	0.3908		Hungarian	20	m
Sample 66	0.3639		Hungarian	23	m
Sample 67	0.3717		Hungarian	28	m
Sample 68	0.3528		Hungarian	21	m
Sample 69	**0.2360**	**63.3 pg/mg**	**Hungarian**	**22**	**f**
Sample 70	0.2912		Hungarian	19	f
Sample 71	0.2632		Hungarian	23	f
Sample 72	**0.2382**	**26.9 pg/mg**	**Hungarian**	**20**	**f**
Sample 73	0.2798		Hungarian	23	m
Sample 74	0.3388		Hungarian	19	m

Sample 75	0.3504		Hungarian	19	m
Sample 76	0.3335		Hungarian	21	f
Sample 77	0.3505		Hungarian	23	f
Sample 78	**0.2535**	**12.7 pg/mg**	**Hungarian**	**20**	**f**
Sample 79	**0.2388**	**33.0 pg/mg**	**Hungarian**	**21**	**m**
Sample 80	0.2647		Hungarian	18	f
Sample 81	0.2693		Hungarian	21	f
Sample 82	**0.2423**	**5.0 pg/mg**	**Hungarian**	**22**	**m**
Sample 83	0.3502		Hungarian	23	m
Sample 84	0.3740		Hungarian	21	f
Sample 85	0.3156		Hungarian	22	f
Sample 86	0.3155		Hungarian	20	f
Sample 87	0.3327		Hungarian	22	m
Sample 88	0.3842		Hungarian	22	m
Sample 89	0.3399		Hungarian	23	f
Sample 90	0.3698		Hungarian	19	f
Sample 91	0.2924		Hungarian	22	f
Sample 92	0.2713		Hungarian	20	f
Sample 93	0.2674		Hungarian	22	m
Sample 94	**0.2407**	**BDL**	**Hungarian**	**22**	**f**
Sample 95	0.3781		UK	27	m
Sample 96	0.4244		UK	30	m
Sample 97	0.4131		UK	21	m
Sample 98	0.3780		UK	42	f
Sample 99	0.4175		UK	29	m
Sample 100	0.6183		UK	17	m
Sample 101	0.5173		UK	18	m
Sample 102	0.4248		UK	18	f
Sample 103	0.4828		UK	20	m
Sample 104	0.5251		UK	18	m
Sample 105	0.5404		UK	24	m
Sample 106	0.4270		UK	31	f
Sample 107	0.5122		UK	34	m
Sample 108	0.4979		UK	31	f
Sample 109	0.5124		UK	29	m
Sample 110	0.4879		UK	23	f
Sample 111	0.5899		UK	25	m
Sample 112	0.6355		UK	18	m
Sample 113	0.5367		UK	19	m
Sample 114	0.5699		UK	18	m
Sample 115	0.4795		UK	25	f
Sample 116	0.5192		UK	22	f
Sample 117	0.5832		Hungarian	19	m
Sample 118	0.5981		Hungarian	20	m
Sample 119	0.3915		Hungarian	24	m
Sample 120	0.5902		Hungarian	25	m
Sample 121	0.3853		Hungarian	22	f

Sample 122	0.5241		Hungarian	18	f
Sample 123	0.5626		Hungarian	23	m
Sample 124	0.4223		Hungarian	18	f
Sample 125	0.3812		Hungarian	18	f
Sample 126	0.5173		Hungarian	22	f
Sample 127	0.5868		Hungarian	22	f
Sample 128	0.4311		Hungarian	21	f
Sample 129	0.5156		Hungarian	19	m
Sample 130	0.5164		Hungarian	20	f
Sample 131	0.4152		Hungarian	23	f
Sample 132	0.6043		Hungarian	23	m
Sample 133	0.5377		Hungarian	21	f
Sample 134	**0.2440**	**40.2 pg/mg**	**Hungarian**	**18**	**f**
Sample 135	0.5614		Hungarian	22	m
Sample 136	0.6150		Hungarian	22	m
Sample 137	0.5875		Hungarian	19	f
Sample 138	0.6373		UK	25	m
Sample 139	0.4301		UK	24	m
Sample 140	0.5090		UK	29	m
Sample 141	0.3848		UK	18	m
Sample 142	0.4369		UK	20	m
Sample 143	0.4971		UK	18	m
Sample 144	0.5759		UK	23	m
Sample 145	0.5781		UK	29	f
Sample 146	0.5721		UK	25	m
Sample 147	0.4349		UK	28	m
Sample 148	0.5404		UK	23	m
Sample 149	0.5330		UK	22	m
Sample 150	0.4258		UK	18	m
Sample 151	0.4552		UK	30	m
Sample 152	0.5244		UK	20	m
Sample 153	0.4206		UK	18	m
Sample 154	0.5786		UK	32	m
Sample 155	0.5462		UK	20	m
Sample 156	0.4762		UK	21	m
Sample 157	0.4683		UK	22	m
Sample 158	0.4977		UK	32	m
Sample 159	0.5077		UK	27	m
Sample 160	0.4281		UK	19	m
Sample 161	0.6754		UK	23	m
Sample 162	0.4482		UK	22	m
Sample 163	0.4780		UK	19	m
Sample 164	0.5115		UK	22	m
Sample 165	0.5622		UK	28	m
Sample 166	0.5121		UK	29	m
Sample 167	0.4516		UK	25	m
Sample 168	0.4638		UK	20	m

Sample 169	0.4612		UK	25	m
Sample 170	0.5307		UK	25	m
Sample 171	0.5071		UK	30	m
Sample 172	0.5398		UK	22	m
Sample 173	0.5132		UK	24	m
Sample 174	0.4416		UK	19	m
Sample 175	0.4653		UK	20	m
Sample 176	0.6079		UK	18	m
Sample 177	0.4177		UK	21	m
Sample 178	0.5187		UK	20	m
Sample 179	0.5442		UK	18	m
Sample 180	0.3842		UK	18	m
Control*	**0.2245**	**47.4 pg/mg**	**UK**	**53**	**m**

BDL means below detection limit, which was found to be 0.5 pg/mg for stanozolol.
BQL means below quantification limit, which was found to be 1 pg/mg for stanozolol.
*Male bodybuilder who admitted use of stanozolol for the past 30 years by intramuscular and oral route.

Hair analysis results for nandrolone (Chapter 3)

For ELISA screening, a positive control was prepared by spiking drug free hair with nandrolone to get a final concentration of 3.8 pg/mg. Drug free hair was used as a negative control. Analysis of the positive and negative controls was carried out as discussed in section 3.2.4. For the positive control, ELISA absorbance at 650 nm was found to be 0.1856, whereas for the negative control it was found to be 0.3221. Samples that gave absorbance values lower than 0.1856 were considered positive. All the positive results are given below in red (Table 3.2A). All positive results were confirmed using LC-MS/MS.

Table 3.2A: Hair analysis results for nandrolone

Sample ID	ELISA Absorbance at 650 nm	LC-MS/MS confirmation pg/mg	Nationality	Age	Sex
Sample 1	0.4013		Hungarian	30	f
Sample 2	0.4052		Hungarian	22	f
Sample 3	0.2358		Hungarian	26	f
Sample 4	0.4175		Hungarian	21	f
Sample 5	0.5617		Hungarian	24	m
Sample 6	0.3667		Hungarian	22	f
Sample 7	0.3615		Hungarian	24	f
Sample 8	0.2767		Hungarian	22	m
Sample 9	0.6151		Hungarian	18	f
Sample 10	0.3764		Hungarian	25	f
Sample 11	0.3137		Hungarian	26	m
Sample 12	0.3622		Hungarian	19	f
Sample 13	0.7612		Hungarian	22	m
Sample 14	0.6334		Hungarian	19	f
Sample 15	0.3813		Hungarian	21	f
Sample 16	0.5484		Hungarian	18	f
Sample 17	0.3958		Hungarian	20	f
Sample 18	0.5876		Hungarian	20	m
Sample 19	0.6660		Hungarian	22	m
Sample 20	0.2979		Hungarian	20	f
Sample 21	0.4914		Hungarian	23	m
Sample 22	0.2625		Hungarian	25	m
Sample 23	0.2617		Hungarian	21	f
Sample 24	0.3261		Hungarian	21	m
Sample 25	0.3749		Hungarian	20	f
Sample 26	0.6785		Hungarian	21	m
Sample 27	0.5628		Hungarian	18	m
Sample 28	0.3600		Hungarian	22	m
Sample 29	0.6436		Hungarian	23	m
Sample 30	0.2075		Hungarian	25	f

Sample 31	0.3348		Hungarian	24	f
Sample 32	0.2425		Hungarian	20	f
Sample 33	0.4621		Hungarian	21	f
Sample 34	0.4578		Hungarian	21	f
Sample 35	0.2233		Hungarian	19	f
Sample 36	0.5573		Hungarian	23	m
Sample 37	0.2770		Hungarian	22	m
Sample 38	0.5561		Hungarian	23	m
Sample 39	0.2247		Hungarian	22	f
Sample 40	0.6484		Hungarian	22	m
Sample 41	0.2377		Hungarian	20	f
Sample 42	0.5559		Hungarian	20	m
Sample 43	0.6439		Hungarian	30	f
Sample 44	0.4465		Hungarian	29	m
Sample 45	0.3726		Hungarian	23	m
Sample 46	0.4846		Hungarian	19	f
Sample 47	0.6174		Hungarian	18	m
Sample 48	0.2634		Hungarian	20	f
Sample 49	0.3109		Hungarian	28	f
Sample 50	**0.1562**	**BDL**	**Hungarian**	**23**	**m**
Sample 51	0.3568		Hungarian	19	f
Sample 52	0.2693		Hungarian	20	f
Sample 53	0.2064		Hungarian	18	f
Sample 54	0.3077		Hungarian	21	f
Sample 55	0.2981		Hungarian	20	m
Sample 56	0.2585		Hungarian	19	m
Sample 57	0.2235		Hungarian	22	m
Sample 58	0.7160		Hungarian	21	m
Sample 59	0.3473		Hungarian	20	f
Sample 60	0.4724		Hungarian	30	m
Sample 61	0.3492		Hungarian	20	f
Sample 62	0.6265		Hungarian	22	m
Sample 63	0.6985		Hungarian	22	m
Sample 64	0.3101		Hungarian	20	f
Sample 65	0.5931		Hungarian	20	m
Sample 66	0.6164		Hungarian	23	m
Sample 67	0.5703		Hungarian	28	m
Sample 68	0.4755		Hungarian	21	m
Sample 69	0.2392		Hungarian	22	f
Sample 70	0.2994		Hungarian	19	f
Sample 71	0.2516		Hungarian	23	f
Sample 72	0.2539		Hungarian	20	f
Sample 73	0.4291		Hungarian	23	m
Sample 74	0.5985		Hungarian	19	m
Sample 75	0.5029		Hungarian	19	m
Sample 76	0.3346		Hungarian	21	f
Sample 77	0.5887		Hungarian	23	f

Sample 78	0.2821		Hungarian	20	f
Sample 79	0.2493		Hungarian	21	m
Sample 80	**0.1081**	**14.04 pg/mg**	**Hungarian**	**18**	**f**
Sample 81	0.2455		Hungarian	21	f
Sample 82	0.2756		Hungarian	22	m
Sample 83	0.4265		Hungarian	23	m
Sample 84	0.5144		Hungarian	21	f
Sample 85	0.2910		Hungarian	22	f
Sample 86	0.3257		Hungarian	20	f
Sample 87	0.3320		Hungarian	22	m
Sample 88	0.3750		Hungarian	22	m
Sample 89	0.2982		Hungarian	23	f
Sample 90	0.5327		Hungarian	19	f
Sample 91	0.2764		Hungarian	22	f
Sample 92	0.3061		Hungarian	20	f
Sample 93	0.2486		Hungarian	22	m
Sample 94	0.2372		Hungarian	22	f
Sample 95	0.3263		UK	27	m
Sample 96	0.3528		UK	30	m
Sample 97	0.3383		UK	21	m
Sample 98	0.3174		UK	42	f
Sample 99	0.3305		UK	29	m
Sample 100	0.4377		UK	17	m
Sample 101	0.4724		UK	18	m
Sample 102	0.2864		UK	18	f
Sample 103	0.2980		UK	20	m
Sample 104	0.4209		UK	18	m
Sample 105	0.5021		UK	24	m
Sample 106	0.2821		UK	31	f
Sample 107	0.4019		UK	34	m
Sample 108	0.2787		UK	31	f
Sample 109	0.3674		UK	29	m
Sample 110	0.3155		UK	23	f
Sample 111	0.6101		UK	25	m
Sample 112	0.6223		UK	18	m
Sample 113	0.2395		UK	19	m
Sample 114	0.3541		UK	18	m
Sample 115	0.3588		UK	25	f
Sample 116	0.3434		UK	22	f
Sample 117	0.5922		Hungarian	19	m
Sample 118	0.3986		Hungarian	20	m
Sample 119	0.2383		Hungarian	24	m
Sample 120	0.4701		Hungarian	25	m
Sample 121	0.2514		Hungarian	22	f
Sample 122	0.3330		Hungarian	18	f
Sample 123	0.5892		Hungarian	23	m
Sample 124	0.3065		Hungarian	18	f

Sample 125	0.2941		Hungarian	18	f
Sample 126	0.5099		Hungarian	22	f
Sample 127	0.6501		Hungarian	22	f
Sample 128	0.3481		Hungarian	21	f
Sample 129	0.5644		Hungarian	19	m
Sample 130	0.2100		Hungarian	20	f
Sample 131	0.2678		Hungarian	23	f
Sample 132	0.4441		Hungarian	23	m
Sample 133	0.3325		Hungarian	21	f
Sample 134	0.5293		Hungarian	18	f
Sample 135	0.6746		Hungarian	22	m
Sample 136	0.6125		Hungarian	22	m
Sample 137	0.5056		Hungarian	19	f
Sample 138	0.5564		UK	25	m
Sample 139	0.2677		UK	24	m
Sample 140	0.3260		UK	29	m
Sample 141	0.4798		UK	18	m
Sample 142	0.2606		UK	20	m
Sample 143	0.2723		UK	18	m
Sample 144	0.3661		UK	23	m
Sample 145	0.4796		UK	29	f
Sample 146	0.4112		UK	25	m
Sample 147	0.3225		UK	28	m
Sample 148	0.3493		UK	23	m
Sample 149	0.3677		UK	22	m
Sample 150	0.2762		UK	18	m
Sample 151	0.3281		UK	30	m
Sample 152	0.3182		UK	20	m
Sample 153	0.2525		UK	18	m
Sample 154	0.2862		UK	32	m
Sample 155	0.4459		UK	20	m
Sample 156	0.2792		UK	21	m
Sample 157	0.2763		UK	22	m
Sample 158	**0.1617**	**BDL**	**UK**	**32**	**m**
Sample 159	0.4230		UK	27	m
Sample 160	0.2597		UK	19	m
Sample 161	0.5541		UK	23	m
Sample 162	0.2478		UK	22	m
Sample 163	0.3179		UK	19	m
Sample 164	0.2783		UK	22	m
Sample 165	0.3887		UK	28	m
Sample 166	0.2642		UK	29	m
Sample 167	0.2860		UK	25	m
Sample 168	0.2686		UK	20	m
Sample 169	0.2851		UK	25	m
Sample 170	0.2832		UK	25	m
Sample 171	0.4074		UK	30	m

Sample 172	0.2837	UK	22	m
Sample 173	0.2992	UK	24	m
Sample 174	0.2425	UK	19	m
Sample 175	0.2854	UK	20	m
Sample 176	0.4050	UK	18	m
Sample 177	0.2451	UK	21	m
Sample 178	0.3169	UK	20	m
Sample 179	0.3729	UK	18	m
Sample 180	0.2130	UK	18	m

BDL means below detection limit, which was found to be 3 pg/mg for nandrolone (LC-MS/MS)

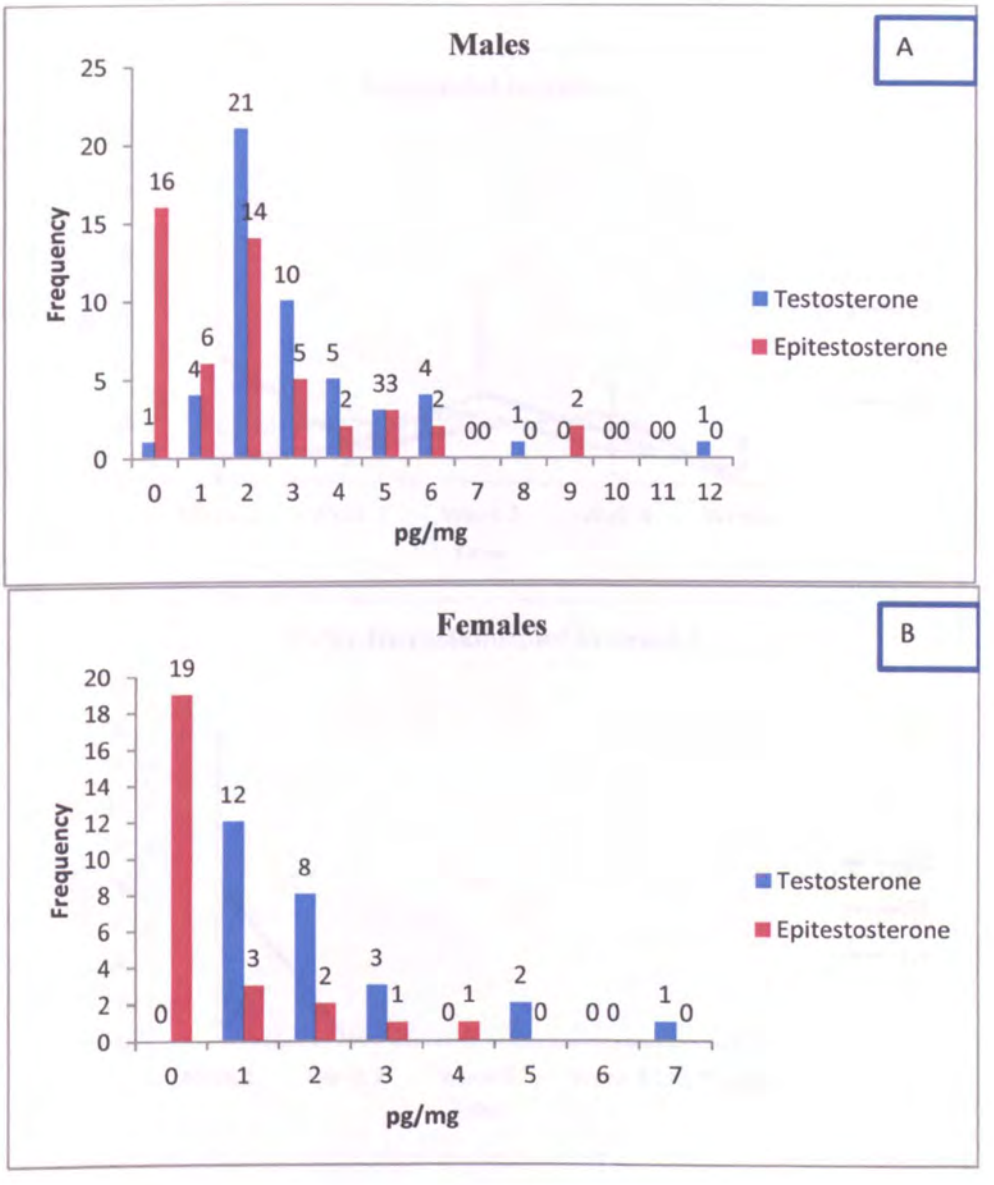

Figure 5.1A: Histograms of results of hair analysis for males (A) and females (B) population (N=75)

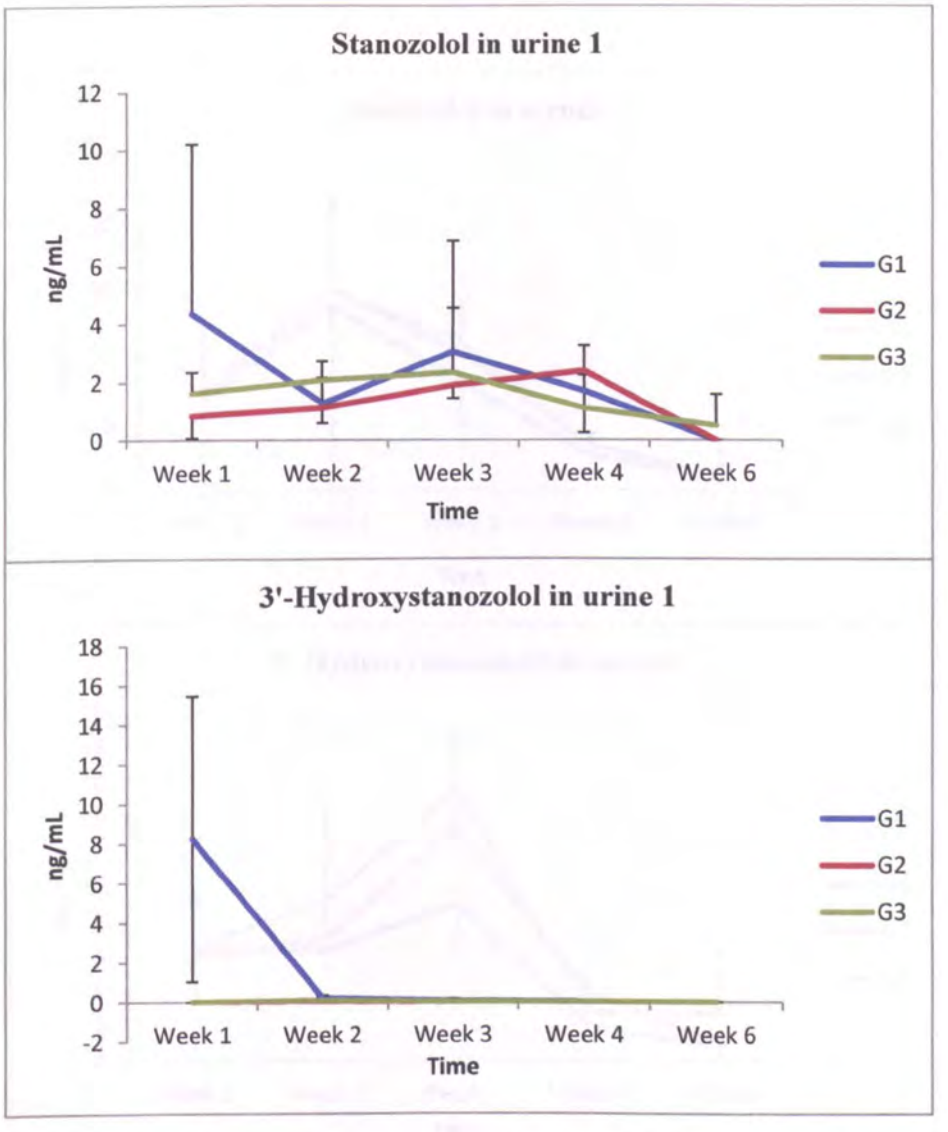

Figure 6. 1A: Concentrations of stanozolol and 3'-hydroxystanozolol in urine (1) samples from groups G1, G2 and G3 during stanozolol treatment (week 1, week 2 and week 3) and poststanozolol treatment period (week 4 and week 6). G1 = 5 mg/kg stanozolol only, G2 = 5 mg/kg stanozolol + 1 mg/kg diclofenac, G3 = 5 mg/kg stanozolol + 5 mg/kg diclofenac

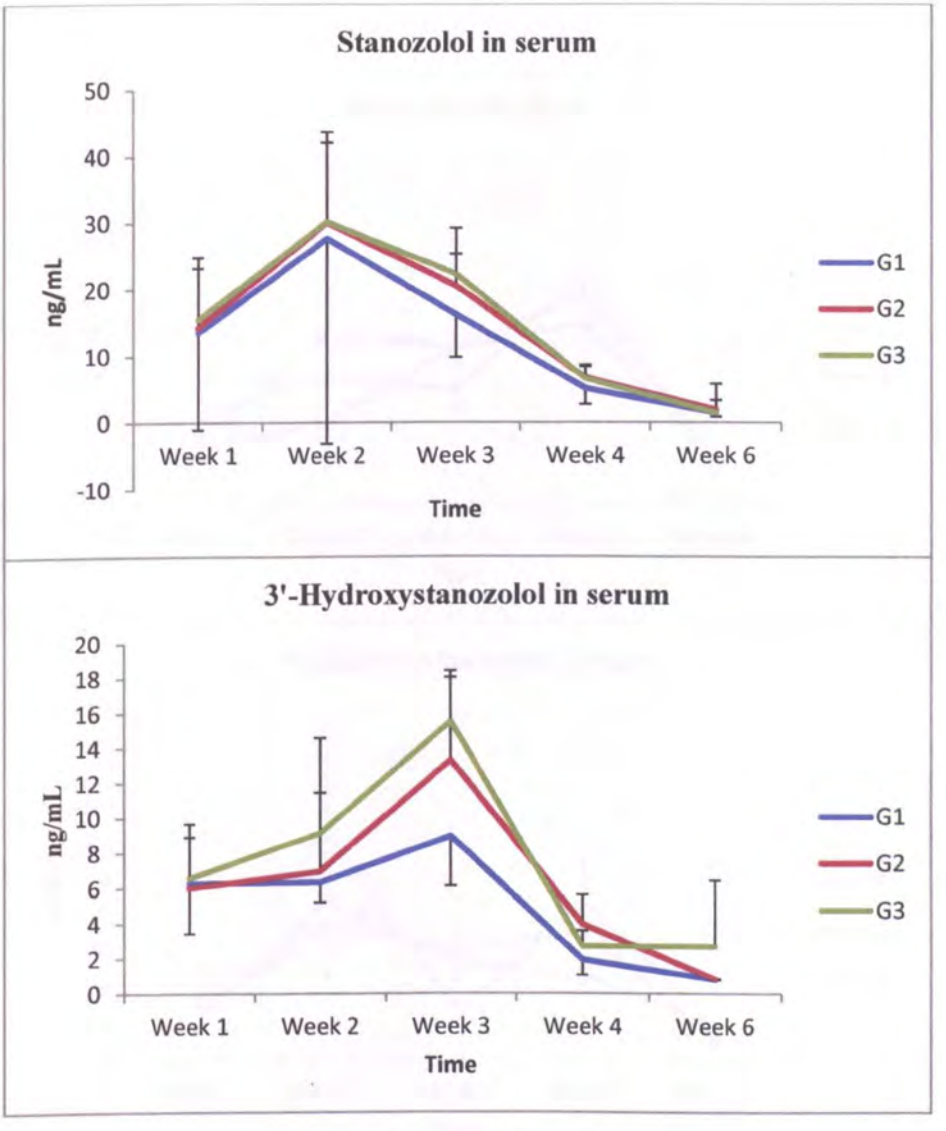

Figure 6. 2A: Concentrations of stanozolol and 3'-hydroxystanozolol in serum samples from groups G1, G2 and G3 during stanozolol treatment (week 1, week 2 and week 3) and poststanozolol treatment period (week 4 and week 6). G1 = 5 mg/kg stanozolol only, G2 = 5 mg/kg stanozolol + 1 mg/kg diclofenac, G3 = 5 mg/kg stanozolol + 5 mg/kg diclofenac

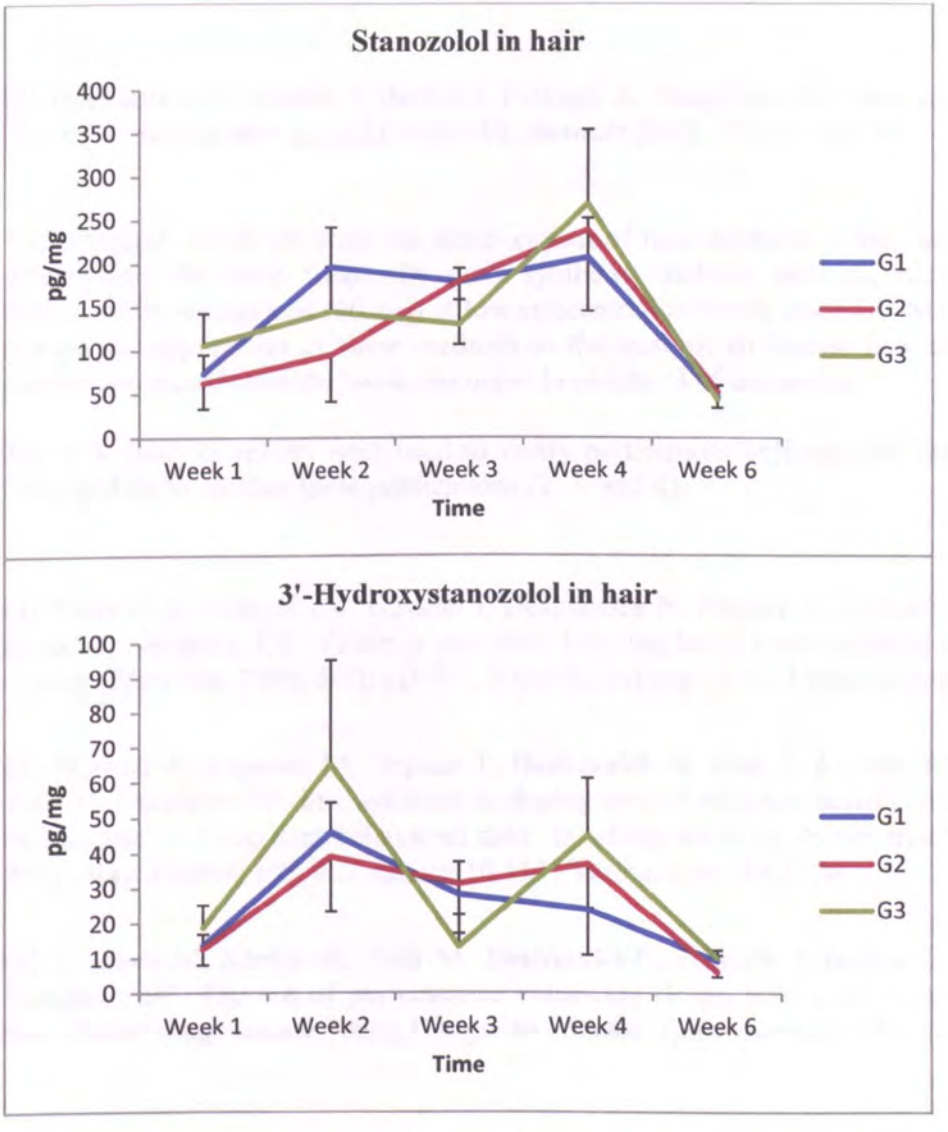

Figure 6. 3A: Concentrations of stanozolol and 3'-hydroxystanozolol in hair samples from groups G1, G2 and G3 during stanozolol treatment (week 1, week 2 and week 3) and poststanozolol treatment period (week 4 and week 6). G1 = 5 mg/kg stanozolol only, G2 = 5 mg/kg stanozolol + 1 mg/kg diclofenac, G3 = 5 mg/kg stanozolol + 5 mg/kg diclofenac

Publications arising from this thesis

[1] **Deshmukh N,** Hussain I, Barker J, Petroczi A, Naughton DP. Analysis of anabolic steroids in human hair using LC-MS/MS. *Steroids* 2010, 75(10):710-714.

This research article presents the development of new methods, which are capable of determining the most frequently used synthetic anabolic steroids, nandrolone and stanozolol in human hair (20 mg) at low concentration levels using LC-MS/MS. It also reports the application of these methods to the analysis of human hair samples. This publication raised from the work discussed in chapter 3 of this thesis.

The hair analysis results were used to verify participants' self-reported doping behaviour, leading to another three publications (2, 3, and 4).

[2] Petroczi A, Aidman EV, Hussain I, **Deshmukh N,** Nepusz T, Uvacsek M, Toth M, Barker J, Naughton DP. Virtue or pretense? Looking behind self-declared innocence in doping. *PLoS One* 2010, 5(5): e10457, http://dx.doi.org/10.1371/journal.pone.0010457.

[3] Petroczi A, Uvacsek M, Nepusz T, **Deshmukh N,** Shah I, Aidman EV, Barker J, Toth M, Naughton DP. Incongruence in doping related attitudes, beliefs and opinions in the context of discordant behavioral data: In which measure do we trust? *PLoS One* 2011, 6(4): e18804, http://dx.doi.org/10.1371/journal.pone.0018804.

[4] Uvacsek M, Ránky M, Tóth M, **Deshmukh N,** Hussain I, Barker J, Petroczi A, Naughton DP. The use of performance enhancing drugs, nutritional supplements and recreational drugs among young Hungarian athletes. *Sporttudomanyi Szemle* 2010 4:20.

[5] **Deshmukh N,** Barker J, Petroczi A, Naughton DP. Detection of stanozolol in environmental waters using liquid chromatography tandem mass spectrometry. *Chemistry Central Journal* 2011, 5:63, http://journal.chemistrycentral.com/content/5/1/63.

This article reports the development of a new method, which is capable of detecting stanozolol in various aqueous matrices using only 5 mL sample. Stanozolol was detected in the hair samples of subjects living in Budapest city (reported in publication 1). Hence this reason this study was initiated to investigate the possible environmental sources of steroid contamination. This publication was raised from the work discussed in chapter 4.

[6] **Deshmukh N,** Hussain I, Barker J, Petroczi A, Naughton DP. Detection of testosterone in human hair using liquid chromatography-tandem mass spectrometry. *Journal of Pharmacy and Pharmacology* 2010, 62(10):1205.

This abstract presents the development of a new method capable of determining testosterone in human hair at low concentration levels using LC-MS/MS. This work was presented at UKPharmSci conference held at Nottingham University. A poster was presented, which was awarded 'The best poster' prize by GlaxoSmithKline. Also, an oral talk was presented.

This abstract was short-listed for a press release and then published online by *LabBulletin* (2010) with the title 'Testosterone doping advance' (http://www.labbulletin.com/ articles/20100906_2). This work was further extended and published as a short communication (publication 7)

[7] **Deshmukh N,** Barker J, Petroczi A, Naughton DP. Detection of testosterone and epitestosterone in human hair using liquid chromatography-tandem mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis* 2012, 67-68:154-158.

This short communication article presents the development of new method, which is capable of determining the levels of testosterone and its inactive epimer, epitestosterone in human hair using only 50 mg hair. This article also reports for the first time the natural levels of T/E ratio in human hair. This method can compliment urinalysis to detect doping with testosterone. This publication arised from the work discussed in chapter 5.

[8] **Deshmukh N,** Zachar G, Petroczi A, Székely AD, Barker J, Naughton DP. Determination of stanozolol and 3'-hydroxystanozolol in rat hair, urine and serum using liquid chromatography tandem mass spectrometry. *Chemistry Central Journal* 2012, 6:162, http://journal. chemistrycentral.com/content/6/1/162.

This methodology paper presents for the first time the development of new methods, which are capable of detecting stanozolol and 3'-hydroxystanozolol in rat hair, urine and blood serum using LC-MS/MS. As discussed in chapter 6, these published methods were used to investigate the inhibitory effect of diclofenac on the urinary excretion of stanozolol and 3'-hydroxystanozolol, *in vivo,* using a rat model.

[9] **Deshmukh N**, Petroczi A, Barker J, Szekely AD, Hussain I, Naughton DP. Potentially harmful advantage to athletes: a putative connection between UGT2B17 gene deletion polymorphism and renal disorders with prolonged use of anabolic androgenic steroids. *Substance Abuse Treatment, Prevention, and Policy* 2010, 5:7, http://dx.doi.org/10.1186/1747-597X-5-7.

This article postulates the association of deletion polymorphism in the UGT2B17 gene with the occurrence of renal disorders on chronic exposure to AASs. Individuals devoid of the UGT2B17 gene are reported to have impaired urinary excretion patterns of AAS. Owing to this the circulating levels of active AAS may get elevated. This may increase the body mass index leading to renal injuries. This article is briefly discussed in chapter 1.

Other publications

[10] Shah SAB, **Deshmukh N**, Barker J, Petroczi A, Cross P, Archer R, Naughton DP. Quantitative analysis of mephedrone using liquid chromatography tandem mass spectroscopy: application to human hair. *Journal of Pharmaceutical and Biomedical Analysis* 2012, 61:64-69.

[11] Petroczi A, Nepusz T, Cross P, Taft H, Shah S, **Deshmukh N**, Schaffer J, Shane M, Adesanwo C, Barker J, Naughton DP. New non-randomised model to assess the prevalence of discriminating behaviour: a pilot study on mephedrone. *Substance Abuse Treatment, Prevention, and Policy* 2011, 6:20, http://dx.doi.org/10.1186/1747-597X-6-20.

[12] Leeder J, Van Someren K, Gaze D, Jewell A, **Deshmukh N**, Shah I, Barker J, Howatson G. Recovery and adaptation from repeated intermittent sprint exercise. Manuscript submitted to *'International Journal of Sports Physiology and Performance'* has been accepted for publication.

SOME OF THE REFERENCES HAVE BEEN EXCLUDED AT THE REQUEST OF THE UNIVERSITY

Virtue or Pretense? Looking behind Self-Declared Innocence in Doping

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Abstract

Background: Social science studies of doping practices in sport rely predominantly on self-reports. Studies of psychoactive drug use indicate that self-reporting is characterised by under-reporting. Likewise doping practice is likely to be equally under-reported, if not more so. This calls for more sophisticated methods for such reporting and for independent, objective validation of its results. The aims of this study were: i) to contrast self-reported doping use with objective results from chemical hair analysis and ii) to investigate the influence of the discrepancy on doping attitudes, social projection, descriptive norms and perceived pressure to use doping.

Methodology/Principal Findings: A doping attitudes questionnaire was developed and combined with a response latency-based implicit association test and hair sample analysis for key doping substances in 14 athletes selected from a larger sample (N = 82) to form contrast comparison groups. Results indicate that patterns of group differences in social projection, explicit attitude about and perceived pressure to use doping, vary depending on whether the user and non-user groups are defined by self-report or objectively verified through hair analysis. Thus, self-confessed users scored higher on social projection, explicit attitude to doping and perceived pressure. However, when a doping substance was detected in the hair of an athlete who denied doping use, their self-report evidenced extreme social desirability (negative attitude, low projection and low perceived pressure) and contrasted sharply with a more positive estimate of their implicit doping attitude.

Conclusions/Significance: Hair analysis for performance enhancing substances has shown considerable potential in validating athletes' doping attitude estimations and admissions of use. Results not only confirm the need for improved self-report methodology for future research in socially-sensitive domains but also indicate where the improvements are likely to come from: as chemical validation remains expensive, a more realistic promise for large scale studies and online data collection efforts is held by measures of implicit social cognition.

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Introduction

The widespread use of performance enhancing drugs [1], along with advances in performance enhancements coupled with the increasing costs of continuous development of the testing methods [2] have led anti-doping strategies to turn to identifying predictors and/or barriers of doping behaviour, over and above sanctioning. The recent debate around the practicalities and moral justification of in- and out of competition testing [1,3] has reinforced the need for preventive measures. Social science doping research has a long standing tradition in investigating social cognition (attitudes, norms, beliefs) and personality traits in a quest to find a set of characters that clearly distinguishes athletes who engage in doping practices and those who do not [4–10]. Based on these differences, past research has strived to establish behavioural models [11–16] with the ultimate aim of being able to predict doping use and to inform anti-doping programmes for potential intervention points and strategies. To date, only a few of these models have been empirically tested [13,15], and they are exclusively based on self-declaration of behavioural intention or behaviour; and explicit assessment of attitudes, beliefs, norms and motivation.

Previously, researchers assumed that social cognitive determinants of behaviour are accessible and explicitly endorsed by individuals, hence relied exclusively on individual's self-reports when investigating thoughts and feelings that underlie human behaviour. However, over the past two decades, convincing evidence has led to suggestions that the human mind operates in dual, conscientious and unconscientious, mode [17–19], therefore key components of the cognitive processes influencing behaviour are partially hidden from people's awareness or under limited

ability to control. Owing to this phenomenon, it has been acknowledged that self-report measures are restricted in capturing the complexity of the cognitive processes that underlie social actions, thus social psychologists have turned to incorporating implicit assessment of the relevant cognitions. This approach has particularly intrigued researchers in socially sensitive domains where it is fair to assume that socially desirable responding is likely to confound explicit assessments [20].

Individual differences in implicit cognition exert a profound influence on social behaviour, including attitudes, stereotypes and self-concept. Their assessment poses one of the most intriguing challenges in psychological measurement. In addition to projective testing and similar interpretive methods traditionally employed to assess 'the unspoken', recent developments in cognitive methodology offer a host of new methods ranging from priming [21] and implicit association [22] through semi-projective techniques [23] to performance based methods such as video-game embedded assessment protocols [24,25].

Recently, the utility of implicit measures of social cognition have been investigated in relation to doping. A recent study [26] showed that the adapted Implicit Association Test (IAT) has the capacity to uncover automatic evaluative bias toward doping among self-confessed users and was able to predict behaviour in hypothetical situations above and beyond the explicit measures. Although the authors concluded that the doping IAT could further benefit from a refined stimuli set and improved protocol, the results indicated that implicit assessment of doping attitude has the ability to make a key contribution to the understanding of cognitive processes behind doping behaviour. A study using an emotional Stroop task with doping words suggested that allocation of attentional resources presents among young adolescents, but the source of this attentional bias has remained speculative [27]. Young people might be tuned for doping related stimuli because of external exposure (media, anti-doping education), and not necessarily internal motivation.

Assessment of doping attitude-behaviour links

The majority of the quantitative research into doping behaviour has been based on self-reports, where athletes are not only asked to report on their own attitudes, perceived injunctive and/or descriptive norms but also asked to confess their compromising behaviour (i.e. taking prohibited substances). Self-reports among athletes in Olympic sports have yielded prevalence data ranging between 1 and 30%, which itself is higher than the yearly rate (~2.%) of adverse analytical findings in the World Anti-Doping Agency accredited laboratories [28]. This 2% constitutes a yearly average of some 3,500 positive tests.

Alternative approaches to self-report methods

Despite the widespread use, self-report techniques come with considerable limitations. With regard to self-reported behaviour, it must be assumed that individuals are willing to disclose this, often discriminating, information to the researcher. When self-reports are used to assess social cognitive processes, it is further assumed that people have introspective access to the construct in question (e.g. attitude) and have no intention of distorting their responses. Violations of either of these two assumptions negate the validity of self-report assessment and conclusions derived solely from self-declared data.

Doping is a decidedly ostracised behaviour. Admitting use or even expressing supportive opinions against the general view is likely to prompt many athletes to conceal their true behaviour and thoughts about doping if they could be discriminating for the person or the group he/she represents. Recently, researchers have recognised this problem and made attempts to use indirect methods to obtain information on doping behaviour. One notable example being the use of the Random Response Technique (RRT) where estimation of doping prevalence is made on aggregated levels [29,30]. Another line of research has made attempts to estimate the likelihood of self-involvement in doping utilising the False Consensus Effect (FCE) which has been evidenced in various socially sensitive situations [31,32]. Despite the advances these latter approaches have brought to doping behaviour research, results still carry the inevitable caveat of being based on self-declarations. Independent validation or calibration [33] of these results remains an issue.

Objective verification of self-reported drug use

Previously reported validity studies of self-reported drug behaviour used chemical analysis for the presence of mainly social drugs in urine, saliva or hair [34–39]. Beyond the expected discrepancies, it was also demonstrated that inconsistencies in self-reported drug use by adolescents are not random but are associated with socioeconomic parameters, personality characteristics and/or underlying social cognitive determinants [35]. For example, reporting and under-reporting of drug use was discordant and driven by social desirability concerns [36]. Discordance between self-reports and objective validation also occurred in the unexpected direction with a considerable proportion (34%) of self-report data unconfirmed by urinalysis [39]. This may be explained by the difference between the time and/or duration of use, drug half-life and the detection window of the chosen chemical validation. To our knowledge, no research has been published that focuses on verifying self-reported performance enhancing drug use with chemical analysis of hair samples which covers prolonged periods.

In spite of the limited validity of self-reports in socially sensitive behaviour being well documented, how this discrepancy affects the conclusions drawn on the differences in social cognitive measures between those involved vs. those who are abstinent remains unknown. Whereas social psychology research routinely considers the effect of social desirability on explicitly assessed data, we are unaware of studies that investigated differences in related social cognition under different scenarios where user vs. non-user groups were established based on self-report admissions, chemical findings or validated self-reports, and used both explicit and implicit assessments. Therefore, the aims of this study were: i) to contrast self-reported doping use with objective results from chemical hair analysis and ii) to investigate the influence of the discrepancy on doping attitudes, social projection, descriptive norms and perceived pressure to use doping.

Aims

Previous research using a larger sample pool, from which the current study sample was selected, investigated the FCE regarding doping and social drug use and provided compelling evidence of the differences in projected use of doping among peers and attitude between those athletes who confessed to having personal experience with doping and those who claimed no use [32]. The differences were in the expected direction with self-confessed doping users giving higher prevalence estimates, showing a more lenient explicit attitude toward performance enhancements than their no-user counterparts. In this study, we expanded the investigation by using hair analysis to verify self-reported doping use or abstinence, and added implicit assessments in a selected group of athletes.

We hypothesised that:

H1: Accurately reported doping use was expected to be associated with more positive explicit doping attitudes and higher estimates of projected use by others; while denied use would lead to lower explicit attitudes but realistic or elevated estimates of doping use in others; and accurately reported abstinence ('clean athletes') would be associated with relatively low scores on both measures.

H2: Doping use was expected to result in greater correlation between explicit and implicit doping attitudes, whereas larger discrepancy between explicit (cognitively controlled) and implicit ('unconscious') measures was expected in those who do not use doping.

Results and Discussion

Verifying self-reported doping behaviour

Hair samples from the participants in our previous study [32] were tested for performance enhancing and social drugs. Of the 82 athletes, 12 (14.6%) reported having personal experience with prohibited performance enhancing substances, one with therapeutic use exemption. Twelve hair samples were positive for anabolic steroids and/or erythropoietin (EPO), of which 10 (12.2%) were confirmed with no overlap between confessed lifetime experience and current use. None of the positives reported medical use of anabolic steroids or EPO. The pattern was very similar for social drugs with 15% overlap between self-reported use (27, 32.5%) and current use (12, 14.6%). Three of the confirmed doping positives also tested positive for social drugs.

The observed discrepancies between self-reports and objectively verified social drug taking behaviour is in line with previous research and although not surprising, they highlight the fact that a significant proportion of respondents simply choose to deny their real current or recent behaviour, even under circumstances when the verification is known to the participants. This phenomenon that has already cast doubt over drug use survey research expands to, or even magnifies the unreliability of doping use epidemiology surveys. The evaluation of anti-doping interventions is seriously hindered by the absence of reliable information on athletes' true behaviour; opens the field to wild guesses and speculations, often about other athletes, sports and nations. Devising more reliable ways to gauge this crucial information is an important issue but beyond the foci of this research and shall be addressed by future research. The present investigation aims to interpolate the tendency of giving misleading information about the behaviour to selected self-reported social cognitive processes.

Hair sample results were combined with self-reported doping use to inform the selection of 14 athletes to populate the groups in **Table 1**. Among the athletes selected for this study, 4 athletes admitted having used performance enhancing substances (PEDs) with no (or undetectable) current use. Of the remaining 10 athletes claiming that they have never used such substance 6 hair samples were positive for steroids, with all samples but one showed above the level for stanozolol, and one for nandrolone. Of these 6 athletes, 2 tested positive for a selection of social drugs despite that they both denied such drug use.

Based on self-report and hair analysis results for doping substances, athletes were categorised into disjoint groups of: i) clean athletes (matching negative self-report and hair screening), ii) denier (negative self-report coupled with positive hair samples), iii) open users (matching positive self-report and hair) and iv) unverified/non-current user (admitted use with currently negative hair sample). Although hair samples were also tested for recreational drugs (5 out of the 10 positive samples for doping were also positive for recreational drugs), parallel psychological testing was only performed in relation to doping, hence the confessed use of recreational drugs and/or positive hair samples for such substances will not be addressed in this report. In our previous study we have shown that whilst self-reported use of recreational drugs and doping substances was not independent, related social cognition were domain specific [32]. That is, self-admitted doping users gave significantly higher estimates of doping prevalence among athletes but not social drug prevalence, and vice versa. Similarly, differences in doping attitude scores were related to doping use but independent of social drug use. However, two athletes in the current sample denied any type of drug use whilst their hair samples contained evidence of both PEDs and social drugs. As this category of athletes demonstrated repeated denial on a single survey, they were treated as a separate group in this study.

Attitudes, perceived pressure and social projection by user groups

Assuming that direct experience increases attitude salience and the level of attitude - behaviour consistency [40], athletes' explicit and implicit attitudes and social projections were contrasted in the four user groups. The relationship between explicit and implicit doping attitude was investigated separately in each group (with repeat deniers excluded from the analysis owing to the insufficient variation in the sample) but included in **Table 1** and **Figure 1**.

Prior to in-depth analysis, it is important to note the distinction between the two types of information. In the questionnaire phase, participants were asked if they have ever used performance enhancing substances or social drugs. Hence an affirmative to this question does not necessary mean current or recent use. Hair analysis covered approximately the last 3 months (minus the last 2 weeks when the hair is still in the body); therefore results reflect relatively recent use. It should be noted that the hair analysis at

Table 1. Mean tests results (±SD) for self-report measures and implicit association effects (implicit doping attitude) by user groups.

Declared group membership	Objectively confirmed group membership	Explicit doping attitude (raw scale score)	Implicit doping attitude (IAT effect, ms)	Perceived pressure to dope (raw scale score)	Social projection (raw scale score)
Non-user	'Clean'	29.00±6.73	−255.98±153.46	2.50±5.00	32.50±26.30
	'Denier'	28.50±4.93	27.48±132.41	0.00	9.50±12.50
	'Repeat denier''	34.00±6.06	−17.91±99.13	0.00	20.00±28.28
Self-reported non-user group:		29.90±5.64	−94 98±185.18	1.00±3.16	20.80±22.12
User	'Unconfirmed self-report'	47.25±6.90	−140.56±129.85	37.50±37.97	52.50±25.00

doi:10.1371/journal.pone.0010457.t001

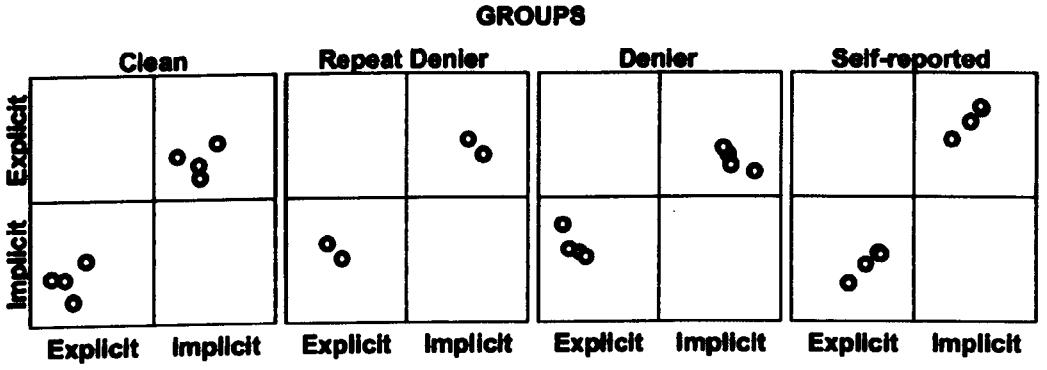

Figure 1. Scatterplot between explicit doping attitude scores ('explicit') as measured by PEAS and implicit doping associations ('implicit') as measured by the Brief IAT-D by doping user groups.
doi:10.1371/journal.pone.0010457.g001

this stage was limited to the list of most often used performance enhancing and social drugs. Contradicting answers can be derived from two legitimate sources: i) respondent answered truthfully about having an experience but the last occasion when drugs were used happened before the 6-month maximum detection window; or ii) the drugs used were not among those tested for. Theoretically there is also a possibility that a respondent did not answer truthfully but there is very little reason to admit a socially unacceptable behaviour when it in fact did not happen. On the contrary, a 'no' answer on the questionnaire coinciding with positive analytical results in the matching hair sample can be seen as a denial on the self-report because the denied 'ever use' is contradicted by the very presence of a drug or drugs in the hair.

Explicit measures. In self-reports, 'deniers' and 'repeat deniers' are classified as 'non-users' with explicit scores and measures below those who admit to doping and close to those who are truly clean. This phenomenon holds clearly for two of the three explicit measures, doping attitude and perceived pressures. Interestingly, social projections were given the lowest percentage by those who denied doping use where hair results indicated otherwise and reached the highest estimation by those who admit using PEDs. Users denying their actions claimed that they feel no pressure at all to use PEDs, followed by the clean athletes (with a low 2.5%) and self-admitted users scoring the highest with 37.5%. Correspondingly, 3 out of 4 of the self-admitted users believed that most high-performing athletes used performance enhancing substances in training and competition with the 4th athlete believing that doping is used by most athletes in training but not in competition. Of those who denied doping use but their hair samples indicated otherwise, half (3/6) agreed that performance enhancing substances are used in both training and competition by most high performing athletes, followed by 2/6 stating that most athletes do not use doping (1 in each 'denier' group) with 1 athlete believing that doping is used by most high performing athletes but used only in competition. This view was generally shared by the clean athletes, where 2 of the 4 thought that doping is used in both training and competition with the remaining 2 votes being split between training only and competition only.

Therefore, relying solely on self-report data, the observed differences in deliberate judgment were in the direction expected from known groups, with differences in three of the four measures reaching statistical significance. These are, in diminishing order of significance: explicit attitude ($|t| = 4.901$), pressure to use PEDs ($|t| = 3.217$) and social projection ($|t| = 2.343$; all $t_s < CV = 1.782$ directed, at $df = 12$, $\alpha = 0.05$). In reality, however, the membership of the self-reported non-user group was seriously confounded by a number of distorted answers about athletes' doping use. When these denials were corrected by hair analysis verification, a considerably different pattern of group differences emerged.

The highest estimation of doping prevalence given by self-confessed users is consistent with previous results [31,32]. The elevated estimation may be explained by the desire to find comfort in big numbers (also called False Consensus Effect) by which people who are involved in a socially disputable act tend to overestimate the number of others doing the same [41]. The opposite trend has also had some support from literature [42], when socially endorsed behaviour may correlate with slight underestimation of the proportion of other well-behaved individuals to reinforce one's uniqueness. Our results are consistent with this observation: our self-reported non-users, overall, gave a considerably lower estimation (21% vs. 52%) of doping prevalence in others. However, past research has mainly based these interpretations on self-reported behaviour. With the added insight from the hair analysis, the description of this phenomenon can further be refined. Contrary to the expectation, those athletes who denied PED use did not give realistic or elevated estimates of doping use in others. In fact, their projection was the lowest among all groups. Those who were determined to create a good impression to hide their real behaviour gave a very low estimate of doping prevalence, scored the lowest on the explicit doping attitude scale (indicating strong disapproval) and claimed that they felt no pressure at all to use PEDs. By contrast, they performed the implicit doping association task with ease when doping words were combined with good words; the task that was more difficult to non users, for whom doping had little or no salience. As would be expected, self-admitted users' performance on the same task fell somewhere in between. All together, the discrepancy of the different inferences that could be drawn under the two scenarios (self-report vs. validated behavioural data) highlights not only the unreliability of self-reports in social sensitive domains but also their effects on related constructs of social cognition.

Theoretically there are two fundamental and mutually exclusive assumptions underpinning the observed low scores on explicit social cognition measures among verified doping users. On the one hand, it may be reduced introspective accessibility of the constructs in question: having no insight into their feelings and biases the respondents produced low scores are no reflection of their actual doping-related cognition, but instead represent an extraneous influence, such as generic social desirability. On the other hand, answers on the explicit tests are consciously and deliberately distorted

in order to create a favourable (but false) impression. Our results, however, suggest that objectively verified doping users had, in fact not only introspective access to the construct (doping attitude) but also had positive feelings toward it. Investigating accessibility effects on varied implicit social cognition, Gawronski and Bodenhausen demonstrated that performance on a latency-based response compatibility task (such as the IAT) is affected by the practiced ease of and subjective feelings about the retrieval of relevant information (i.e. valence attached to doping) from memory [43].

Implicit measures. By contrast, the implicit association test was more revealing. Responding to pairings of positive-connotation words (the 'good' category) with doping substance words was fastest among those who currently use doping but denied it, followed by those who are currently using doping and admit it. Not surprisingly, responding to the same word pairings was slowest for those who claimed to have no experience with doping, followed by those who reported having used PEDs. Interestingly, athletes with doping experience performed the task quite well, indicating a closer association of doping with positive connotations than observed in those who have not used doping. Current users, as indicated by their hair analysis results, performed the *good+doping* pair the fastest with the results being close to the *good+nutritional supplement* pairing. These differences, however, are very small with large variance, based on small groups, and hence should be treated as preliminary observations, rather than definite conclusions.

Relationship between explicit and implicit measures: indicators for method development

The triangulation of self-reported explicit measures and objective verification of behaviour data using hair analysis with an implicit measure provided some preliminary evidence that the reason behind underreporting explicit cognitions is not a genuine effect but more likely a strategic response. In order to take a step forward to identifying deniers without the advantage of hair sample analysis, we examined the correlation between explicitly and implicitly assessed doping attitudes separately for the four groups. In the literature, the correlation between explicit and implicit measures of the same construct tends to be small [44]. This is especially true when social desirability is thought to confound explicit responses. In several studies, implicit measures had incremental predictive power in criterion validity over and above self-reports in socially sensitive domains [45]. Scatterplots by user groups depicted in **Figure 1** and corresponding correlation coefficients in **Table 2** suggest that the relationship between the parallel explicit and implicit measures is indicative of deliberate distortion. We assume that the implicit association is close to the true reflection of people's feelings toward the attitude object. For example, those who endorse doping would be able to perform the lexical sorting task of doping words when they share the same key with positive-connotation words faster compared to those who associate doping with negative connotations.

Table 2. Correlation between explicit and implicit doping attitudes by user groups.

	PEAS * Brief IAT-D (average time diff)	PEAS * Brief IAT-D(d-score)
'Confirmed clean' (n = 4)	.281	.270
'Self-reported user' (n = 4)	.991	.942
'Denier' (n = 4)	−.868	−.951

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Athletes who honestly admitted PED use performed congruently on the explicit and implicit measures. The more they endorsed doping in self-report and deliberate judgement, the faster they performed the *good+doping* pair test. Note that this is a trend between the two measures. In terms of sign of their attitudes, even these athletes were negative towards doping, albeit not as negative as their non-user counterparts.

Interestingly, trends expected and observed in research using self-reports change dramatically when the behavioural categories are based on objective measures (chemical analysis) and not on self-reports. Whilst patterns of explicitly assessed social cognition and tend to be consistent with self-reported behaviour, data from hair analysis revealed that distorted responses tend to bias these results. It can be argued that cultural context (i.e. doping use is unaccepted, un-sportsmanlike behaviour) influenced the athletes' automatic associations when performing the IAT task, as evidenced by the general trend of doing better on the *good+nutritional supplements* pair compared to the task when the *good+doping* shared the same response key [44]. If that is the case, athletes who denied PEDs use appeared to be less affected by this, showing little differences in response latencies between these two tasks.

The fact that self-reports on behaviour are very consistently associated with explicit social-cognitive outcomes is indeed informative. It is also consistent with mainstream social cognition literature [44–46] linking self-report to consciously controlled, deliberate outcomes – as distinct from more automatic and less controlled outcomes linked to implicit attitudes and dispositions. In our context this could indicate how athletes want to be seen to the outside word. In future studies, the strength and effect of this desire should be taken into account in explicitly measured doping-related constructs. Self-reports reflect what respondents want to reveal about themselves in that particular context, which has a non-trivial relationship to their actual feelings, thoughts or behaviour.

Limitations

Following the recommendation [44], we use the terms explicit and implicit with reference to measurement, not the construct (e.g. attitude). Based on the implicit doping attitude data at hand, no assertions can be made about the level of awareness among the selected athletes, especially in the denier group, of their own attitude demonstrated in the IAT. Rather, the considerable discrepancy between explicitly and implicitly measured attitudes in the denier group only differ qualitatively in their doping behaviour and their willingness to disclose this information suggest that athletes, indeed, were aware of their attitudes but owing to the sensitive nature of the issue, they made a deliberate effort to conceal their feelings about doping when it was under their cognitive control (i.e. explicitly measured) and deliberated. By contrast, automatic activation of these attitudes during the implicit association test was something that is very difficult to manipulate at will. The fact that the task was presented as a timed exercise to respondents who were competitive athletes may have further enhanced the validity of the test. That is, athletes were likely to be focused on performing fast and accurately on the task, instead of pondering about what the test might be measuring.

Limitations of this study arise from the sample size. Whilst the number of hair samples screened and positives samples confirmed are considerably higher than what is typically used in publications focusing on the chemical analyses for steroids [47,48], it is somewhat below the typical sample size in similar experimental psychology studies [45]. Results from this study were presented as evidence for the need for chemical validation of self-reports and mixed methodology, rather than drawing firm inferences regarding user vs. non user groups. In order to do this, similar

investigations need to be conducted on sufficiently large samples to establish representative groups and improve confidence with which practically meaningful differences/relationships can be observed. All together, the discrepancy of the different inferences that could be drawn under the two scenarios (self-report vs. validated behavioural data) highlights not only the questionable validity of self-reports in social sensitive domain but also their profound effects on related social cognitive outcomes. Sample descriptions (e.g. means and standard deviations) in this study are only indicative and presented here to assist in estimating the required sample sizes for future studies.

Conclusion

Incorporating developments in hair sample analysis for the detection of performance enhancing substances, this initial study examines the prospects of objective validation of athletes' doping attitude estimations and admissions of use. Overall the results indicate that patterns of group differences in deliberately expressed attitudinal outcomes, such as social projection, explicit attitude to doping and perceived pressure to use, vary depending on whether the user and non-user groups are defined by self-report or by objective verification such as hair sample analysis. When user and non-user groups were defined by self-report, the differences between them on several attitudinal outcomes were observed in the expected direction (i.e. self confessed user groups scored higher on social projection, explicit attitude to doping and perceived pressure to use). However, data from hair analysis revealed that deliberate response distortion may have biased these results. Subjects, whose hair sample returned positive for doping but who denied doping use in self-reports, were observed to manipulate their questionnaire responses to a greater degree than all other groups. Implicit doping attitude and its correlation to the explicit attitude towards doping are indicative of this distorted responding.

Therefore, the observed discrepancy between self-report and objectively (e.g. chemically) validated behavioural data needs to be considered when drawing conclusions from self-report findings. Our results pose a challenging question about the veracity of studies where doping-related behaviours and attitudinal outcomes are examined through group or individual differences that are themselves based on self-report. Our findings not only confirm the need for improved self-report methodology for future research in socially-sensitive domains but also indicate where the improvements are likely to come from: as chemical validation remains expensive, a more realistic promise for large scale studies and online data collection efforts is held by measures of implicit social cognition.

Owing to the time and resource-intensive nature of chemical validation (including equipment, personnel and know-how), large scale adoption of such validation for self-reported behaviour data across doping research does not seem feasible. However, improving self-report methodology remains imperative. One possible avenue is incorporating implicit assessments to gain

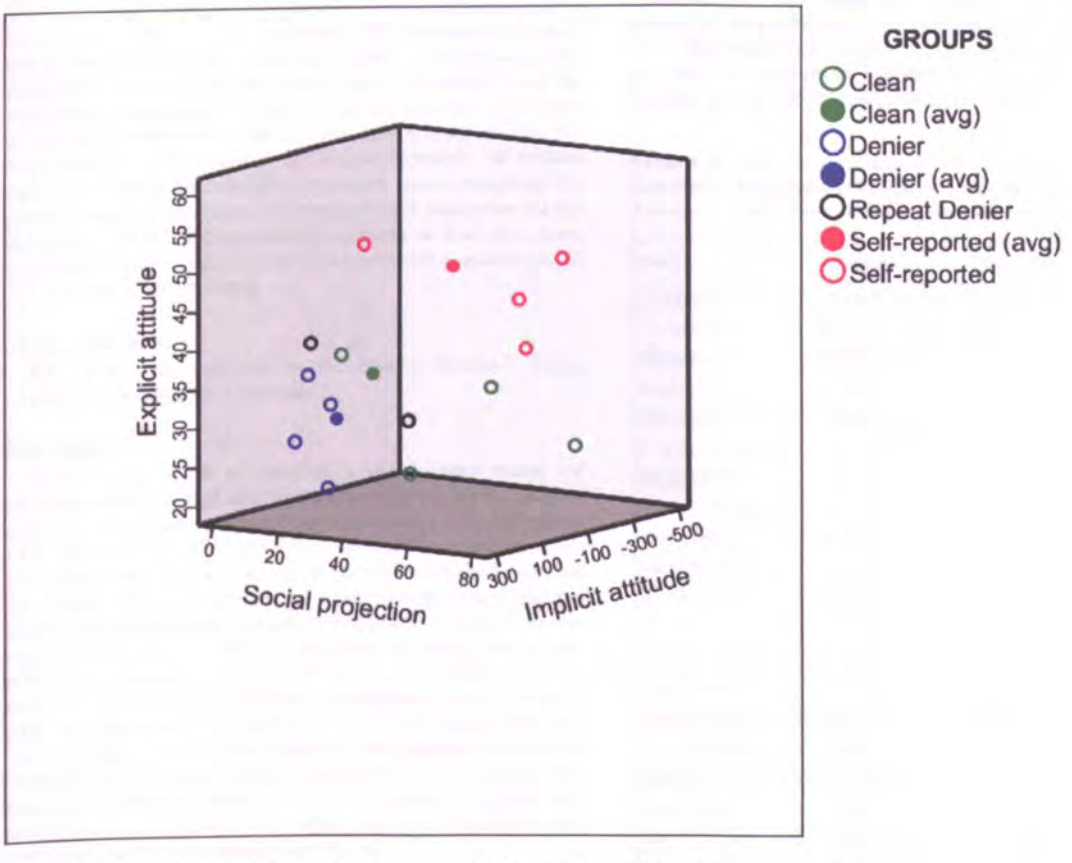

Figure 2. Sample characteristics and group means for 2 explicit and 1 implicit assessments.
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incremental predictive validity over and above explicit self-report measures. This approach has also been advocated by Greenwald et al. [45] upon meta-analysis of 122 empirical studies using explicit and implicit measures to predict behavioural, judgemental and physiological outcomes.

Methods

The chemical validation of self-reported information on doping and drug taking behaviour was part of a multi-centre study investigating social projection in doping and social drug use [31,32]. This part of the study aimed to detect the presence of selected drugs and metabolites in hair in order to investigate the validity of self-reports and the effect on any expected discrepancy between self-declaration and objective behavioral data on doping related social cognition.

Design

This study was based on mixed methods using a questionnaire, computerised psychological test and hair analysis for selected performance enhancing drugs. Self-report questionnaire results on doping behaviour were compared to the data gleaned from hair sample analyses for 14 selected athletes (4 per group plus 2) based on their self-reported behavior and hair sample results from the ELISA screening. In groups with more than 4 athletes (e.g. 'clean', 'denier' and 'self-reported'), 4 athletes were randomly selected for confirmation and further testing. The sample pools were as follows: 61/115 clean athletes, 11/115 self-reported users (only 1 was confirmed), and 12/115 deniers (2 erythropoietin and 9 steroids users, one was not confirmed). The representativeness of this random selection is shown in **Figure 2**. Participants with unconfirmed positive ELISA results were eliminated from the sample pool. Participation was anonymous, voluntary and based on fully informed written consent. Participants were told that the hair samples will be analysed for various chemicals. All athletes were aware of the hair sampling procedure before completed the questionnaire and performed the computerised assessment. As the completion of the testing protocol required at least one hour, participants were compensated for their time with a small payment (value of less than 10 Euros).

Ethics statement

The study was approved by the Faculty Research Ethics Committee in Kingston University.

Procedure

Athletes were asked to complete a web browser based test consisting of the explicit and implicit attitude measures, complemented with a paper-and-pencil questionnaire. A brief self-esteem IAT with 'good', 'bad', 'self' or 'others' stimuli set separated the two doping measures and served as method practice. Results for the implicit self-esteem test are not reported in this study. Implicit assessments preceded the explicit questionnaire measures (including questions about PED use), separated by other, non-doping related computerised tests, hence explicit did not influence the implicit assessment [44]. Although respondents were presented with an Information Sheet detailing the hair sampling procedure when seeking consent, the emphasis of the research was not on doping but investigating resource depletion in executive functioning, where doping appeared to be one avenue of evoking self-control and was mixed with other tasks (e.g. Donders' task switching and Stroop response inhibition).

Testing took place in a well-lit, quiet room containing two desktop computers. One or two athletes were present and completed the task at a time under supervision. The data collection was conducted between 8 am and 6 pm during weekdays.

Validation of self-reports

Validation of self-report was conducted using hair samples. The key advantage of using hair, as opposed to blood, urine or saliva, is its wide detection window, coupled with being non-invasive, easily stored and free of biohazards. The selection of drugs for screening was based on frequency of detection in WADA reports over the past five years [28]. Thus, along with testosterone, stanozolol, nandrolone and boldenone are frequently used anabolic steroids which differ in their licensing status [49]. In addition, tests were conducted for Naltrexone and most commonly used recreational drugs (for the full list, see **Table 3**). This research has adhered to the WADA CODE for laboratories [50]. Proper chain of custody was followed for hair samples collection, storage and disposal. Any unusual conditions like colour, pH and specific gravity were recorded.

Hair samples

The hair sample consisted of a lock of untreated hair with a diameter of 3 to 4 mms (approximately 50 hairs), minimum 3 cm in length (equal 100 mg in weight), cut directly at the skin surface at the vertex posterior whenever possible. The sample was stored individually in labelled, sealable paper envelopes, according to the protocols established and approved by the Kingston University Faculty Research Ethics Committee.

Chemicals and reagents. ELISA kits for nandrolone, stanozolol, amphetamine, methamphetamine, cocaine, delta-9-tetrahydrocannabinol (THC), ketamine, erythropoietin (EPO), and their metabolites, were obtained from Neogen Corporation (Lexington KY 40511 USA), with enzyme immunoassay (EIA)

Table 3. Limits of detection (LOD) and WADA general Minimum Required Performance Limit (MRPL) values.

Drugs	Category	ELISA LOD	MRPL value
Nandrolone	Anabolic steroid	0.07 ng/ml	2 ng/ml
Testosterone	Anabolic steroid	0.5 ng/ml	2 ng/ml
Naltrexone	Anabolic steroid	1.3 ng/ml	2 ng/ml
Boldenone	Anabolic steroid	6 ng/ml	2 ng/ml
Stanozolol	Anabolic steroid	1 ng/ml	2 ng/ml
3-HydroxyStanozolol	Anabolic steroid	12 ng/ml	2 ng/ml
Amphetamine	Stimulant	11.5 ng/ml	500 ng/ml
N-Desmethylselegiline	Stimulant	1.27 ng/ml	500 ng/ml
Ephedrine	Stimulant	23.4 ng/ml	500 ng/ml
Methamphetamine	Stimulant	9.5 ng/ml	500 ng/ml
Δ^8 THC	Stimulant	0.6 ng/ml	500 ng/ml
Δ^9 THC	Stimulant	0.5 ng/ml	500 ng/ml
Cocaine	Stimulant	5.1 ng/ml	500 ng/ml
Cocaethylene	Stimulant	5.5 ng/ml	500 ng/ml
Benzoylecgonine	Stimulant	6.8 ng/ml	500 ng/ml
m-Hydroxycocaine	Stimulant	7.1 ng/ml	500 ng/ml
Ketamine	Stimulant	7 ng/ml	500 ng/ml
Norketamine	Stimulant	137 ng/ml	500 ng/ml
EPO	Peptide hormone	1.2 mU/ml	5 mU/ml or 40 pg/ml

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Table 4. Limits of detection using LC-MS/MS.

Drugs	Category	MRPL value	LC-MS LOD		Calibration curve in hair pg/mg
Nandrolone	Anabolic steroid	2 ng/ml	1 ng/ml	2.5 pg/mg	3 to 400
Testosterone	Anabolic steroid	2 ng/ml	0.1 ng/ml	0.25 pg/mg	1 to 400
Stanozolol	Anabolic steroid	2 ng/ml	0.2 ng/ml	0.5 pg/mg	1 to 400

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being part of the ELISA kits. Drugs, their metabolites and internal standard (stanozolol D3) were obtained from LGC standards (Teddington, UK). All chemicals and silanized amber glassware were from Sigma Aldrich (UK). Blank hair was obtained from healthy non-athlete volunteers.

Screening by ELISA

The hair sample was rinsed twice with 5 ml dichloromethane for 2 minutes. After complete drying, hair was finely cut into circa 1 mm segments. Hair segments (ca 50 mg) were weighed in a glass tube. Calibrants and controls for each kit were prepared by spiking blank hair with the required amount of drug. Hair samples, calibrants and controls were then incubated in 1 mL of 1 M NaOH at 95°C for 15 minutes. After cooling, the homogenate was neutralized (pH 7) with required amount of 1 M HCl (approx 1 mL) and then diluted with equal amount of enzyme immunoassay (EIA) buffer (1:1 v/v). Screening methods were fully validated in accordance with the WADA Code of validation for urine and plasma which was extended to hair samples. Neogen Corp. (USA) forensic ELISA kits were used on a Biotek-ELx808 (USA) and Varian Cary 50 MPR Microplate Reader (UK). The full range of drugs and their metabolites are given in **Table 4**. In addition to the steroid results, the application of the Neogen ELISA methods have been extended from biofluids to hair samples for the detection of EPO and the most frequently used drugs of abuse that are on the WADA 2009 List of Prohibited Substances [51]. These include amphetamine, methamphetamine, cocaine, marijuana and ketamine (currently not prohibited) and their selected metabolites. This process involved developing extraction methods along with devising a protocol for analysis. Methods for extraction of the drugs from hair were developed and subsequent ELISA analyses were validated in-house.

For all non-threshold and threshold substances appropriate controls near the appropriate threshold levels were included in the initial screening, although uncertainties of measurements were not taken into account. **Table 4** shows the detection limit of ELISA kits supplied by Neogen Corporation (USA) and general MRPL levels set by WADA.

Confirmation

The ELISA results were confirmed by liquid chromatography-mass spectrometic (LC-MS/MS) methods using a ThermoScientific LC-MS/MS Accela UPLC coupled with Triple Quadrupole TSQ™ Quantum Access system. These confirmatory quantitative methods are more sensitive than the initial screening procedures with the LOD's of the three key substances in hair are shown in **Table 4**. There are no therapeutic use exemptions (TUE) for the prohibited substances detected.

Analyses by LC-MS/MS. After decontamination, hair was finely cut into 1 mm segments. Following a previously established method [52], hair segments (ca 20 mg) were weighed in a glass tube and incubated in 1 ml of 1 M NaOH at 95°C for 15 minutes in the presence of stanozolol D3 as an internal standard (I.S). After cooling, the homogenate was neutralized with approximately 1 mL of 1 M HCl followed by addition of 0.2 M phosphate buffer (pH 7.0). Liquid – Liquid extraction was employed for all three steroids analyzed. Pentane (3.5 ml) was added to the homogenate. After agitation and centrifugation (4 minutes at 1257 g) the organic layer was separated and evaporated to dryness under a stream of nitrogen gas at 60°C. The dried residue was reconstituted with 100 µL acetonitrile. An aliquot (4 µL) of reconstituted extract was injected into the ThermoScientific LC-MS/MS system.

LC-MS/MS conditions. An Agilent ZORBAX column (SB-C18, 2.1×50 mm, 1.8 µm) was used. Formic acid (0.1%) and acetonitrile were used as mobile phase. The LC mobile phase gradient flow used was: A: acetonitrile (%), B: 0.1% formic acid; start: 50% A, after 10 min: 80% A–20%B, after 11 min: 100%A, after 12 min: 50%A. Total flow rate through the column was set at 100 µl/min using gradient flow. Column temperature was set at 60°C. The mass spectrometer was operated in the positive electrospray ionisation mode. SRM (single reaction monitoring) was used to confirm each analyte as shown in **Table 5**. A standard calibration curve and quality controls were prepared by spiking negative control of hair (blank hair) with the required amount of drug and internal standard.

Psychological assessments

Psychological assessment consisted of computerized word sorting task (used to assess implicit associations) and a paper-and-pencil questionnaire seeking information on explicit doping attitude, and basic demographic information (gender, age, ethnicity, sport, level of competition, nationality). In order to protect athletes' anonymity, only mean age and gender distribution is reported.

Implicit doping attitude (the brief version [53]). In this test block, respondents were presented by words falling into four categories (good, bad, nutritional supplements or doping). The stimuli used in each category are shown in **Table 6**. Two of those four category names were shown on the left hand side of the screen during the test. Respondents were asked to press 'E' if the stimulus word matches either of the categories or to press 'I' if it does not match them. Words were presented in 24pt Arial font. Each

Table 5. Main qualifier ions of analytes used for steroid analysis.

Analyte	Parent mass	Product mass
Nandrolone	275.2	109.2
Stanozolol	329.2	81.2
Testosterone	289.2	109.2, 97.2
Stanozolol D3 (I.S)	332.2	81.2

doi:10.1371/journal.pone.0010457.t005

Table 6. Stimuli of the Brief Implicit Doping Attitude test.

Category	Words
Good	peace, joy, love, smile
Bad	sick, hell, poison, fail
Doping	nandrolone, stanozolol, testosterone, amphetamine
Supplements	vitamins, ginseng, garlic, calcium

doi:10.1371/journal.pone.0010457.t006

stimulus was preceded by a fixation cross which stayed on-screen for 400 ms. Stimuli stayed on-screen until the respondent pressed either 'E' or 'I'. A large red X was shown on the bottom of the screen for 400 ms when the answer was wrong; respondents had to press the correct button to proceed.

The Brief Doping IAT test consisted of two blocks. In the first block, categories 'good' and 'nutritional supplement' were assigned to the 'E' key; the second block used categories 'good' and 'doping'. Each block consisted of 32 stimuli and each word was presented twice. Brief instructions were presented before each block; the instructions specified the words of the categories that were selected as target categories (i.e. good and nutritional supplement in the first block; good and others in the second block) but not the other two. The 'good' combinations (good + nutritional supplement and good + doping) were fixed as focal categories. Respondents were instructed to proceed as fast as they could. The order of the two blocks was counterbalanced.

The Doping IAT effect was calculated as the difference time difference between the two focal test blocks as shown in **Figure 3**. The difference was also divided by the variance to derive the D-scores [54]. Because the difference was calculated as: [*Good + Nutritional Supplement*] − [*Good + Doping*], difference time>0 means that completion of the good + nutritional supplement combination task took longer, whereas difference time<0 suggests that the [*Good + Doping*] completion took longer.

The computerised test application also included an explicit measure of doping attitude using the Performance Enhancement Attitude Scale (PEAS). The PEAS consists of 17 statements related to performance-enhancing drugs. Respondents were asked whether they agree with the statements. Answers were recorded using a 6-point Likert-type scale (1 = strongly disagree, 6 = strongly agree). The PEAS has shown good evidence for scale reliability and validity [55].

The anonymous questionnaire included key questions on drug and doping taking behaviour: *Have you ever used a social drug?* (Yes/No); *Have you ever used a banned substance?* (Yes/No) and *Do you use nutritional supplements?* (Yes/No). The question regarding nutritional supplement use (beyond and above the normal diet and taken in a concentrated form) was included as a control (not reported). At the beginning of the questionnaire, athletes were presented with clear definitions: 'doping' or 'banned substances' were those substances that are prohibited by the World Anti-Doping Agency or other governing body in training and/or competition (e.g. steroids, EPO). 'Social' or 'recreational' drugs were defined as psychoactive drugs (e.g. stimulants, opiates, cannabis, cocaine, etc.) used for recreational purposes rather than for work, medical or spiritual reasons with caffeine, alcohol and tobacco excluded. Nutritional supplements were vitamins, minerals, and non-vitamin non-mineral substances including herbals and botanicals. Exemplars were given for all three groups. In addition to these key questions, athletes were also asked about the perceived pressure to use doping (0–100%), estimated prevalence of doping among fellow athletes (0–100%) and their general belief about the doping use pattern (descriptive norm). For the exact wording and answer options of these questions, see **File S1**.

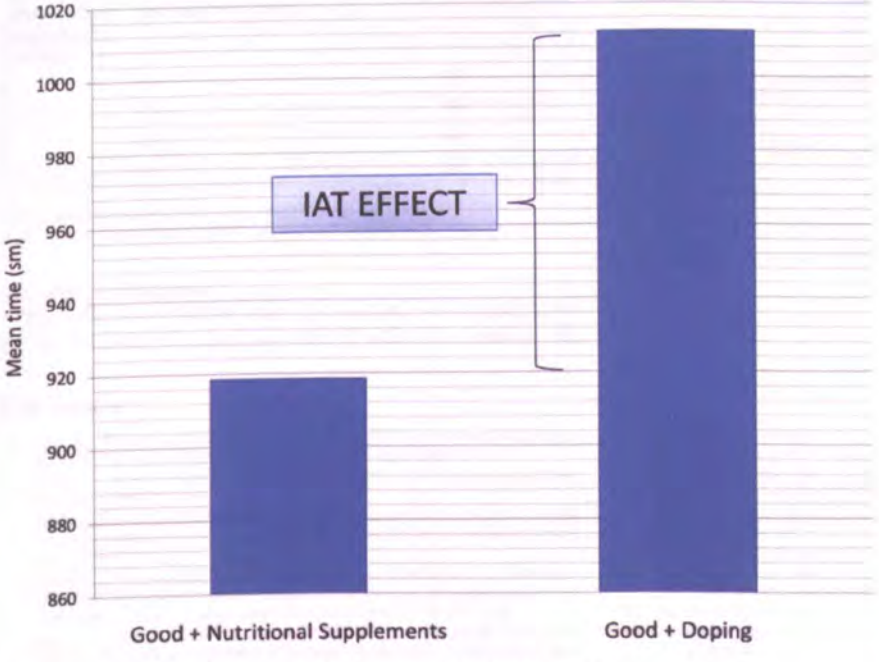

Figure 3. Illustration of the Implicit Association Test (IAT) effect.
doi:10.1371/journal.pone.0010457.g003

Table 7. Cross-tabulated data of self-reports and parallel hair analysis (n = 14).

	Self-report: never used doping		Concentration (pg/mg)
Hair analysis negative for doping	**'clean' (n = 4)**		
	A1	–	-VE
	A2	–	-VE
	A3	–	-VE
	A4	–	-VE
Hair analysis positive for doping	**'denier' (n = 4)**		-VE
	A5	*Stanozolol, 3'OH Stanozolol*	12.65 (Stanozolol)
	A6	*Stanozolol, 3'OH Stanozolol*	11.24 (Stanozolol)
	A7	*Stanozolol, 3'OH Stanozolol*	40.24 (Stanozolol)
	A8	*Stanozolol, 3'OH Stanozolol*	56.08 (Stanozolol)
Hair analysis positive for doping and social drugs	**'repeat denier' (n = 2)**		
	A9	*Stanozolol, 3'OH Stanozolol, Amphetamine, Methampetamines, N-Desmethylselegiline, Ephedrine, MDMA, MDA, Δ^8 THC THC, Δ^9 THC*	9.82 (Stanozolol)
	A10	*Nandrolone, Boldenone,Testosterone, Naltrexone, Amphetamine, Methampetamines,N-Desmethylselegiline, Ephedrine, MDMA, MDA, Δ^8 THC, Δ^9 THC*	14.04 (Nandrolone) -VE (Testosterone)
	Self-report: admit using doping		**ELISA**
Hair analysis negative for doping	**'self-reported' (n = 4)**		
	A11	–	ND
	A12	–	ND
	A13	–	ND
	A14	–	ND

-VE = negative results, ND = - not detectable.
doi:10.1371/journal.pone.0010457.t007

Sample characteristics

The hair samples of the selected 14 athletes were analysed for PEDs. Athletes competed in track & field (5), triathlon (4), volleyball (2), orienteering (1), basketball (1) and karate (1). The mean age was 20.43±3.18 years, 10 females and 4 males in the sample. In this small sample, age and gender appear to be unrelated to doping use.

Table 7 summarised the self-report and hair analysis results for the selected 14 athletes. Note that positive hair samples for social drugs were not confirmed beyond the ELISA screening at this stage. The focus of the paper was performance enhancing drugs and social cognition relating doping, hence the test did not contain explicit or implicit measures of social cognition about social drugs. **Figure 2** shows the selected athletes' position in relation to the group mean for the full sample (N = 482).

Analyses

Group differences in and relationship between explicit doping attitude and implicit doping associations and social projection were compared for groups based on self-reports and hair analyses. Group means are reported with standard deviation. Independent samples t-tests were used to compare scores achieved on social cognitive measures, where user vs. non-user groups were formed by the self-reported PEDs taking. Graphs and statistical analysis were conducted by SPSS 17.0 and Excel 2007.

Supporting Information

File S1 This file contains the questionnaire used to collect data regarding athletes' drug and doping behaviour, doping attitude, descriptive norm, social projection and perceived pressure.
Found at: doi:10.1371/journal.pone.0010457.s001 (0.06 MB DOC)

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Author Contributions

Conceived and designed the experiments: AP DPN. Performed the experiments: MU MT. Analyzed the data: AP EVA TN. Contributed reagents/materials/analysis tools: AP EVA IH ND TN JB DPN. Wrote the paper: AP EVA IH ND JB DPN. Developed the implicit doping attitude test: AP EVA. Contributed to the design of the study and behavioural data analysis: EVA. Developed the methods for chemical analysis and performed the chemical analyses: IH ND. Developed the computerised test: TN. Contributed to the data acquisition and statistical analyses: TN. Contributed to drafting the manuscript: MU MT JB. Contributed to the chemical analysis: JB. Contributed to the method development for chemical analysis and interpretation of the results: DPN.

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Incongruence in Doping Related Attitudes, Beliefs and Opinions in the Context of Discordant Behavioural Data: In Which Measure Do We Trust?

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Abstract

Background: Social psychology research on doping and outcome based evaluation of primary anti-doping prevention and intervention programmes have been dominated by self-reports. Having confidence in the validity and reliability of such data is vital.

Methodology/Principal Findings: The sample of 82 athletes from 30 sports (52.4% female, mean age: 21.48±2.86 years) was split into quasi-experimental groups based on i) self-admitted previous experience with prohibited performance enhancing drugs (PED) and ii) the presence of at least one prohibited PED in hair covering up to 6 months prior to data collection. Participants responded to questionnaires assessing a range of social cognitive determinants of doping via self-reports; and completed a modified version of the Brief Implicit Association Test (BIAT) assessing implicit attitudes to doping relative to the acceptable nutritional supplements (NS). Social projection regarding NS was used as control. PEDs were detected in hair samples from 10 athletes (12% prevalence), none of whom admitted doping use. This group of 'deniers' was characterised by a dissociation between explicit (verbal declarations) and implicit (BIAT) responding, while convergence was observed in the 'clean' athlete group. This dissociation, if replicated, may act as a cognitive marker of the denier group, with promising applications of the combined explicit-implicit cognitive protocol as a proxy in lieu of biochemical detection methods in social science research. Overall, discrepancies in the relationship between declared doping-related opinion and implicit doping attitudes were observed between the groups, with control measures remaining unaffected. Questionnaire responses showed a pattern consistent with self-reported doping use.

Conclusions/Significance: Following our preliminary work, this study provides further evidence that both self-reports on behaviour and social cognitive measures could be affected by some form of response bias. This can question the validity of self-reports, with reliability remaining unaffected. Triangulation of various assessment methods is recommended.

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Introduction

Epidemiological and social science research assessing social cognitions linked to doping behaviour has been constrained by the almost exclusive use of self-report methodology [1]. Anti-doping prevention programmes are also evaluated via self-reported changes in attitudes and willingness to use doping substances, anabolic steroids in particular [2–5]. However, recent research has drawn attention to a potential distorting effect of social desirability observed in self-reported social cognitive measures related to doping [6,7].

In a recent project, benefitting from a multidisciplinary approach combining social and analytical sciences [8], evidence has emerged that potentially could change the landscape of social science research into doping. We have shown, albeit on a small sample, that taking self-reports at face value could lead to misleading conclusions about the social cognitive processes that underlie doping behaviour. For the first time in social science doping research, our results showed that not only the information on doping behaviour but also self-reported social cognitive measures could also be affected by some form of response bias. Whilst differences in explicit (self-reported) social cognitive measures between user and non-user groups were observed in the expected direction when groups were created from self-report, generally the reverse was evidenced when the user status was based on hair analysis results (i.e. based on the presence of at least one prohibited performance enhancing drug in hair). Implicit measures were consistent with the grouping based on hair analysis.

The outcome of this project suggested that respondents may consistently manipulate their answers on all related measures in order to maintain the image they wish to project, although the possibility that this response bias might stem, at least partially, from self-deception (as opposed to strategic responding for impression management) cannot be ruled out.

The strikingly different patterns in self-reports and implicit associations in the context of behavioural data inevitably lead to the question of: Which data should we trust? The self-report methodology has endured a mix of support and criticism in the past. Whilst a plethora of literature suggests that self-report can yield a valid assessment of substance use behaviour, it has also drawn equally strong criticism. For example, the Timeline Follow-Back procedure is one of the widely used methods in clinical and research settings to accrue quantitative retrospective estimates of substance use or risky behaviour covering 7 days to 2 years prior to the interview date [9]. It has demonstrated utility in assessing alcohol, marijuana and tobacco use, as well as sexual behaviour involving taking risks of HIV infection, vis-a-vis face-to-face and over the phone interview or internet application [10-12] when covering a shorter period, but produces increasingly less accuracy as the timeframe increases [13]. In a similar vein, alternative questionnaire methods have been developed and reviewed for validity and reliability in identifying substance abuse. These include, but are not limited to, the Lifetime Drug Use History [14], Risk Behavior Assessment of lifetime and recent use of amphetamine [15], Drug Abuse Screening Test [16], the CAGE [17] and Cannabis Use Problems Identification Test [18].

Whilst these tests have provided adequate evidence of validity and reliability, other studies using various biomarkers to validate self-reported behaviour data have put convincing evidence forward for a considerable under-reporting of substance use [19-22]. A systematic review [23] revealed that this bias is not limited to socially undesirable behaviours; it also extends to simple measures such as height and weight with a tendency towards over- and under-reporting, respectively [23]. The effect of gender, race, age and contextual contingencies such as drug type and seriousness of the offence on over- and under-reporting substance use has also been investigated [24] with race as the only factor so far demonstrating an effect on admitting drug taking behaviour.

Although laboratory experiments manipulating the conditions from neutral to high demand for socially desirable responding are abundant [25], evaluating the facets of potential response bias in field research is seriously compromised by the absence of available independent objective verification [26]. Therefore, the present project aimed to address this gap by triangulating two behavioural measures and two distinct assessment procedures. Specifically, the current paper repeats the previous analysis [8] on a larger sample to verify our preliminary results and seek further evidence to ascertain whether responses given by the different user groups show a distinct pattern. The importance of this question is underscored by the fact that whilst all of these options distort the data obtained from self-reports, they lead to fundamentally different consequences for the inferences made from these data. Random untruthful answers are likely to lead to statistically non-significant results and potentially low internal consistency in measures. Consistently manipulated answers, regardless if the cause is unconscious or strategic, evidently lead to incorrect, but entirely believable conclusions about doping users vs. non-users.

To further explore issues that emerged from our previous study and were reported as preliminary findings [8], we formulated the following hypotheses.

To examine to what extent self-reported doping use is influenced by strategic responding (response bias), and to what extent the implicit assessment remains unaffected by the same bias, we hypothesise that:

H_1: self-reports align with self-declared use, and the doping BIAT aligns better with user grouping based on hair analysis;

H_2: we expect strong divergence between explicit and implicit attitude measures among deniers, and small convergence in the clean athlete group.

Based on a previous study showing domain specificity in the behaviour – social projection relationship where self-declared doping users overestimated the prevalence of doping but not social drugs and vice versa [27], we hypothesise that non-doping related variables are unaffected by the behavioural context.

H_3: No difference exists in self-reported non-doping variables across doping user groups (including both self-declaration and hair-analysis.

As literature precedence shows gender difference in areas prone to socially desirable responding such as dietary intake [28,29], it was plausible that the level of honesty/dishonesty about sensitive or discriminating behaviour could be affected by gender. Although findings to date are inconclusive regarding the direction of this effect, females generally find lying less acceptable then males [30–32]. A recent study investigating dental hygiene found that admission of insufficient dental hygiene doubled for females when a less intrusive method was used, but increased only by 46% among males [33], indicating not only gender but interaction between gender- and method effect. Therefore, regarding potential gender differences, we hypothesise that

H_4: a gender effect exists on self-reported data for doping behaviour and social cognitive determinants of doping.

Methods

The study utilised a mixed method design to afford triangulation between explicit measures through self-reported questionnaire, implicit associations using a computerised test for latency measures, and bioanalysis via hair specimens.

Participants

We expanded the previously used small sample of 14 [8] to the full sample of 82 male and female athletes using convenience sampling. Inclusion criteria were limited to i) holding registration as an athlete in sport clubs/teams and active current participation in organised sports' competitions and ii) the ability to provide 3 cm of a head hair sample. No specific athlete group was targeted. Competition levels were ranging from university club to international level.

Recruitment

After securing ethical approval, athletes were recruited via personal contacts to ensure good rapport and trust between the researchers and participants. Athletes received a small payment (value<10€) as compensation for their time and inconvenience. Details on recruitment, inclusion-exclusion criteria, and sample characteristics are provided in Petroczi et al. [8]. The project was approved by the Kingston University Faculty of Science Research Ethics Committee. Participation was voluntary with implied consenting procedure. The Participant Information Sheet, as well as the first page of the questionnaire clearly stated that the completion and return of the questionnaire and submission of the

computerised test was taken as consent. The rationale behind omitting written consent was justified on the basis that given the sensitivity (doping use) ensuring complete anonymity was necessary. Data were linked via computer-generated alphanumerical codes. The data collection with implied consent was approved by the Faculty Research Ethics Committee.

Measures

Behaviour. Independent categorical variables were created by dividing the sample into quasi-experimental groups on the basis of i) self-admitted previous experience with prohibited performance enhancing substances and ii) the presence of at least one prohibited performance enhancing drug in hair covering the last 6 months, depending on hair length. This led to four theoretically possible groups as presented in Table 1 along with basic demographic information. Known groups were formed based on self-reports (we refer to this as 'self-report doping scenario') or hair analysis (we refer to this as 'hair analysis doping scenario'). For self-report measure of doping behaviour, athletes were asked whether they have ever used a prohibited performance enhancing substance. The combined approaches (self report and hair analysis together) yield a group where participants denied their experience with doping despite the fact that their hair analysis indicated fairly recent use. For the purpose of this study, we used three groups: self-admitted doping use (group A), self-declared clean athletes (group B) and deniers (group C).

Self-reported doping behaviour was established from the answers given to the question: *Have you ever used a banned substance?*, with a dichotomous answer option. At the beginning of the questionnaire, clear and concise definitions were given for doping, social drugs and nutritional supplements with exemplars given for all three groups. The social drug category excluded caffeine, alcohol and tobacco.

Hair samples were cut at scalp with a blunt tip scissor and stored in paper envelopes at room temperature until analysis. It is generally accepted that hair analysis is not suitable for detecting single (possibly accidental) exposure [34]. Incorporation of these performance enhancing drugs requires sustained use during the period the hair sample (length) covers.

Psychological assessments. Dependent variables included a wide range of self-reports on social cognitive determinants of doping coupled with a modified version of the Brief Implicit Association Test (BIAT) procedure [35] to doping. The doping BIAT pairs performance enhancement related stimuli in doping (*nandrolone, stanozolol, testosterone, amphetamine*) and nutritional supplement (*vitamins, ginseng, garlic, calcium*) target categories with 'good' attributes (*peace, joy, love, smile*). The opposite evaluative term ('bad': *sick, hell, poison, fail*) was non-focal [8]. Categories, attributes and their stimuli were shown to participants before the test. Upon incorrect answer, a large red X appeared for 400 ms at the bottom of the page prompting participants to correct their answers. Mean latency difference and D-score were calculated according to the scoring algorithm recommended by Greenwald et al. [36]. Latency time difference and D-score <0 indicates relatively stronger associations for nutritional supplements with good attributes; whereas latency difference time and D-score >0 suggests the opposite.

Explicit social cognitive measures were: doping attitude, using the Performance Enhancement Attitude Scale, PEAS [37]; social projection of doping and nutritional supplement (NS) use prevalence; beliefs whether doping should be allowed in competition and opinion on what proportion of athletes would be found guilty if samples taken today would be analysed in 10 years time. The PEAS is a 17-item, unidimensional measure of general doping attitude with good reliability across several studies [37]. The PEAS is scored on a 6-point Liker-type scale indicating levels of agreement with the attitude statements. Response options were anchored at each point ranging from strongly disagree (1) to strongly agree (6). Example items are: "*doping is an unavoidable part of competitive sport*", "*health problems related to rigorous training and injuries are just as bad as from doping*" and "*there is no difference between drugs, fibreglass poles, and speedy swimsuits that are all used to enhance performance*". In this study, the Hungarian version of PEAS with previously established psychometric properties was used [27,37]. Social projection was solicited by using independent single questions asking respondents to estimate the proportion of the general population using nutritional supplements and illicit drugs; as well as the proportion of athletes in their own sport using nutritional supplements and doping. Estimation was given in percentage between 0 (nobody) to 100% (everyone). These, and the other non-standard questions regarding perceived pressure, preferred competitive situation and doping opinion are provided (Table S1) along with their scoring method. Non-doping related dependent variables (social projection of NS and social drug use

Table 1. Athlete groups and demographic information based on self-reports and hair analysis.

	Self report: No doping	Self report: Yes doping
	GROUP C	**GROUP D[b]**
Hair Positive	Male: 2, Female: 8	
	Mean age: 19.50±1.354	
	Individual sports: 6, team sports: 3; unspecified[c]: 1	
	Olympic sports: 7/10	
	GROUP B	**GROUP A**
Hair Negative[a]	Male: 29, Female: 32	Male: 8, Female: 3
	Mean age: 21.75±3.026	Mean age: 21.73±2.240
	Individual sports: 33, team sports: 16; unspecified[c]: 12	Individual sports: 10, team sports: 1
	Olympic sports: 47/61	Olympic sports: 5/11

[a]For selected performance enhancing drugs.
[b]Owing to the different timeframe (lifetime vs. last 6 months) and limited scope of the hair analysis we found no athletes in this group.
[c]Sports that can be either (e.g. rowing).
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among members of the general population) were used as control variables.

Bioanalysis. Approximately 100 mg untreated head hair, cut at scalp, was screened using ELISA kits for the presence of the most commonly used anabolic steroids (stanozolol, nandrolone and boldenone). Positive samples for anabolic steroids were confirmed and quantified using liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods [8,38]. Erythropoietin (EPO) was detected using quantifiable ELISA. Hair digestion and analytical methods using LC-MS/MS were developed in house to increase sensitivity and to reduce the amount of hair required [38].

Data analysis

Group comparisons are first performed by factorial ANOVA to allow the testing of main and interaction effects. Owing to the unequal, and in some cases too small, group sizes, main effects were then confirmed by non-parametric (Mann-Whitney) tests. Owing to the small sample size for 'user' groups, inferential statistics should be read with caution. Means and standard deviations are provided for all dependent variables. Relationships were tested using the chi-square test for independent variables and correlation coefficients (Pearson's r and Kendall's tau) for dependent variables. Effect size and minimum required sample size for statistical significance were calculated using G*Power 3.1 software. Interaction terms were calculated by multiplying the z-scores for the two continuous explicit and implicit attitude measures. The BIAT effect was shown by latency difference (raw scores in ms) and D-scores [36] with negative values representing a longer latency time on the *good + doping* task (vs. *good + nutritional supplement* task). The doping BIAT task was run on a standard desktop computer (AMD Athlon™ 64X2 Dual Core Processor 4400+) under Windows XP operating system, using a bespoke test developed in-house. Response options were assigned to keyboard letters. Statistical analyses were performed using PASW Statistics v18.

Results

The mean age of the samples was 21.48±2.86 years (range 18–30). Gender distribution of the sample was close to equal (52.4% female). Athletes in the sample represented 30 sports with track & field: 14, triathlon: 7, gymnastics: 7 and soccer: 6 appearing more than five times. Competitive levels ranged from university to national level. Scale reliability (Cronbach alpha) for the PEAS in this study was .806.

Prevalence rate of the use of performance enhancing substances and nutritional supplements

Nutritional supplement use was reported by 60%, whereas those having personal experience with doping constituted 13.4% of the athletes. Admitted doping appears to be independent of self-reports on NS or social drug use. Eight hair samples were positive for stanozolol and two for EPO, giving a 12% prevalence rate for prohibited performance enhancing substance use in the sample. Detected Stanozolol levels in hair are reported in Petroczi et al., 2010 and Deshmukh et al., 2010. EPO levels were 13.35 pg/mg and 12.53 pg/mg for the two positives. Frequencies by gender are shown in Table 2. Interestingly, more males admitted having experience with doping than females (20.5% vs. 7.0%, respectively) with a reversed pattern for positive hair analysis (5.3% vs. 18.6%, respectively).

None of the athletes who returned positive hair samples admitted doping use. Conversely, no self-admitted doping was confirmed by current hair sample tests (Table 1). Owing to the evident uncertainty (i.e. whether the mismatch between self-reports and analytical results were due to false reporting or limits in time and/or scope of the hair analysis) around this latter group (group A in Table 1), these athletes were excluded from further analyses but their results are provided in Tables S2 and S3 to inform future research.

Table 2. Self-reported use of nutritional supplements and doping in comparison to prevalence of Stanozolol/EPO use based on detection in hair in the sample by gender (presented as frequencies).

	Use	Male	Female	Total[a]
Nutritional supplements self-report	Yes	28	21	49
	No	11	22	33
Doping self-report	Yes	8	3	11
	No	31	40	71
Stanozolol or EPO detected in hair[b]		2	8	10

[a]one reported prohibited performance enhancement use for medical reason with TUE.

[b]one Stanozolol level was below the level of quantification (0.5 pg/mg of hair), Deshmukh et al., 2010.

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Social cognitive factors

Social projections for NS, doping and social drug use showed a positive, statistically significant but relatively weak relationship between fellow athletes using prohibited performance enhancing drugs and NS ($r = .385$, $p < .001$) and social drugs by the general population ($r = .231$, $p = .036$).

Based on self-declared doping behavior, an interaction effect between user group and gender was only found for social projection of doping ($F = 4.454$, $p = .038$) and NS ($F = 4.379$, $p = .040$) use by fellow athletes. No gender difference was evidenced for any of the outcome variables except the pressure to use banned substances, where the t-test showed a statistically significant difference ($t(57.67) = -2.093$, $p = .041$), but the non-parametric test failed to confirm this ($p = .071$). On the scale of 100% representing maximum pressure, the estimation given by males was higher (18.46%, SD = 28.40 vs. 7.72%, SD = 15.58). Gender difference or interaction effect was not detected in comparing 'clean' athletes and 'deniers' within the self-declared clear group.

A statistically significant difference was found between admitted doping users and non-users in explicit attitude ($p < .001$) and social projection of doping use ($p = .024$), with a borderline significance for pressure to use doping ($p = .062$). As expected, those who admitted having personal experience with doping exhibited more of a lenient attitude (shown by higher PEAS score) towards doping and gave higher estimated proportions of doping users among athletes and reported higher perceived pressure to use doping. Means, SDs and test statistics are provided in Table 3. These findings are in keeping with literature precedents.

Triangulating explicit and implicit measures with objective behavioural data

Adjusting the criteria for establishing user groups with hair analysis results, a new group emerged within self-declared clean athletes, namely those who denied being involved in doping practices yet the drug was present in their hair. Analysis

Table 3. Groups by self-declared doping behaviour (means, SDs and test statistics for main effects).

Dependent variable	Doping use	Mean ± SD	Mann-Whitney U significance (p)
Explicit doping attitude (PEAS)	Yes	48.00±12.24	
	No	34.04±8.12	p<.001
Perceived pressure to use doping	Yes	28.64±35.58	
	No	10.38±19.78	p=.062[a]
Fellow athletes use doping	Yes	35.45±27.29	
	No	16.97±19.08	p=.024
Fellow athletes use supplements	Yes	62.18±31.22	
	No	59.49±24.73	p=.682
General public use supplements	Yes	35.73±19.21	
	No	35.30±21.29	p=.859
General public use social drugs	Yes	54.36±21.36	
	No	41.59±20.52	p=.067
BIAT doping (latency in ms)	Yes	−171.90±223.51	
	No	−92.31±156.91	p=.168
BIAT doping (D score)	Yes	−0.280	
	No	−0.249	p=.649

[a]t-test showed significant difference (t(57.67) = −2.093, p = .04; equal variances not assumed).

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comparing clean athletes and deniers yield, yet again, a very different picture than the one that was obtained based on self-reports. For comparison, we provide means, SDs and test statistics of the main user group effect for the same set of dependent variables in Table 4. Owing to the uncertainty around the self-admitted doping users, these athletes have been excluded. Figure 1 illustrates the effect of the behavioural information on observed differences in latency measures and D-scores derived from the implicit association task (BIAT). In panels A and B, user groups were based on self reported information on doping. As revealed by subsequent hair analysis, a proportion of athletes (shown in pink) among those who were classified as non-users based on self-declaration (shown in green) were, in fact, users. Consequently, the results of self-declared non-users are confounded by the not-insignificant cohort of athletes who used doping but denied this key information on the self-reported questionnaire.

Once a new grouping was used, it was notable that the 'denier' group performed quite differently on the BIAT, resulting in a smaller latency difference and diminishing IAT-effect (Figure 1C and 1D). In practical terms, those athletes who denied doping use found the *good + doping* pairing less difficult (shown by decreased latency). The same pattern holds for the D-scores, which are derived from latency measures, but after having taken the individual variations in cognitive ability into account. Latency measures and D-scores are detailed in Tables 3 and 4. The lack of significance is owing to the relatively large individual variations combined with the small sample size. The effect size for comparing 'clean' athletes and 'deniers' was d = 0.581 which would require at least 48 athletes in each group to reach statistical significance. The observed difference, however, is notable and it offers a valuable avenue for future research.

As the means indicate in Table 4, athletes who denied doping contrary to the evidence in their hair samples exhibited less lenient attitudes toward doping. These results support our previous observation that athletes who deny their doping use behaviour gave answers on social cognitive measures that are consistent with a typical non-user. In other word, they are 'faking good' in a consistent manner. These results are not surprising, considering that the outcome measures were exclusively based on self-declarations. The picture, however, has changed for the implicit associations. Albeit the differences in latency or D-scores did not reach statistical significance, the sample means suggest that performance on the BIAT was revealing for deniers. That is, latency measures and D-scores set deniers apart from clean and self-admitted users, but did not differentiate between the latter two groups. There are several plausible explanations for fact that the IAT did not differentiate between admitted doping users and self-reported clean athletes (Figures 1A and 1B). First of all, the 'clean' athletes' group level measure was confounded by those who denied doping, pulling the mean latency and related D score toward a more neutral position. Secondly, the self-reported doping user is a dubious group membership.

In order to find further evidence that triangulating self-reports and implicit assessments could be a rewarding approach to identify dishonest participants, we calculated and compared the interaction terms between these two measures. The interaction effect (Table 5) showed moderate divergence in the denier group and slight convergence among clean athletes. These results are in keeping with the hypothesis that discrepancies between explicit and implicit measures are greater in groups that have 'something to hide' by societal standards.

Finally, we looked at information on preferred competitive situations, beliefs about the necessity of doping to win, opinion regarding the prohibition of doping for elite and all athletes and a projection of the proportion of athletes who would be found positive if samples taken today would be analysed in 10 years was also looked at in the context of behavioural data. The majority of the athletes (89%) indicated that they would prefer to compete in a doping free environment. The remaining 11% opted for a scenario in which both players use doping. The fact that no athletes opted for a unilateral use of doping suggests that there is a proportion of athletes who might be more motivated about enhancing performance in general (including using prohibited means) than

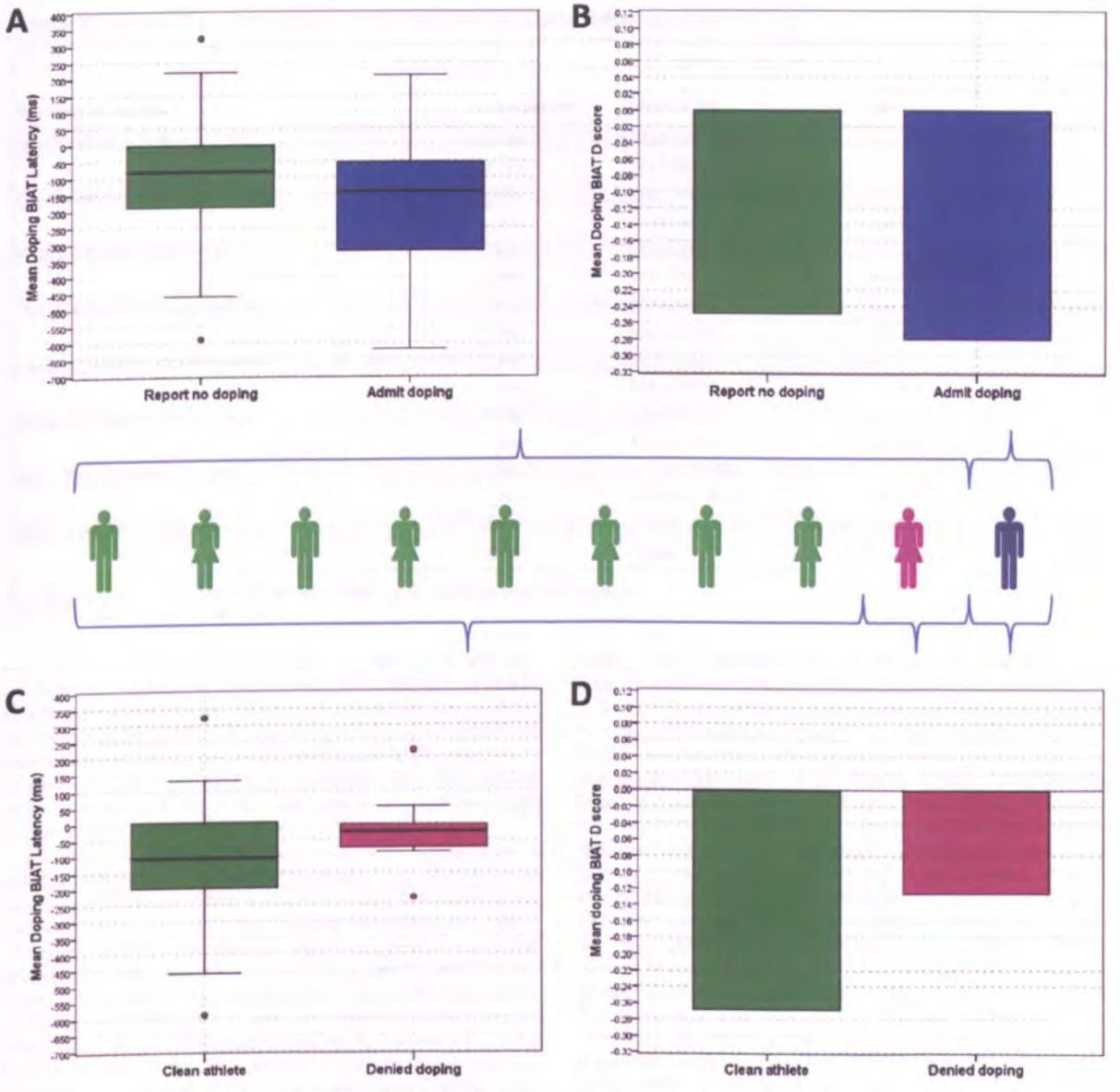

Figure 1. Implicit doping associations by user groups. 'Persons' in the middle represents 100% of the sample with persons in green representing clean athletes (n = 61); blue depicts self-reported doping (n = 11); pink shows the proportion of athletes who denied doping use (n = 10), hence would be classified as non-user according to self-reports. Panels are: (A) latency measure on the doping BIAT task by self-reported user groups; (B) D scores of the doping BIAT task by self reported user groups; (C) latency measure on the doping BIAT task based on hair analysis; (D) D scores of the doping BIAT task by self reported user groups. Circles in panels A and C represents outliers.
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gaining competitive advantage against opponents. Congruent with previous findings, 45% of those who admitted doping expressed a preference for a situation with mutual doping use, followed by 10% of those denied doping and 6% of the clean athletes. Interestingly, 62% of all respondents believed that athletes use performance enhancing substances in training and competition. As expected, athletes who denied doping use consistently expressed opinions regarding doping use, necessity and status similar to or even more rigid than those of clean athletes. Detailed analysis is shown in Table 6.

Discussion

The aim of this study was to expand on and re-examine our previous findings suggesting that self-report regarding doping is contaminated by a response bias whereas the implicit doping-related associations are less affected by it. A pattern similar to that described in Petroczi et al [8] was observed in the extended data set, thus confirming our preliminary findings. In terms of the extremes of the user spectrum (clean athletes and those who denied doping but with hair analysis showing the presence of a

Table 4. Means, SD and test statistics in the hair analysis doping behaviour scenario.

Dependent variable	Doping use	Mean ± SD	Mann-Whitney U significance (p)
Explicit doping attitude (PEAS)	Deny	30.78±6.85	
	Clean	34.55±8.23	p=.221
Perceived pressure to use doping*	Deny	1.00±3.16	
	Clean	11.92±20.92	p=.038
Fellow athletes use doping	Deny	13.60±14.59	
	Clean	17.52±19.77	p=.588
Fellow athletes use supplements	Deny	56.20±24.70	
	Clean	60.02±24.90	p=.589
General public use supplements	Deny	35.00±25.93	
	Clean	35.34±20.69	p=.784
General public use social drugs	Deny	44.70±24.98	
	Clean	41.08±19.90	p=.522
BIAT doping (latency in ms)	Deny	−21.23±119.90	
	Clean	−103.34±159.91	p=.127
BIAT doping (D score)	Deny	−0.126	
	Clean	−0.269	p=.221

*t-test showed significant difference (t(68.96) = 3.818, p<.001, equal variances not assumed).

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performance enhancing drug), self-reports aligned well with self-declared use, whereas the implicit association test (doping BIAT) results aligned better with user grouping based on hair analysis. We further hypothesized a strong divergence between explicit and implicit attitude measures among deniers; and small convergence in the clean athlete group. These assumptions have been verified although perhaps owing to the small sample and relatively large variance, the strength of divergence was not high.

In addition to confirming the previously found discrepancies in self-reports as a function of group identification (i.e. whether it was based on self-reports or hair analysis), we have found that whilst males dominated the self-declared doping user group, the number of females were disproportionately high among deniers, suggesting some gender effect on both self-admissions and denials. Intriguingly, gender effect was not found on any of the dependent variables, indicating that despite the observed pattern in the discrepancy between self-reported and verified behaviour, gender had no systematic effect on self-reported data for social cognitive determinants of doping. We have also found further evidence that distorted social projection (false consensus) is only present for the doping related sensitive issues, not for general population social drug use or NS. Non-doping related variables are unaffected.

Table 5. Interaction between measured variables (mean, minimum and maximum, respectively).

	Clean athletes	Deniers
Explicit attitude x Implicit association	.2331	−.1734
Implicit association x Pressure	.0782	−.1724
Implicit association x Social projection	.0078	.2928
Explicit attitude x Social projection	.0056	.1682
Social projection x Pressure	.1795	.1547
Explicit attitude x Pressure	.1098	.2966

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The similar performance on the implicit association test (BIAT) by clean athletes and self-admitted users is somewhat unexpected. Further investigation is required to untangle this phenomenon, but one possible explanation is that the two groups hold dissimilar beliefs about and attitude toward both NS and doping substances, resulting in different latency on both pairings but similar latency difference and D score. As the implicit test required sorting words into categories where either doping or supplements were, as targets, paired with the 'good' attribute category difference in the IAT effect can only be expected if members of one group hold a different view about the pair of interest (e.g. *good + doping*) and thus perform the task with relative ease in comparison to the other group, but all athletes hold similar views and perform similarly on the comparing task (e.g. *good + nutritional supplements*). In a theoretical case where implicit associations differ on both targets, it is possible to derive a similar IAT effect score with very different performance on the paired tasks. Group mean latency times on each task pairing appear to support this assumption. Self-admitted doping users performed significantly slower on both task pairs than their self-declared clean counterparts (data not shown). Our implicit association tasks contrasted performance enhancing substances and nutritional supplements. Both are often used by athletes with the only difference being in their legal status (i.e. prohibited in sport competition or not). Future studies looking into contrasting deliberately distorted responses on explicit measures and implicit association among 'deniers' should incorporate alternative implicit association tasks independent of potential (non-prohibited) performance enhancements. However, our results suggest, with considerable confidence, that the 'denier' group is characterised by a pattern of dissociation between explicit and implicit responding. This dissociation is, in fact, likely to be a cognitive marker for this group, which may lead to a promising application of the combined explicit-implicit cognitive protocol used in this study as a proxy for the less readily available

Table 6. Doping related opinion by user groups (% is the proportion within the respective group, rounded to the closest full number).

Questions	Answer options	Clean athlete n = 61	Denied doping n = 10
Perceived doping use	Training and competition	38 (62%)	4 (40%)
	Training only	11 (18%)	0
	Competition only	4 (7%)	3 (30%)
	Not used	8 (13%)	3 (30%)
Possible to win without doping?	Yes	41 (68%)	8 (80%)
	No	10 (16%)	2 (20%)
	Do not know	10 (16%)	0
Legalising doping for top level athletes	Yes, without restrictions	0	0
	Yes, but with restrictions	10 (16%)	0
	Absolutely not	51 (84%)	10 (100%)
Legalising doping for all athletes	Yes, without restrictions	0	0
	Yes, but with restrictions	14 (23%)	2 (20%)
	Absolutely not	47 (77%)	8 (80%)
		n = 58	**n = 9**
Proportion of athletes 'clean' today but 'guilty' in 10 years	None	0	0
	A few	8 (14%)	3 (33%)
	A solid minority	7 (12%)	2 (22%)
	Half	18 (31%)	1 (11%)
	Majority	24 (41%)	3 (33%)
	All of them	1 (2%)	0

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biochemical detection methods for large scale social science research on doping.

General population NS and social drug use estimates were used as controls (i.e. non-sensitive issue). However, whilst it served its main purpose, estimations also showed an interesting pattern, hinting that response bias observed in the socially sensitive domain of doping might be linked to the perception of doping as 'drug' (as oppose to functional aid). This is consistent with recent theorising that doping attitudes and behaviours may depend on how doping is represented in the athlete's mind - as a drug use (doping as an illicit behaviour) or as an ergogenic aid (doping as functional use) [39,40]. Investigating contextual influences, Smith et al. [41] concluded that athletes' views on doping were first and foremost influenced by the 'legality' of the substance, then on performance. In this, athletes interviewed considered prohibited performance enhancing substances as cheating but acceptable enhancers as essential. This conclusion maps neatly to our results on nutritional supplements and doping agents but also calls for further investigation as our results on the implicit association task might have been affected by the target stimuli [42]. Following Payne and Gawronski's recommendation [43], future doping research should aim to understand the roots of performance differences in the doping-related explicit and implicit assessment protocols in the context of external validation criteria (e.g., behavioural and bio-markers).

Limitations

Limitations associated with this study include convenience sampling and partly the study design itself. With regard to the prevalence rate, available prevalence statistics are either based on adverse analytical findings or some type of self-reports, which makes direct comparison with our results impossible. Furthermore, owing to the different timeframes and the limited scope of the hair analysis, the extent of a possible overlap between the two subsets of positives is unknown, hence the two prevalence rates (13.4% and 12%) cannot be combined for a lifetime prevalence estimate. We have opened up the self-reports to any prohibited performance enhancing substances in order to obtain a reasonable sample size for self-declared doping users and although this has led to the omission of Group A from part of the analysis, it did not have an effect on the key group (deniers). Future studies may recruit strategically and restrict both the timeframe and the substance list for a complete overlap between self-reports and bioanalysis. Future investigations could also benefit from using more detailed questions and alternative indirect methods regarding doping use. Longer hair samples (if available) may be sectioned by time (e.g. 2-week or 1-month segments) to potentially identify those who may have just started to use doping as this may influence their willingness to admit this behaviour. However, this approach would also require validation studies showing the time for which the drug in question remains in the hair and drug mobility along the hair shaft *in vivo*.

Conclusion

The results of this study draw attention to the discrepancy in doping related explicit attitudes, beliefs and opinions in the context of behavioural data; and to the unique, but perhaps revealing patterns observed in implicit cognition. The most important contribution that our results can add to drug use research is the observed distinct patterns of explicit and implicit responding

among self-declared doping users and deniers which may lead to significant advances in both detection and treatment interventions for these groups. Our findings question the validity of self-reports which may have significant implications in interpreting previous and future doping research. A combination of self-report and implicit cognitive measures seems to hold the strongest promise for future doping research. It is this combination that is likely to produce, with attendant methodology refinements, robust cognitive markers of denial. Objective verification using biomarkers or chemical analysis may not be a feasible approach in all social science research. However, our results suggest that triangulating results obtained on the same or related constructs but using different methodologies could be a cost-effective avenue.

Hence, further research into the methods of combining self-report methodology, with indirect, implicit methods is warranted. Assuming that social desirability has a root in contextual contingencies, research among different user groups could be beneficial. Doping social science research, particularly quantitative research, is seriously lacking in studies using samples drawn from athletes banned from competition owing to doping offences and longitudinal research. Research in this field would benefit from looking beyond doping and having a greater use of direct and indirect methods from social psychology, particularly those used successfully in substance use and addiction research. Incorporating implicit social cognition is one promising avenue for doping social science research. Although it is still debated whether implicit social cognitions reveals something about the individual or the individual's environment, implicit social cognition research is among the thriving areas in social psychology. Doping research, owing to the unique nature of doping (i.e. being positioned between illicit behaviour and functional use of ergogenic aids) provides an excellent testing field for developing a better understanding of the explicit and implicit social cognition and the environment.

Supporting Information

Table S1 Non-standard questions used in the athlete survey.
(DOC)

Table S2 Means and SD for the dependent variables in the self-declared doping user group (Group A).
(DOC)

Table S3 Doping related opinion (% is the proportion within the respective group, rounded to the closest full number) in the self-declared doping user group (Group A).
(DOC)

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Author Contributions

Conceived and designed the experiments: AP DPN. Performed the experiments: MU MT. Analyzed the data: AP TN. Contributed reagents/materials/analysis tools: ND IS JB. Wrote the paper: AP DPN. Developed the implicit association test: EA AP. Developed the software used in implicit assessment: TN.

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RESEARCH ARTICLE Open Access

Detection of stanozolol in environmental waters using liquid chromatography tandem mass spectrometry

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Abstract

Background: Owing to frequent administration of a wide range of pharmaceutical products, various environmental waters have been found to be contaminated with pharmacologically active substances. For example, stanozolol, a synthetic anabolic steroid, is frequently misused for performance enhancement as well as for illegal growth promoting purposes in veterinary practice. Previously we reported stanozolol in hair samples collected from subjects living in Budapest. For this reason we initiated this study to explore possible environmental sources of steroid contamination. The aim of this study was to develop a method to monitor stanozolol in aqueous matrices using liquid chromatography tandem mass spectrometry (LC-MS/MS).

Results: Liquid-liquid extraction using pentane was found to be an efficient method for the extraction of stanozolol from water samples. This was followed by direct detection using LC-MS/MS. The method was capable of detecting 0.25 pg/mL stanozolol when only 5 mL water was processed in the presence of stanozolol D3 as internal standard. Fifteen bottled waters analysed were found to be negative for stanozolol. However, three out of six samples from the Danube river, collected from December '09 to November '10, were found to contain stanozolol at concentrations up to 1.82 pg/mL. In contrast, only one sample (out of six) of urban tap water from Budapest city was found to contain stanozolol, at a concentration of 1.19 pg/mL.

Conclusion: The method developed is efficient, rapid, reproducible, sensitive and robust for the detection of stanozolol in aqueous matrices.

Background

Regular and widespread use of pharmaceuticals, which are frequently excreted as non- metabolized parent compounds, has led to growing concerns for the safety of drinking water [1]. The vast range of pharmaceutical products that have been detected in sewage, surface, ground and drinking waters include bronchodilators, oral contraceptives, antidepressants, beta-blockers, antibiotics, anti-inflammatories and analgesics [2-7]. Even modern sewage treatment works are not constructed to specifically eliminate pharmaceuticals [1] from potable water supplies.

Stanozolol, an anabolic steroid is a synthetic derivative of the endogenously-produced male-sex hormone testosterone. It is commonly misused as a performance enhancement drug because of its ability to enhance muscular strength. The World Anti-Doping Agency (WADA) has banned its use in- and out-of-competition [8]. Despite the restriction, stanozolol is one of the most commonly misused synthetic, anabolic steroids in sport [9] and in veterinary practice, where it is used for growth promoting purposes [10].

In humans, stanozolol is mainly metabolized by undergoing hydroxylation to form mono- and di-hydroxylated metabolites. The majority of these are excreted in urine in the form of conjugates. Less than 5% are excreted as non-conjugated fractions [10]. According to WADA, doping with stanozolol is confirmed if the urinary concentration of its major metabolite, 3-hydroxystanozolol exceeds 2 ng/mL [11]. Unlike testosterone, the synthetic stanozolol and/or its main metabolite should only appear in environmental waters if the former is used for veterinary purposes, taken under medical

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supervision or illegally by athletes for performance enhancement or if either one or both of these compounds are accidently discharged into environmental waters. In previous studies, we reported the detection of stanozolol in hair samples collected from subjects living in Budapest [12,13]. For this reason, we initiated this study to explore possible environmental sources of steroid contamination.

The aim of this study was to develop a methodology for the detection of stanozolol in aqueous matrices. To achieve this, liquid - liquid extraction (LLE) was employed for purification and concentration followed by direct determination using LC-MS/MS. Extraction recovery was evaluated for aqueous matrices spiked with stanozolol at pg/mL levels.

Experimental

Reagents and chemicals

Stanozolol and stanozolol D3 (internal standard) were obtained from LGC standards (Teddington, London, UK). Pentane, deionised water, formic acid, and acetonitrile were obtained from Sigma Aldrich (Poole, Dorset, UK). All chemicals and reagents were of HPLC grade. Environmental water samples were obtained from Budapest (Hungary) and collected from the River Danube and an urban tap (drinking water) in clean, amber bottles. Water samples were collected periodically from December 2009 to November 2010. Samples from Lake Balaton and spring water (Rózsika forrás, Solymár, near Budapest) were also collected for comparison. Some commonly-consumed, bottled non-carbonated, natural mineral water samples were purchased from local supermarkets. The majority of the analysed, commercially available, bottled natural mineral waters are recognised by the European Union [14]. All water samples were stored at -20°C and protected from light prior to analysis.

Extraction procedure

Liquid-liquid extraction (LLE) using pentane was employed for the extraction of stanozolol from water samples. Suspended particles were not filtered from the river water so that the drug adsorbed on them could also be extracted efficiently. A 5 mL aliquot of each water sample was spiked with stanozolol D3 (internal standard, 50 μL of 10 ng/mL) [13] followed by the addition of 3 mL pentane. The contents were vortex mixed vigorously for 10 seconds followed by centrifugation at 3500 × g at ambient temperature for 5 minutes. The pentane layer was separated and collected in a silanized glass tube. To ensure good recovery, the extraction procedure was performed twice. Both organic fractions were pooled and dried by evaporation at 45 °C under a gentle stream of nitrogen gas. The dried residue was then reconstituted with 50 μL acetonitrile. A 5 μL aliquot of the reconstituted solution was injected into the LC-MS/MS system for analysis.

Instrumentation

The LC-MS/MS system consisted of an Accela LC system (Thermo Scientific, UK) coupled to a TSQ Quantum triple quadrupole mass spectrometer (Thermo electron, UK) without a flow splitter. The LC system was comprised of a quaternary pump, automatic solvent degasser, column heater and an auto-sampler equipped with tray chiller. Chromatographic separation was obtained on an Agilent Zorbax SB-C18 column (2.1 mm × 50 mm, 1.8 μm) maintained at 60 °C. Water and acetonitrile both containing 0.1% formic acid were used as mobile phase solvents. The total flow rate through the column was 100 μL/minute. The gradient flow composition is shown in Table 1.

The mass spectrometer was equipped with an electrospray ionization (ESI) source operated in positive ion mode. The capillary temperature was maintained at 350 °C. An ion spray voltage of 4000 V was essential for optimum ionization of stanozolol and stanozolol D3 (internal standard). The protonated molecules, $[M+H]^+$, of stanozolol (*m/z* 329.2) and stanozolol D3 (*m/z* 332.2), were used as precursor ions for collision induced dissociation (CID) for MS-MS analysis. Selective reaction monitoring (SRM) was used to monitor the precursor ions and diagnostic product ions for unambiguous quantification of stanozolol. The collision energies and SRM, *m/z* transitions for stanozolol and internal standard (I.S.) are shown in Table 2.

The Thermo Scientific Xcalibur software (version 2.1) was used to control the LC system and mass spectrometer. Data analysis and assay performance was also evaluated using the same software. The performance of the analytical method was validated for the following set of parameters: linearity, specificity, accuracy, lower limit of detection (LLOD), lower limit of quantification (LLOQ), inter-day precision and intra-day precision. Calibration samples and quality control samples at low, medium and high concentration levels were prepared by fortifying 5 mL HPLC grade water with known concentrations of stanozolol and I.S. followed by LLE and LC-

Table 1 LC mobile phase gradient composition

LC run time (minutes)	0.1% Formic acid in acetonitrile (%)	0.1% Formic acid in water (%)
0	50	50
4	100	0
6.5	100	0
7	50	50
10	50	50

Table 2 Retention times, SRM transitions and collision energies of stanozolol and stanozolol D3 (internal standard)

Analytes	Retention time (min)	Transition (m/z)	Collision energy (eV)
Stanozolol	3.58	329.2 → 81.2	42
		329.2 → 121.2	50
Stanozolol D3	3.56	332.2 → 81.2	42

MS/MS analysis. The analyte-to-internal standard ratio was calculated by dividing the area of analyte peak by the area of the I.S. peak.

A calibration curve was constructed by plotting the analyte-to-internal standard ratio versus the known concentration of stanozolol in each sample. Linear regression analysis using the least squares method was employed to evaluate the calibration curve of analyte as a function of its concentrations in water samples. The LLOQ or lowest point on the calibration curve was defined as the lowest concentration of analyte which could be quantified with a precision < 20% (CV). To determine the lower limit of detection (LLOD), a number of serial 1:2 dilutions were made from the low standard (LLOQ). The lowest concentration which gave a response equivalent to three times the background noise was considered as the LLOD. The accuracy and intra-day precision was assessed by injecting QC samples in replicates at 3 different concentrations. This was repeated on three consecutive days to evaluate the inter-day precision of the assay. The average extraction recovery for the analyte was determined by comparing the analyte to internal standard peak area ratio obtained after extracting negative control water samples fortified with stanozolol at a final concentration of 2 pg/mL in presence of I.S with the un-extracted standard working solutions at the same concentrations. The matrix effects were assessed by comparing the responses of analyte and I.S. obtained from the extracted blank water samples (HPLC grade, tap water and river water) spiked with known concentrations of stanozolol and stanozolol D3 after extraction to those obtained from neat standard solution at the same final concentrations. Validation results are shown in Table 3.

Results and discussion

Method validation

Stanozolol was unambiguously analysed on the basis of its SRM transition and retention time (Figures 1 and 2) via the method proposed and validated herein. Regression analysis indicated that the assay showed excellent linearity within the quantification range of 0.5 to 200 pg/mL water for stanozolol. The LLOQ for stanozolol was found to be 0.5 pg/mL. The correlation coefficients were found to be greater than 0.996 during the method validation procedure. Under the optimized LC-MS/MS conditions, the assay was capable of detecting (LLOD) stanozolol, without any interference, at a concentration as low as 0.25 pg/mL water when 5 mL water was processed. The analytical characteristics of this method including accuracy, linearity, LLOD, LLOQ, inter-day precision, intra-day precision and extraction recoveries from HPLC grade, river and tap water are summarized in Table 3. The relative standard deviation (RSD) was used to assess method precision and it indicated good reproducibility.

Matrix effects in river water led to a reduction in peak areas of stanozolol and stanozolol D3 by 22.3% and 18.4%, respectively. Comparatively, tap water and HPLC water showed lesser matrix effects. The reduction in peak areas was possibly attributed to ion suppression in the ESI source. However, after internal standard correction, the matrix effects in all three types of water samples were comparable and in the range 95.4-97.3% as shown in Table 4. Thus, stanozolol D3 was used as an internal standard to: i) compensate for matrix induced changes in ionization of analyte, ii) correct any loss of analyte during sample preparation, iii) compensate for any variations in the instrument response from injection to injection. The absolute extraction recoveries (with I.S. correction) in three water types; namely HPLC grade water, Danube river water and tap water were in the range 95.3% to 98.4%. The relative extraction recoveries (with I.S. correction) in all three water types were found to be in the range 94.2 to 95.5% for stanozolol as shown in Table 5. This indicated that the method is capable of detecting stanozolol in different types of aqueous matrix when only 5 mL water was processed. The analytical prerequisites for efficient detection of stanozolol at low

Table 3 Summary of assay validation results for stanozolol

Analytes	Linear range (pg/mL)	Concentration (pg/mL)	Precision RSD (%)		Accuracy (%)
			Intraday N = 6+6+6	Interday N = 18+18+18	
Stanozolol	0.5 to 200	2	8.5	7.4	91.7
		16	3.7	8.8	100
		100	6.1	7.4	106.6

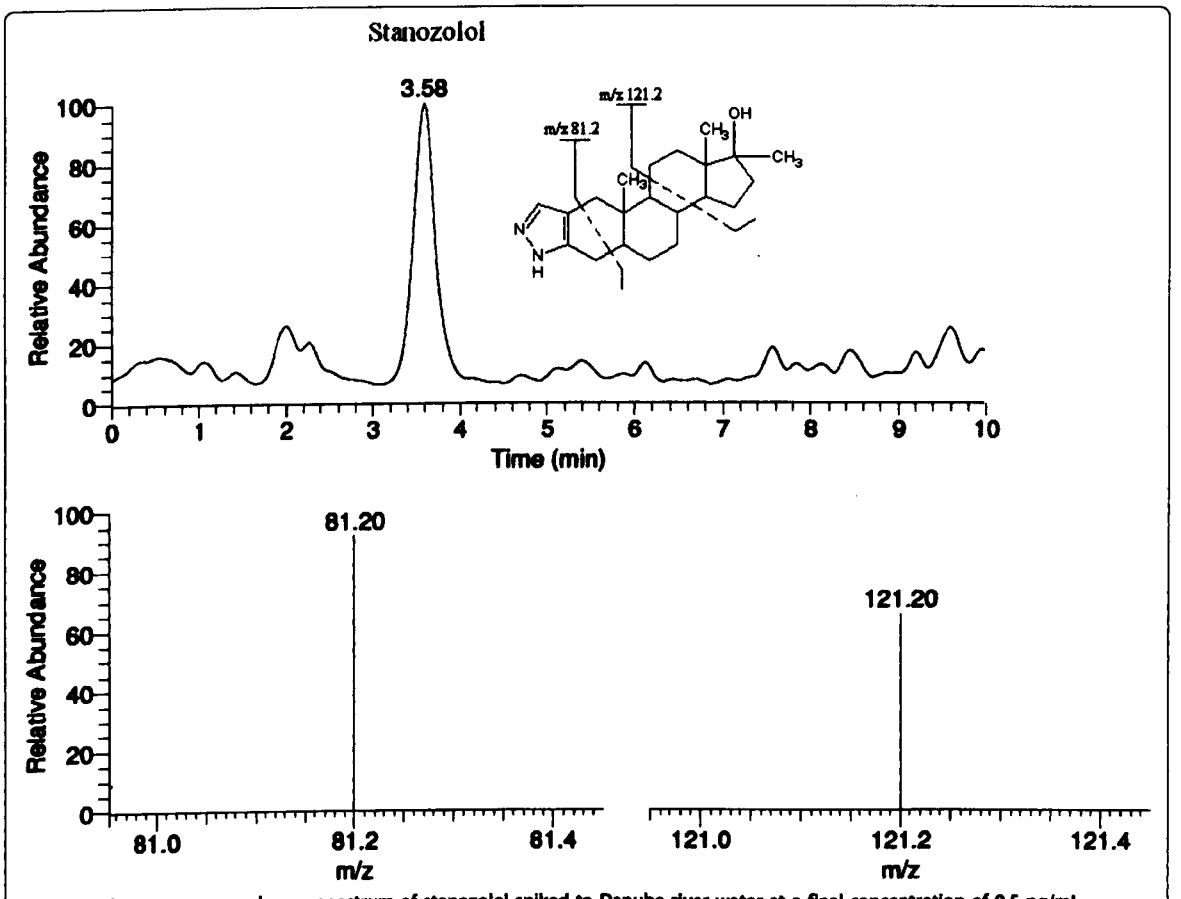

Figure 1 Chromatogram and mass spectrum of stanozolol spiked to Danube river water at a final concentration of 0.5 pg/mL.

levels in aqueous matrix were, purification of water samples using liquid-liquid extraction in presence of a deuterated internal standard followed by injecting only 5 μL aliquot through the column combined with the optimized LC-MS/MS conditions employed for analysis.

Gas chromatography mass spectrometry (GC-MS) and high performance liquid chromatography (HPLC) coupled to fluorescence and UV detectors have been commonly employed to analyse steroids [15]. However, HPLC coupled to fluorescence detection involves laborious sample preparation steps and GC-MS requires a complicated sample derivatization step which makes the method more time consuming and expensive [15,16]. Hence, use of LC-MS/MS for analyzing steroids is a feasible approach as the sample preparation step involved is facile, economical and does not require any additional derivatization step. Compared to previous methods for detecting steroids in environmental waters [15,17-19], the major advantage of our method is that less volume (only 5 mL, opposed to up to 1000 mL) of water sample is required for analysis. Another advantage includes the use of liquid-liquid extraction for purification of water samples, which is less time consuming and more economical in comparison to the solid phase extraction processes employed in previous studies [15,17-20].

Water analyses results

No stanozolol was detected in any of the fifteen bottled waters investigated. In three out of six samples from the Danube river, collected since December '09, stanozolol was detected with levels up to 1.82 pg/mL. In contrast, only one sample of urban tap drinking water from Budapest city was found to contain stanozolol at a concentration of 1.19 pg/mL. The results for stanozolol analysis in different water samples are shown in Table 6.

The possible sources of stanozolol entering the river are unknown, but may be from human or animal consumption and excretion of un-metabolized drug or due to accidental discharge of the parent compound. It should be noted that stanozolol was only found once in

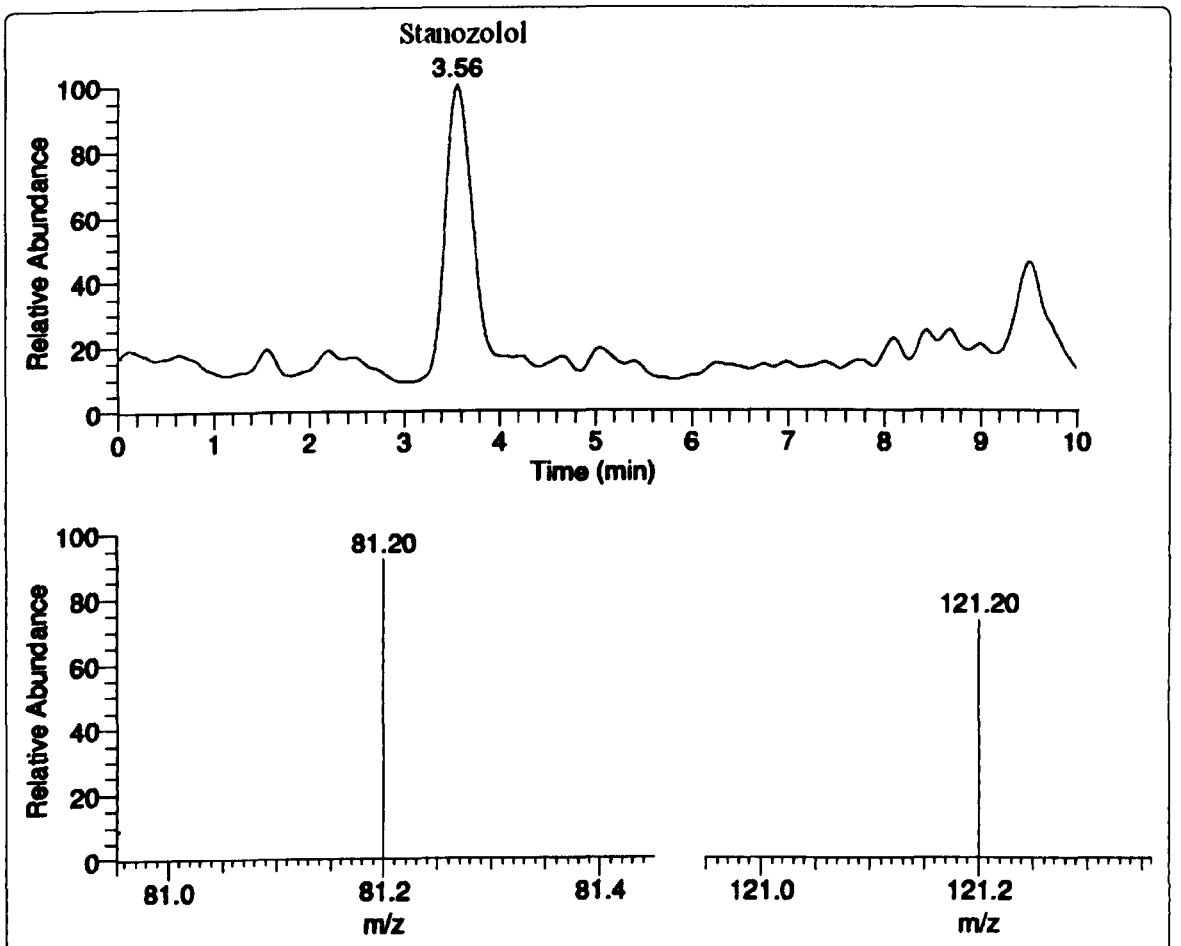

Figure 2 Chromatogram and mass spectrum of stanozolol spiked to tap water at a final concentration of 0.5 pg/mL.

tap water and that this level does not present a threat to health based on recommended intake levels. Water samples from river and tap were collected periodically until November and stanozolol concentrations were found to be reducing over time as shown in Table 5. The possible reasons for a gradual reduction in concentration could be due to: i) variations in rates of contamination, ii) dilution of river water due to rise in water levels, (see Additional file 1), iii) degradation of the steroid in the river water due to other constituents in the river or photolysis, or deposition in the sediment.

The pH values of all the river and tap water samples collected were found to be in the neutral range. The pH

Table 4 Matrix effect results for stanozolol and stanozolol D3 in HPLC water, tap water and river water

Matrix	ME[1] (%)		ME[2] (%)
	Stanozolol	Stanozolol D3 (I.S.)	
HPLC water (N = 6)	95.9	98.7	97.3
Tap water (N = 6)	88.8	92.3	96.2
River water (N = 6)	77.7	81.6	95.4

ME[1] is matrix effect expressed as the ratio of mean peak area of analyte spiked postextraction to the mean peak area of the same analyte standard multiplied by 100. A value less than 100 indicates ion suppression. ME[2] is matrix effect corrected with internal standard.

Table 5 Extraction recovery of stanozolol (I.S. corrected) at 2 pg/mL

Matrix	Absolute extraction recovery (%)	Relative extraction recovery (%)
HPLC water (N = 6)	97.2	95.5
Tap water (N = 6)	98.4	94.5
River water (N = 6)	95.3	94.2

Table 6 Determination of stanozolol in environmental and domestic water samples

Environmental water sample (N = 3)	Average concentration pg/mL					
	31st December 2009	18th April 2010	21thJuly 2010	01st September 2010	24th October 2010	05th November 2010
River Danube	1.82 ± 0.19	0.71 ± 0.06	0.54 ± 0.03	ND	ND	ND
Budapest Tap	1.19 ± 0.03	0.31 (BLQ)	ND	ND	ND	ND
Lake Balaton	-	ND	-	-	-	-
Spring 'Rózsika'	-	ND	-	-	-	-

BLQ means below limit of quantification
ND means not detectable

values of bottled water analysed are summarized in Additional file 2. Stanozolol being basic in nature due to the presence of a pyrozole ring is found to be stable in neutral to slightly basic pH. Further investigation needs to be carried out for determining the source of stanozolol and reasons for gradual decrease in its concentration.

In recent years, numerous reports on steroids found in environmental waters have appeared. Stanozolol has been detected (qualitatively) in sludge samples collected from Huiyang and Meihu waste water treatment plants [17]. Chang *et al.* have also reported the presence of stanozolol in Beijing influent waste water at a concentration of *ca.* 0.54 pg/mL [18]. Recently, Tölgyesi *et al.* have reported the presence of the steroids cortisol, dexamethasone, flumethasone, prednisolone and epitestosterone in Danube river water [15], but their selection of analytes did not include stanozolol.

Our results indicate that stanozolol was present in the River Danube and Budapest tap water in the month of December 2009, when the water level in the river was low (Additional file 2). The National Health Service (NHS) recommends a minimum water intake of 1.2 litres every day [21]. Hence, individuals drinking stanozolol contaminated urban tap water (1.19 pg/mL) will involuntarily consume approximately 1.43 ng stanozolol per day. Since the effective doses of stanozolol for men and women are 50-100 mgs/day and 2.5-10 mgs/day respectively [22], such low levels detected in drinking water may not cause significant harm to the general public, especially as they were found only at one time point. In addition, a new biological sewage treatment plant opened in July 2010 in Budapest in order to treat most of the water supplied to the city (in contrast to only 30-40% water being treated in the past). This major environmental protection investment will potentially contribute to a further decrease in levels of stanozolol compared to those we previously observed. Future studies, sampling from various river and tap water sites should, in due course, be able to provide evidence for this.

Conclusions

In conclusion, a rapid, highly sensitive, robust and reproducible method has been developed to detect stanozolol in different types of water samples. The assay is capable of detecting stanozolol at a concentration as low as 0.25 pg/mL water when only 5 mL water is processed. The performance of this method gives acceptable relative recoveries for stanozolol river and tap water samples. The method can be extended to detect other chemicals and pharmaceutical drugs which may be hazardous to human health and environment.

Additional material

Additional file 1: Danube water level. The supporting document reports the water level, volume, and temperature of River Danube from December 2009 to November 2010.

Additional file 2: Details of bottled drinking water analysed. The supporting document reports the pH, EU recognition and origin of the bottled natural mineral water analysed.

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Authors' contributions
DPN initiated and all authors designed the study. The extraction and method developments were conducted by NIKD who prepared the draft paper. All authors contributed to data analyses and to finalizing the manuscript. All authors have read and approved the final version.

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

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METHODOLOGY Open Access

Determination of stanozolol and 3′-hydroxystanozolol in rat hair, urine and serum using liquid chromatography tandem mass spectrometry

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Abstract

Background: Anabolic androgenic steroids, such as stanozolol, are typically misused by athletes during preparation for competition. Out-of-competition testing presents a unique challenge in the current anti-doping detection system owing to logistic reasons. Analysing hair for the presence of a prohibited drug offers a feasible solution for covering the wider window in out-of-competition testing. To assist *in vivo* studies aiming to establish a relationship between drug levels detected in hair, urine and blood, sensitive methods for the determination of stanozolol and its major metabolite 3′-hydroxystanozolol were developed in pigmented hair, urine and serum, using brown Norway rats as a model system and liquid chromatography tandem mass spectrometry (LC-MS/MS).

Results: For method development, spiked drug free rat hair, blood and urine samples were used. The newly developed method was then applied to hair, urine and serum samples from five brown Norway rats after treatment (intraperitoneal) with stanozolol for six consecutive days at 5.0 mg/kg/day. The assay for each matrix was linear within the quantification range with determination coefficient (r^2) values above 0.995. The respective assay was capable of detecting 0.125 pg/mg stanozolol and 0.25 pg/mg 3′-hydroxystanozolol with 50 mg hair; 0.063 ng/mL stanozolol and 0.125 ng/mL 3′-hydroxystanozolol with 100 μL of urine or serum. The accuracy, precision and extraction recoveries of the assays were satisfactory for the detection of both compounds in all three matrices. The average concentrations of stanozolol and 3′-hydroxystanozolol, were as follows: hair = 70.18 ± 22.32 pg/mg and 13.01 ± 3.43 pg/mg; urine = 4.34 ± 6.54 ng/mL and 9.39 ± 7.42 ng/mL; serum = 7.75 ± 3.58 ng/mL and 7.16 ± 1.97 ng/mL, respectively.

Conclusions: The developed methods are sensitive, specific and reproducible for the determination of stanozolol and 3′-hydroxystanozolol in rat hair, urine and serum. These methods can be used for *in vivo* studies further investigating stanozolol metabolism, but also could be extended for doping testing. Owing to the complementary nature of these tests, with urine and serum giving information on recent drug use and hair providing retrospective information on habitual use, it is suggested that blood or urine tests could accompany hair analysis and thus avoid false doping results.

Keywords: Anabolic androgenic steroid, Doping, Hair analysis, Urinalysis, Serum analysis, LC-MS/MS

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Background

Laboratory statistics of the World Anti-doping Agency (WADA) show that anabolic-androgenic steroids (AAS) account for around 53.6% (average from 2005 to 2010) of all adverse analytical findings in sports [1-6]. Among these, stanozolol is one of the most frequently identified AAS. Stanozolol is a synthetic derivative of the male sex hormone testosterone. According to 'The 2013 Prohibition List' of the WADA code, stanozolol belongs to class S1.1a and its use is prohibited both in- and out-of-competition [7]. Doping with stanozolol is suspected if the urinary concentration of stanozolol and/or its metabolites exceeds 2 ng/mL [8]. Three of the major metabolites of stanozolol are reported to be 3′-hydroxystanozolol, 4β-hydroxystanozolol and 16β-hydroxystanozolol (Figure 1), which are excreted in urine mainly as glucuronide conjugates [9]. Amongst these, the urinary level of 3′-hydroxystanozolol, post deglucuronidation, is routinely used for screening stanozolol misuse [8-10].

Since stanozolol and 3′-hydroxystanozolol are structurally different from most AAS, they can be more difficult to detect in urine than other AAS [11], and thus require bespoke methods. Depending on the dose administered, once in the body, stanozolol gets rapidly metabolised and the metabolites are generally detected in urine until *ca.* 6 days [11]. Thus, urinalysis generally fails to determine the long term history of an individual's drug use [12], which is a major hindrance in cases of performance-enhancing drugs used in preparation for competition. Stanozolol, along with other AAS, is a so called 'training drug' which is taken for a prolonged period, typically in cycles, during preparation, in order to obtain the desired performance-enhancing effects [13,14]. Furthermore, urinalysis also fails to distinguish between chronic use and single, accidental exposure of drugs [15].

The major elimination and deactivation pathway of AAS and their phase I metabolites is through glucuronide conjugation (phase II metabolism), mainly catalysed by the enzyme UGT2B17, followed by excretion in urine [16-19]. However, inter-individual and inter-ethnic variations in the prevalence of deletion polymorphism in the gene coding of the UGT2B17 enzyme have been reported, which eventually influence the urinary excretion of AAS and potentially lead to false-negative doping results [20,21]. It has also been reported that the glucuronidation activity of UGT2B17 and other UGTs towards AAS is inhibited by commonly used anti-inflammatory drugs like diclofenac and ibuprofen, *in vitro* [22-26]. Common dietary substances such as red wine [27], white tea and green

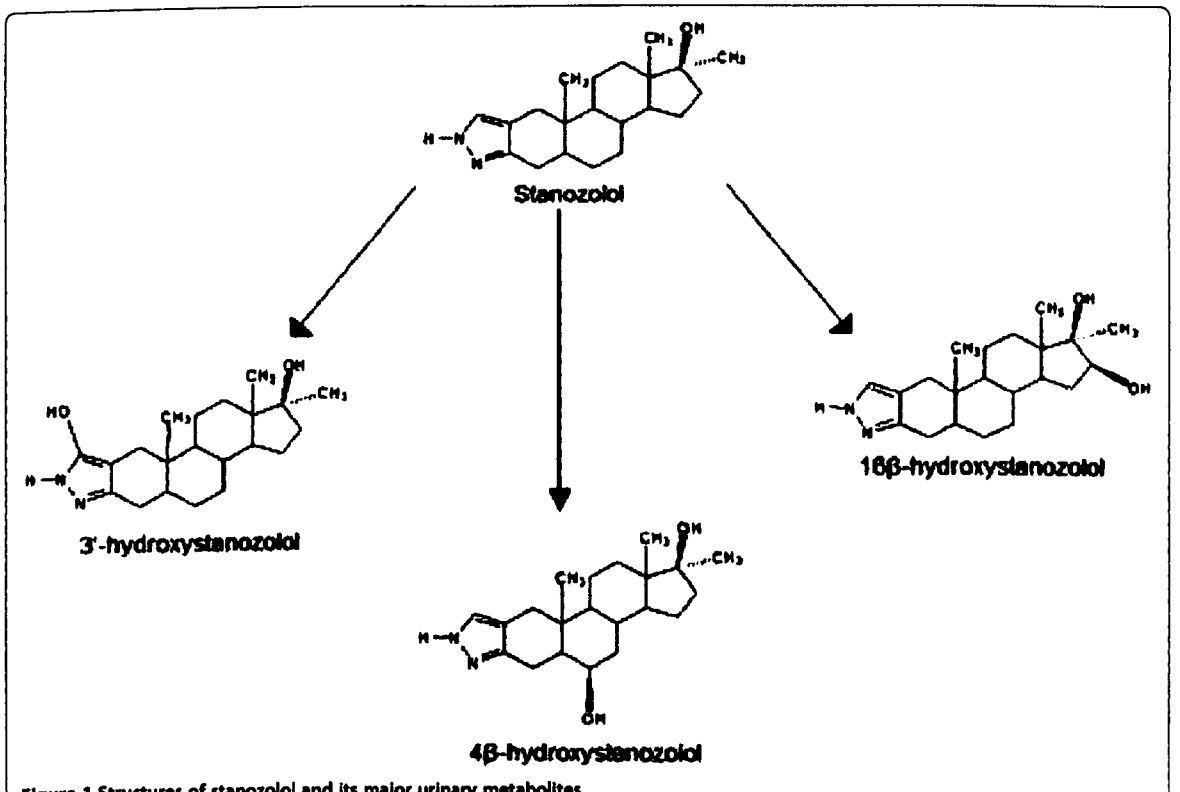

Figure 1 Structures of stanozolol and its major urinary metabolites.

tea [28] have also shown similar inhibitory effects in *in vitro* studies. Although the inhibitory effect is yet to be examined and reported *in vivo*, these *in vitro* results indicate that concomitant use of such over-the-counter medication or common dietary products with AAS may lead to impaired urinary excretion of AAS and their metabolites.

Considering that such genetic and metabolic variations may limit the efficacy of urinalysis in testing doping, it can be suggested that urinalysis, if used as a stand-alone test, is susceptible to confounding doping results [11-13,16-21]. Owing to the growing number of doping cases with AAS [1-6], there is an ever-increasing need to develop new methods to detect drug doping. The current anti-doping regime can be reinforced by employing additional biological samples like blood and hair analysed in tandem with urine. Since impaired glucuronidation leads to reduction in the urinary excretion rate of AAS, it can be assumed that the levels of unconjugated AAS and their phase I metabolites in the systemic circulation will be elevated and thus higher levels of AAS and their phase I metabolites will be available to get incorporated into hair and other body tissues [21]. Hair analysis has been used in the past for detecting drug use [29-32] as it predominantly favours the direct detection of parent AAS and determines a retrospective history of drug use. Thus, hair analysis and blood analysis [33] can provide complementary information to urinalysis to prevent false doping results.

However, to investigate this option further, *in vivo* studies are required to establish a relationship between the drug levels detected in hair, urine and blood. To the best of our knowledge, such studies for the determination of stanozolol and its major metabolite, 3′-hydroxystanozolol in the three matrices together are, as yet, not reported in the literature. Thus, the aim of this work was to take a step forward by developing liquid-chromatography tandem mass spectrometry (LC-MS/MS) based methods which are capable of determining the concentrations of stanozolol and 3′-hydroxystanozolol in pigmented hair, urine and blood serum samples of stanozolol-treated rats.

In the past, *in vivo* studies have been reported where administration of a single high dose of stanozolol (60 mg/kg) to guinea pigs afforded the detection of stanozolol in hair [34,35], whereas it was not possible to detect 3′-hydroxystanozolol [34]. Since, metabolites are generally difficult to detect in hair, it is reasonable to assume that a single-dose treatment may not be sufficient to investigate whether levels of metabolites can be determined in hair. However, multiple doses of stanozolol along with sensitive analytical methods can provide this key information. Thus, as a preliminary step, a 6 day treatment period was used in this study to improve the potential for detecting the metabolites in hair. Athletes typically administer AAS at doses ranging from 3 mg/kg to 25 mg/kg to increase muscle mass, which are 10 to 100 fold higher than the therapeutic doses [36]. Thus, in line with previous steroid-abuse rat studies [36-39], the present study was designed with a daily dose of 5.0 mg/kg for 6 consecutive days, followed by analysing hair, urine and sera samples using newly developed LC-MS/MS methods.

Experimental

Chemicals, reagents and consumables

Reference standards for stanozolol, 3′-hydroxystanozolol, 3′-hydroxystanozolol glucuronide, 3′-hydroxystanozolol D3 and stanozolol D3 were purchased from LGC standards (Teddington, UK). Sodium hydrogen phosphate heptahydrate, sodium phosphate monobasic dihydrate, sodium hydroxide, formic acid, hydrochloric acid, LC-MS grade water, acetonitrile, methanol, HPLC grade dichloromethane, pentane, chloroform and ethylacetate were purchased from Sigma Aldrich (Poole, UK). β-glucuronidase from *E. Coli* (Cat. No. 03707598001, Lot No. 12438921) was purchased from Roche Diagnostics (Burgess Hill, UK). All chemicals were of analytical-reagent grade and were used without further purification. For the animal experiment, stanozolol, ketamine (2.5%) and xylazine (Rompun, 2%) were purchased from Desma (Madrid, Spain), Kőbányai Gyógyszerárugyár (Budapest, Hungary) and Haver-Lockhart laboratories (Kansas, US) respectively. 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). Silanised glass inserts were purchased from Capital Analytical (Leeds, UK). Silanised, amber, glass vials were purchased from Sigma Aldrich (Poole, UK).

Animals

Male, brown Norway rats were purchased from Charles River laboratories (Sulzfeld, Germany). Each animal weighed around 280–340 g and was approximately 5 months old. All animals were kept in an animal house located in Semmelweis University, Budapest, Hungary. Animals were housed in groups of three individuals in standard laboratory cages. Rats were kept in a constant room temperature environment with an alternating 12-h light–dark cycle. Food and water were available *ad-libitum*.

Administration of stanozolol and sample collection

Five rats kept in standard lab cages under 12/12 light/dark cycle were administered with stanozolol (in saline) intra-peritoneally [34], at a dose of 5.0 mg/kg/day for six consecutive days. The dose of stanozolol selected was in line with previous steroid studies using rat models [36-39]

and considered equivalent to levels abused by humans on a milligram per kilogram of body weight basis [36,37]. Hair, urine and blood samples were collected on the 7th day of the study, *i.e.* one day after stopping the stanozolol treatment.

The growth rate of rat hair was tested prior to the treatment regime by shaving the back of the experimental animals and the sampling protocol was adjusted accordingly.

Urine was collected by gently pressing the abdomen. Blood was taken from the tail vein. Blood samples were left to clot for 45 to 60 minutes and then centrifuged (at 1000 × g for 10 minutes at room temperature) to harvest serum. Before collecting blood and urine samples, the animals were anaesthetised with a mixture of ketamine and xylazine. Two weeks before the experiment, the entire dorsal surface of the animal was shaved to the skin with an electric shaver and drug-free control hair was collected and preserved. Exactly the same dorsal surface was sampled on the 7th day of the experiment to avoid any diluting effect of the hair grown before the stanazolol treatment period. Drug-free blood and urine samples were also collected before the experiment was initiated. Serum and urine samples obtained were stored at −80°C. Hair samples were stored in sealed, clean envelopes at room temperature. The administration of stanozolol and sample collection were conducted under the institutional license of Department of Anatomy, Histology and Embryology, Semmelweis University, Budapest, Hungary in accordance with the EC Council directives on laboratory animals (86/609/EEC). Samples were analysed in Kingston University.

Sample preparation

Hair samples

Hair samples were initially decontaminated by rinsing twice with 2 mL dichloromethane for two minutes at room temperature. After decontamination, hair samples were allowed to air dry and then pulverised using a ball mill. Fifty milligrams of decontaminated hair powder was incubated with 1 mL 1 M sodium hydroxide at 95°C for 10–15 minutes in the presence of deuterated internal standards (ISs) stanozolol D3 and 3′-hydroxystanozolol D3. After cooling, the homogenate was neutralised with 1 M hydrochloric acid, followed by addition of 2 mL of 0.2 M phosphate buffer (pH 7.0).

Serum and urine samples

Serum and urine samples were thawed and vortex mixed. A 100 μL aliquot of each was used for analysis.

Enzymatic hydrolysis of glucuronide conjugates

The enzyme β-glucuronidase was used for the enzymatic hydrolysis of glucuronide conjugates to determine the total concentration (glucuronide conjugated + unconjugated) of stanozolol and 3′-hydroxystanozolol in each matrix (hair, urine and serum). For this step, and, in a similar manner to the hair samples, the serum and urine samples were also neutralised by mixing with 1 mL of 0.2 M phosphate buffer (pH 7.0). The neutralised solutions of hair, serum and urine were hydrolysed by incubation with 50 μL of β-glucuronidase at 50°C for two hours in the presence of internal standards [9]. After cooling, the samples were purified by performing liquid–liquid extraction (LLE).

Sample purification

LLE was carried out by using a mixture of pentane, chloroform and ethylacetate (4 mL in total) in the ratio 3:2:1 v/v/v. The mixture was vortex mixed for 20 seconds and then centrifuged at 4000 × g for 20 minutes at 4°C. The organic layer was transferred into a clean, silanised, glass vial and evaporated at 40°C using a gentle stream of nitrogen gas. The dried residue was reconstituted with 100 μL methanol. The reconstituted solution was filtered through a 0.2 micron PTFE membrane filter, prior to injecting (3 μL) into the LC-MS/MS system.

Liquid chromatographic-tandem mass spectrometry

The analysis of stanozolol and its metabolite 3′-hydroxystanozolol was carried out using an LC-MS/MS system, which comprised of a 1260 infinity LC system (Agilent, Wokingham, UK) coupled to a 6430 triple quadrupole mass spectrometer (Agilent, Wokingham, UK). The LC system comprised of a binary pump, automatic degasser, column heater and 1290 infinity thermostated autosampler. The analytical column used was a SB C-18 column (2.1 mm, 50 mm, 1.8 μm), kept in a column oven at 45°C. A 0.2 micron inline filter was installed prior to the column to prevent the analytical column from blocking. Mobile phase solvents comprised of water with 0.001% v/v formic acid as solvent A and 50:50 mixture of acetonitrile and methanol as solvent B. The flow rate of mobile phase through the column was 300 μL/min. The gradient flow composition is shown in Table 1.

The mass spectrometer was equipped with an electrospray ionisation (ESI) source, which was operated in positive ion mode. The protonated molecules, $[M + H]^+$, of stanozolol (*m/z* 329.5), 3′-hydroxystanozolol (*m/z* 345.5), stanozolol D3 (*m/z* 332.5) and 3′-hydroxystanozolol D3 (*m/z* 348.5) were used as precursor ions for collision induced dissociation (CID) for MS-MS analysis. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode to monitor the precursor ions and the diagnostic product ions of each analyte and IS. The MRM transitions, collision energies and retention times of each analyte and internal standard are detailed in Table 2.

Table 1 Chromatograms of stanozolol and 3′-hydroxystanozolol extracted from (a) hair, (b) urine and (c) serum at LLOQ concentration levels

LC run time (minutes)	Solvent A Water (0.001% formic acid)	Solvent B Acetonitrile: methanol (50:50)
0	60	40
1	60	40
2	15	85
5	0	100
6	0	100
7	60	40
15	60	40

For the optimum ionisation of analytes, the following mass spectrometric conditions were applied: 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 125 V. The mass spectrometric parameters were optimised using the Masshunter optimizer software (version B.03.01). The LC-MS/MS system was controlled by the Masshunter workstation software (LC/MS data acquisition, version B.03.01).

Method validation

The validation of the analytical methods was performed according to the Food and Drug Administration (FDA) guidelines [40], by determining accuracy, precision, lower limits of quantification (LLOQ), lower limits of detection (LLOD), linearity, selectivity, and extraction recoveries [41,42]. Drug-free rat hair, urine and serum samples were used for method development and validation. Samples for calibration curves were prepared by spiking known amounts of stanozolol, 3′-hydroxystanozolol and ISs (stanozolol D3 and 3′-hydroxystanozolol D3) to drug-free hair, urine and serum. Quality control (QC) samples were prepared similarly at three concentration levels (for each matrix) distributed over the linear range. Calibration curves were prepared for each matrix by plotting the analyte to IS ratio against the known concentrations of analyte in each sample. The analyte to IS ratio for each analyte was obtained by dividing the peak area of analyte by the peak area of the IS. Samples for calibration curves and quality controls were treated in a way similar to unknowns. The linearity of the method was investigated by using linear regression analysis.

The accuracy of each assay was determined by analysing QC samples at three concentration levels in replicates (N = 6, per concentration level) and comparing the mean calculated values with the respective nominal concentration values. Intra-day precision was determined by measuring 6 replicates per concentration level, on the same day. Inter-day precision was assessed by analysing 6 replicates per concentration level, on three consecutive days. Intra-day and inter-day precision of the method was characterised in terms of relative standard deviation (RSD, %). The limits of acceptable variability were set at 15% for all the concentrations, except at LLOQ, for which 20% was accepted. LLOD was defined as the lowest concentration of the analyte which gave a peak response equivalent to three times the background noise [i.e. signal to noise ratio (S/N) ≥ 3]. LLOQ was defined as the lowest amount of analyte which gave a peak response with a S/N ≥ 10 and which could be measured with adequate precision and accuracy (RSD less than 20% and an inaccuracy ±20%) [40].

The selectivity of the method was determined by analysing the drug-free samples of hair, urine and serum in replicates and confirming the absence of any detectable peaks at the retention times of stanozolol, 3′-hydroxystanozolol and ISs. The extraction recovery for each analyte was determined at three concentration levels by replicate analysis (N = 6) of blank matrices (urine, serum and hair) spiked with known concentrations of analytes and ISs and

Table 2 Retention times, MRM transitions and collision energies of stanozolol, 3′-hydroxystanozolol, stanozolol D3 and 3′-hydroxystanozolol D3

Compounds	Retention time (min)	MRM transitions	Collision energy (eV)
Stanozolol	6.0	329.5 > 81.1	50
		329.5 > 121.1	46
3′-Hydroxystanozolol	5.6	345.5 > 97.1	50
		345.5 > 121.1	42
Stanozolol D3	6.1	332.2 > 81.2	50
3′-Hydroxystanozolol D3	5.8	348.5 > 97.1	50

then extracted as described above. The analyte to internal standard peak area ratios obtained after extraction were then compared with analyte to internal standard peak area ratios of standard solutions prepared in methanol at the same final concentrations. To determine matrix effects, blank hair, urine and serum samples from different animals were extracted as described above. In order to consider only the matrix effect and not losses during the extraction procedure, the blank extracts were spiked with known concentrations of analytes and ISs after the extraction step, followed by analysis. The resulting peak areas of stanozolol, 3′-hydroxystanozolol and ISs were then compared with the peak areas of standard solutions of stanozolol, 3′-hydroxystanozolol and ISs at the same theoretical concentrations.

Results and discussion

Method development

Both stanozolol and 3′-hydroxystanozolol were detected and quantified on the basis of their retention time and MRM transitions (Table 2). The most abundant product ions that were monitored for stanozolol were m/z 81.1 and 121.1, whereas for 3′-hydroxystanozolol, the most abundant product ions that were monitored were m/z 97.1 and 121.1. Figure 2 represents the product ions mass spectra (full scan) of stanozolol and 3′-hydroxystanozolol. Operating the mass spectrometer in MRM mode enhanced the method selectivity, sensitivity and specificity. Stanozolol D3 and 3′-hydroxystanozolol D3 were used as internal standards for stanozolol and 3′-hydroxystanozolol respectively. Internal standards were used to compensate for any: i) ionisation suppression, ii) variations in the instrument response from injection to injection and iii) loss of analytes during sample preparation.

Use of different mobile phase solvents was investigated. For instance, use of water as solvent A in combination with methanol or acetonitrile or a mixture of methanol and acetontrile (50:50) as solvent B was examined. Different gradient and isocratic mobile phase compositions were investigated. Addition of formic acid (0.001% v/v, 0.01% v/v and 0.1% v/v) to solvent A and/or solvent B was also investigated. Optimum sensitivity and excellent peak shapes for all analytes and ISs were obtained when water with formic acid (0.001% v/v) was used as solvent A and a mixture of acetonitrile and methanol (50:50) was used as solvent B under the gradient conditions shown in Table 1. It was observed that when formic acid was added to solvent A and/or solvent B at concentrations ≥ 0.01% v/v, there was a drastic reduction in the sensitivity of all analytes and ISs (up to 50%). However, when formic acid was added only to water (solvent A) at a concentration of 0.001% v/v, there was no effect on the sensitivity and peak shapes.

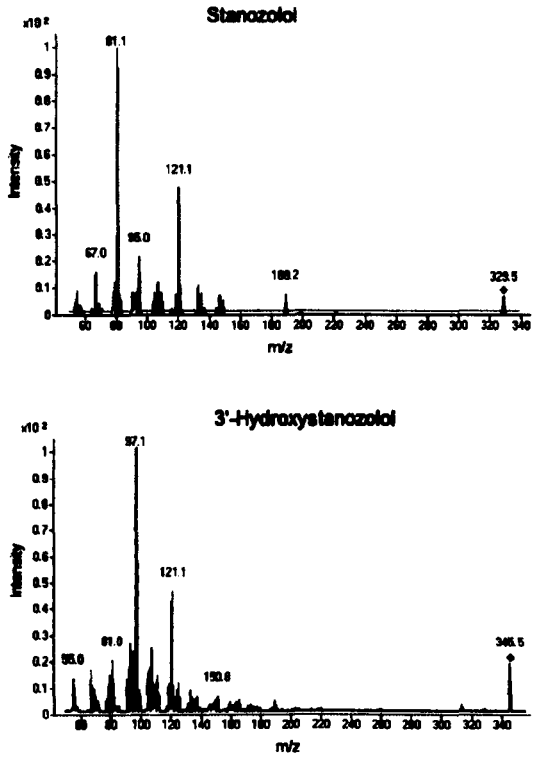

Figure 2 Product ion mass spectra (full scan) of stanozolol and 3′-hydroxystanozolol.

For hair analysis, alkali digestion was employed for the extraction of drugs from hair matrix. Alkali digestion ensures complete dissolution of the hair matrix and hence it is generally known to give good recoveries of drugs entrapped in the hair matrix. However, a potential drawback of complete dissolution of hair is that the components of hair matrix in solution may interfere with the analysis. Thus, to reduce the unwanted matrices that may affect the analysis, sample purification was carried out using LLE. The extraction efficiencies of different solvents like pentane, hexane, chloroform, ethyl acetate and ethanol, and their combinations were investigated. It was found that a mixture of pentane, chloroform and ethyl acetate in the ratio 3:2:1 v/v/v facilitated maximum recovery of analytes and ISs. Also, owing to the hair decontamination step employed (using dichloromethane), no external interferences were observed. The LLE step employed was also efficient for the extraction of stanozolol, 3′-hydroxystanozolol and ISs from the urine and serum samples. Under the analytical conditions employed, there were no matrix interferences that affected the analysis of stanozolol and 3′-hydroxystanozolol in hair, urine and serum. The enzymatic hydrolysis of glucuronide conjugates of stanozolol and 3′-hydroxystanozolol was

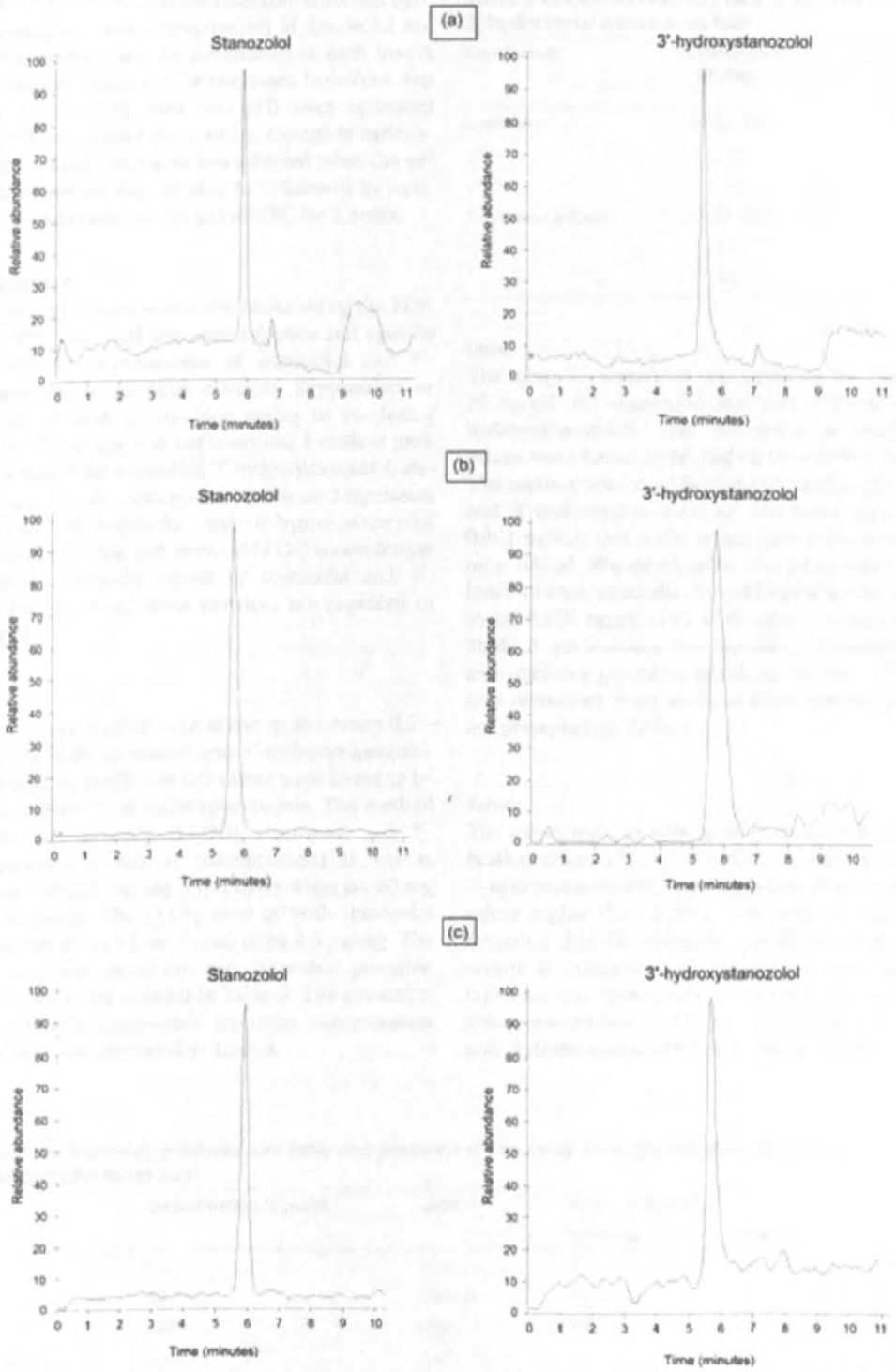

Figure 3 Chromatograms of stanozolol and 3′-hydroxystanozolol extracted from (a) hair, (b) urine and (c) serum at LLOQ concentration levels.

carried out. This ensured that the total concentration (glucuronide conjugated plus unconjugated) of stanozolol and 3′-hydroxystanozolol could be determined in each matrix [9]. The conditions employed for enzymatic hydrolysis step (incubation temperature, time and pH) were optimised using 3′-hydroxystanozolol glucuronide. Complete hydrolysis of the glucuronide conjugate was achieved when the pH of the sample solution was adjusted to 7, followed by incubation with β-glucuronidase (50 μL) at 50°C for 2 hours.

Method validation

The validation results are within the limits set by the FDA guidelines [40]. The methods were selective and specific for unambiguous determination of stanozolol and 3′-hydroxystanozolol in all three matrices. Suppression or enhancement of analyte ionisation owing to co-eluting components of matrices was not observed. Excellent peak shape was achieved for stanozolol, 3′-hydroxystanozolol, stanozolol D3 and 3′-hydroxystanozolol D3. Figure 3 represents chromatograms of stanozolol and 3′-hydroxystanozolol extracted from hair, urine and serum at LLOQ concentration levels. Typical calibration curves of stanozolol and 3′-hydroxystanozolol in all three matrices are provided in Additional file 1.

Hair

The assay for hair analysis was linear in the range 0.5 - 400 pg/mg for both stanozolol and 3′-hydroxystanozolol. The determination coefficient (r^2) values were found to be higher than 0.9986 for all calibration curves. The method was capable of detecting (LLOD) stanozolol and 3′-hydroxystanozolol in hair at concentrations as low as 0.125 pg/mg and 0.25 pg/mg respectively when *ca.* 50 mg hair was processed. The LLOQ level of both stanozolol and 3′-hydroxystanozolol was found to be 0.5 pg/mg. The accuracy, intra-day precision and inter-day precision results of the assay are detailed in Table 3. The extraction recoveries of both compounds (at three concentration levels) from hair are presented in Table 4.

Table 4 Extraction recovery results of stanozolol and 3′-hydroxystanozolol from hair

Compounds	Concentration (pg/mg)	% Extraction recovery (N = 6)
Stanozolol	0.5 (LLOQ)	100.84
	2.5	103.53
	10	105.39
3-Hydroxystanozolol	0.5 (LLOQ)	88.51
	2.5	107.99
	10	102.64

Urine

The assay for urinalysis was linear in the range 0.125 - 25 ng/mL for stanozolol and 0.25 - 25 ng/mL for 3′-hydroxystanozolol. The determination coefficient (r^2) values were found to be higher than 0.9959 for all runs. The method was capable of detecting (LLOD) stanozolol and 3′-hydroxystanozolol at concentrations as low as 0.063 ng/mL and 0.125 ng/mL urine respectively, when only 100 μL aliquot of urine was processed. The LLOQ levels of stanozolol and 3′-hydroxystanozolol were found to be 0.125 ng/mL and 0.25 ng/mL urine respectively. Table 5 summarises the accuracy, intra-day precision and inter-day precision results of the assay. The extraction recoveries from urine at three concentration levels are presented in Table 6.

Serum

The serum assay showed good linearity within the quantification range 0.25 - 100 ng/mL for both stanozolol and 3′-hydroxystanozolol, with determination coefficient (r^2) values higher than 0.9981. The method was capable of detecting (LLOD) stanozolol and 3′-hydroxystanozolol in serum at concentrations as low as 0.063 ng/mL and 0.125 ng/mL respectively when only 100 μL aliquot of serum was processed. The LLOQ level of both stanozolol and 3′-hydroxystanozolol was found to be 0.25 ng/mL.

Table 3 Accuracy, intra-day precision and inter-day precision of the assay for detecting stanozolol and 3′-hydroxystanozolol in rat hair

Compounds	Concentration (pg/mg)	Level	Precision RSD (%)		Accuracy (%)
			Intra-day	Inter-day	
Stanozolol	2.5	Low	3.4	2.8	105.0
	20	Medium	1.5	1.3	103.4
	100	High	1.2	2.8	100.7
3′-Hydroxystanozolol	2.5	Low	1.5	4.1	97.3
	20	Medium	3.6	3.4	101.9
	100	High	3.8	5.3	100.5

Table 5 Accuracy, intra-day precision and inter-day precision of the assay for detecting stanozolol and 3′-hydroxystanozolol in rat urine

Compounds	Concentration (ng/mL)	Level	Precision RSD (%)		Accuracy (%)
			Intra-day	Inter-day	
Stanozolol	0.5	Low	10.4	6.3	90.6
	2.5	Medium	4.6	7.0	105.6
	5	High	4.1	4.0	111.4
3′-Hydroxystanozolol	0.5	Low	4.9	6.2	89.7
	2.5	Medium	7.1	5.0	109.2
	5	High	6.4	5.1	104.2

The accuracy, intra-day precision and inter-day precision results of the assay are detailed in Table 7. The extraction recoveries are presented in Table 8.

Application of the method to real samples

The developed methods were employed for determining the total (glucuronide-conjugated plus un-conjugated) concentration of stanozolol and its metabolite 3′-hydroxystanozolol in rat hair, urine and serum samples. Table 9 represents the average concentrations (three replicates) of stanozolol and 3′-hydroxystanozolol in hair, urine and serum samples of each rat. The average concentrations of stanozolol and 3′-hydroxystanozolol in rat hair were found to be 70.18 ± 22.32 pg/mg and 13.01 ± 3.43 pg/mg respectively. The average ratio of concentrations of stanozolol to 3′-hydroxystanozolol in hair was found to be 5.38 ± 0.93. Variations observed in the concentration of stanozolol and 3′-hydroxystanozolol amongst individual animals could be owing to differences in their metabolic pattern. Furthermore, difference in the amount of water consumed by animals can also lead to variations in the levels of drugs in their body. The results indicate that stanozolol gets preferentially incorporated in hair relative to its metabolite 3′-hydroxystanozolol. These findings are in agreement with previous reports [32]. In the past, researchers have found it difficult to detect 3′-hydroxystanozolol in hair. Cirimile *et al.* reported the detection of stanozolol in scalp hair of a bodybuilder who declared to be a regular user of stanozolol [43]. However, 3′-hydroxystanozolol was not detectable in hair under their analytical conditions. Similarly, in another study carried out by Shen *et al.*, stanozolol was detectable in guinea pig hair after administering stanozolol at a single high dose of 60 mg/kg, whereas, 3′-hydroxystanozolol was not detectable [34]. However, the method presented here was capable of detecting stanozolol and 3′-hydroxystanozolol in rat hair after administering stanozolol for 6 days at a dose of 5.0 mg/kg/day that is considered equivalent to those levels abused by athletes [36-39]. Thieme *et al.* have reported a case where both stanozolol and 3′-hydroxystanozolol were detectable in the hair of a bodybuilder using gas chromatography high resolution mass spectrometry (GC-HRMS), after sample derivatisation [44]. In the past GC-MS and GC-HRMS have been frequently employed for the detection of AAS [43-46]. The major disadvantage of such technique is that it requires a laborious and expensive sample derivatisation step. Generally, the derivatives are unstable and susceptible to thermal decomposition during analysis, thus affecting the reproducibility of the method. In contrast, LC-MS/MS normally does not require any additional derivatisation step. Thus, LC-MS/MS can be considered as a more economical and feasible approach for analysing AAS [29,30,47].

Table 6 Extraction recovery results of stanozolol and 3′-hydroxystanozolol from urine

Compounds	Concentration (ng/mL)	% Extraction recovery (N = 6)
Stanozolol	0.125 (LLOQ)	107.87
	2.5	111.77
	10	109.64
3-Hydroxystanozolol	0.25 (LLOQ)	91.44
	2.5	107.92
	10	111.82

In urine, the average concentrations of stanozolol and 3′-hydroxystanozolol were found to be 4.34 ± 6.54 ng/mL and 9.39 ± 7.42 ng/mL respectively. The average urinary ratio of stanozolol concentration to 3′-hydroxystanozolol concentration was found to be 0.37 ± 0.32. The results indicate that the urinary concentrations of stanozolol are comparatively lower than 3′-hydroxystanozolol, as expected. However, in serum the average ratio of concentrations of stanozolol to 3′-hydroxystanozolol was found to be 1.09 ± 0.37. Thus, suggesting that both compounds can be detected in serum at similar concentration levels and with equal ease.

The results suggest that the newly developed LC-MS/MS based methods are capable of detecting and quantifying total concentration (glucuronide conjugated plus

Table 7 Accuracy, intra-day precision and inter-day precision of the assay for detecting stanozolol and 3′-hydroxystanozolol in rat serum

Compounds	Concentration (ng/mL)	Level	Precision RSD (%)		Accuracy (%)
			Intra-day	Inter-day	
Stanozolol	1.25	Low	4.4	3.0	111.0
	10	Medium	1.4	1.6	108.4
	50	High	1.7	1.4	90.4
3′-Hydroxystanozolol	1.25	Low	4.3	6.4	98.0
	10	Medium	3.5	3.9	102.3
	50	High	4.8	5.3	91.9

unconjugated) of stanozolol and its major metabolite, 3′-hydroxystanozolol in hair, urine and serum samples of brown Norway rats after administering stanozolol for 6 days at a dose (5.0 mg/kg/day), and this is in line with steroid studies using rat models [36-39]. Future studies may expand the stanozolol treatment period to 3 or more weeks to mimic typical athlete use, along with experimenting with different stanozolol doses and conditions. These newly developed methods can assist *in vivo* studies designed to further investigate the metabolism of stanozolol. Urinalysis can provide information on whether UGT substrates/inhibitors and deletion polymorphism in the UGT2B17 gene reduce the glucuronidation rate (phase II metabolism) of stanozolol and 3′-hydroxystanozolol, as impaired glucuronidation has been reported to reduce the urinary concentrations of AAS. It can be assumed that, owing to compromised urinary excretion, the serum levels of unconjugated stanozolol and 3′-hydroxystanozolol can get elevated [21]. Thus, potentially greater amounts of stanozolol and 3′-hydroxystanozolol will be available to get incorporated in hair. Hence, these methods can assist in investigating the potential application of hair analysis and serum analysis to provide complementary information when the urinary excretion of stanozolol and 3′-hydroxystanoozlol is impaired.

Table 8 Extraction recovery results of stanozolol and 3′-hydroxystanozolol from serum

Compounds	Concentration (ng/mL)	% Extraction recovery (N = 6)
Stanozolol	0.25 (LLOQ)	113.24
	2.5	101.92
	10	109.42
3-Hydroxystanozolol	0.25 (LLOQ)	95.36
	2.5	111.03
	10	105.29

Conclusions

To our knowledge, the detection of stanozolol and 3′-hydroxystanozolol in rat hair, urine and serum at such low concentration levels using LC-MS/MS, has been reported here for the first time. Using the newly developed methods presented here, future research can carry out *in vivo* studies to further investigate stanozolol

Table 9 Concentrations of stanozolol and 3′-hydroxystanozolol in rat hair, urine and serum samples

Matrix	Animal	Stanozolol	3′-Hydroxystanozolol	Ratio
Hair (pg/mg)				
	1	46.57 ± 0.17	10.96 ± 0.15	4.25
	2	47.25 ± 1.07	9.13 ± 0.12	5.18
	3	82.58 ± 2.67	16.92 ± 0.37	4.88
	4	77.98 ± 1.03	11.72 ± 0.30	6.65
	5	96.54 ± 1.02	16.29 ± 0.07	5.93
	Mean	70.18 ± 22.32	13.01 ± 3.43	5.38 ± 0.93
Urine (ng/mL)				
	1	5.20 ± 0.09	13.54 ± 0.43	0.38
	2	0.20 ± 0.01	0.36 ± 0.02	0.56
	3	0.17 ± 0.02	5.07 ± 0.01	0.03
	4	0.70 ± 0.01	8.51 ± 2.41	0.08
	5	15.41 ± 0.08	19.49 ± 0.22	0.79
	Mean	4.34 ± 6.54	9.39 ± 7.42	0.37 ± 0.32
Serum (ng/mL)				
	1	5.91 ± 0.00	7.25 ± 0.29	0.82
	2	4.69 ± 0.01	7.62 ± 0.27	0.62
	3	13.01 ± 0.01	8.76 ± 0.67	1.49
	4	5.24 ± 0.06	3.81 ± 0.27	1.38
	5	9.90 ± 0.03	8.38 ± 0.22	1.18
	Mean	7.75 ± 3.58	7.16 ± 1.97	1.09 ± 0.37

metabolism, thus making an important step towards understanding of the array of factors that may confound urinalysis results. Also, these methods can be extended by analysing human hair, urine and serum samples in tandem to provide a pattern of drug use and this can be useful for testing doping with stanozolol and other commonly abused AAS. Hair can provide retrospective information on an individual's drug use and this can be used in out-of-competition testing. However, information of current drug-use, if important, can be obtained by urine and blood serum analyses. Thus, when the three tests are used in combination, useful information on an individual's drug use can be obtained and false doping results can be prevented.

Additional file

Additional file 1: Figure S1. Calibration curves of stanozolol and 3'-hydroxystanozolol in (**a**) hair, (**b**) urine and (**c**) serum.

Abbreviations

AAS: Anabolic androgenic steroids; CID: Collision induced dissociation; FDA: Food and Drug Administration; GC-HRMS: Gas chromatography high resolution mass spectrometry; GC-MS: Gas chromatography mass spectrometry; ISs: Internal standards; LC-MS/MS: Liquid chromatography tandem mass spectrometry; LLE: Liquid-liquid extraction; LLOQ: Lower limit of quantification; LLOD: Lower limit of detection; MRM: Multiple reaction monitoring; QC: Quality control; RSD: Relative standard deviation; UGT2B17: Uridine diphosphate-glucuronosyltransferase 2B17; WADA: World Anti-Doping Agency.

Competing interests

The authors declare that they have no competing interests.

Authors' contribution

AP, JB and DPN initiated the study. GZ, NIKD and AP designed the study. GZ and ADSz conducted the animal experiments. The method development and sample analyses were conducted by NIKD who prepared the draft paper. All authors contributed to data analyses and to finalising the manuscript. All authors have read and approved the final version.

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SUBSTANCE ABUSE TREATMENT, PREVENTION, AND POLICY

METHODOLOGY Open Access

New non-randomised model to assess the prevalence of discriminating behaviour: a pilot study on mephedrone

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Abstract

Background: An advantage of randomised response and non-randomised models investigating sensitive issues arises from the characteristic that individual answers about discriminating behaviour cannot be linked to the individuals. This study proposed a new fuzzy response model coined *'Single Sample Count'* (SSC) to estimate prevalence of discriminating or embarrassing behaviour in epidemiologic studies.

Methods: The SSC was tested and compared to the established Forced Response (FR) model estimating Mephedrone use. Estimations from both SSC and FR were then corroborated with qualitative hair screening data. Volunteers (n = 318, mean age = 22.69 ± 5.87, 59.1% male) in a rural area in north Wales and a metropolitan area in England completed a questionnaire containing the SSC and FR in alternating order, and four questions canvassing opinions and beliefs regarding Mephedrone. Hair samples were screened for Mephedrone using a qualitative Liquid Chromatography-Mass Spectrometry method.

Results: The SSC algorithm improves upon the existing item count techniques by utilizing known population distributions and embeds the sensitive question among four unrelated innocuous questions with binomial distribution. Respondents are only asked to indicate *how many* without revealing *which* ones are true. The two probability models yielded similar estimates with the FR being between 2.6% - 15.0%; whereas the new SSC ranged between 0% - 10%. The six positive hair samples indicated that the prevalence rate in the sample was at least 4%. The close proximity of these estimates provides evidence to support the validity of the new SSC model. Using simulations, the recommended sample sizes as the function of the statistical power and expected prevalence rate were calculated.

Conclusion: The main advantages of the SSC over other indirect methods are: simple administration, completion and calculation, maximum use of the data and good face validity for all respondents. Owing to the key feature that respondents are not required to answer the sensitive question directly, coupled with the absence of forced response or obvious self-protective response strategy, the SSC has the potential to cut across self-protective barriers more effectively than other estimation models. This elegantly simple, quick and effective method can be successfully employed in public health research investigating compromising behaviours.

Keywords: random response technique, non-random model, Mephedrone, survey, illicit substances, epidemiology

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dards are in place to identify those who violate the anti-doping rules worldwide and to deter others from doing so [2,3]. The use of performance-enhancing substances and methods deemed to be prohibited is an anti-doping rule violation that is established by the presence of a prohibited substance or its metabolites or markers in the athlete's sample, typically urine or blood [1].

Aside from an ongoing ethical debate on various facets of doping [4], including moral reasoning [5] and medical ethics [6-9], recent critical analyses of the current anti-doping approach have claimed that the current anti-doping is not fit for purpose [10-13]. Reasons for this criticism encompassed ethical issues around constant surveillance [14,15], analytical difficulties and costs [16,17], as well as marked inter-individual differences [18-20].

Anabolic androgenic steroids (AASs) are synthetic derivatives of the endogenously produced male sex hormone, testosterone, which exhibits both anabolic (protein synthesizing) and androgenic (masculinising) effects. Steroids are one of the most potent and the most widely used performance-enhancing substances both amongst Olympic athletes [21-23] and also those outside of the auspices of the World Anti-Doping Agency (WADA), such as competitive and recreational body builders, professional players or even non-athlete adolescent boys [24,25]. The use of AASs is widespread particularly amongst athletes because such drugs can improve their performance in sports by accelerating muscle growth, increasing aggressiveness and enhancing a sense of well-being. Chronic use of AASs has been known to cause serious adverse effects such as virilization, feminization, liver disorders, neuropsychiatric disorders, adverse blood lipid profiles (increased LDL and decreased HDL), cardiovascular disorders and renal complications [26-28]. Among these, renal diseases have received less attention, most likely because renal disorders are infrequent among AAS users in comparison to other, more prevalent diseases. Throughout the last decade, the literature sporadically presented cases of severe renal disorders among AASs users, especially with elevated and prolonged use [29-34]. The number of incidents presented is well below the estimated number of AAS users, however, the user profiles described in these case studies do not differ significantly from those AASs abusers who do not develop renal complications. Thus, there may be a connection between deletion mutation in a steroid conjugating enzyme and occurrence of renal diseases with chronic use of AAS.

AASs are commonly excreted in urine mainly as glucuronide conjugates, the formation of which is catalyzed by various uridine diphosphate-glucuronosyltransferase (UGT) enzymes. The UGTs 2B7, 2B15 and 2B17 are found to be the principal enzymes involved in glucuronidation of androgens and their metabolites in humans. Glucuronidation of steroids and their phase I metabolites is an important detoxification and deactivation metabolic pathway which is catalyzed mainly by *UGT2B17* and to a minor extent by *UGT2B15* [35-38]. In 2008, a few months before the Beijing Olympics, Schultze and colleagues discovered that the occurrence of deletion polymorphism in the gene coding of *UGT2B17* enzyme affected the urinary excretion patterns of testosterone [18]. As the current doping testing regime relies on detecting the steroid metabolites in urine, *UGT2B17* deficient drug abusers are likely to test negative despite the use of the drug. Although the enzyme deficient athletes who are aware of their own genetic profile may benefit from evading doping testing, they may be vulnerable to serious health consequences due to inadequate deactivation and elimination of steroids. The potential consequences of this discovery has triggered the WADA and sport governing bodies to examine their testing regimes.

However, it is inevitable that this phenomenon has significant implications beyond the sporting arena. Juul et al. [39] observed in pubertal boys, that the homozygous deletion in the *UGT2B17* gene, in line with Schulze et al. [18], affected the urinary excretion pattern of androgen metabolites, but not circulating androgen levels. A deficiency of the *UGT2B17* enzyme decreases the rate of steroid deactivation and elimination, and owing to this, the bioavailability of steroids may be enhanced to a certain extent. Hence, elevated and prolonged use of AAS may predispose the enzyme deficient individuals to detrimental effects, particularly relating to kidney damage.

Therefore, in this paper we hypothesise that the observed renal disorders among AAS users is connected to the genetic profiles of these users and functional polymorphic deletion of the *UGT2B17* gene significantly increases the chance of developing kidney complications.

Inter-individual variations in the frequency of UGT2B17 gene deletion polymorphism

The occurrence of the *UGT2B17* gene varies amongst individuals of various ethnic groups. Wilson et al. carried out a study on African Americans and the Caucasian population, and found that the occurrence of deletion mutation in the *UGT2B17* gene was five time more frequent in Caucasians than in African Americans [40].

Several comparative studies of urinary steroid concentrations amongst various ethnic groups have been reported and it has been observed that individuals lacking this enzyme have negligible excretion of steroids. Sjöqvist et al. examined the association of androgen excretion with *UGT2B17* deletion in a population based study comprising of Korean and Swedish participants [22]. It was found that the absence of the *UGT2B17* gene was seven times more frequent in Koreans than in the Swedish population. On examining the association

between deletion polymorphism and urinary levels of androgens it was revealed that testosterone excretion was 16 times higher in Swedish people compared to the Koreans. These findings indicate the importance of this gene in the excretion of steroids.

Baume et al. reported inter-individual variations in the excretion patterns and kinetics of nandrolone and its metabolites after administration of [^{13}C] nandrolone to volunteers and noted the possible natural production of nandrolone and its metabolites [20]. However, it can be postulated that the variations in nandrolone excretion could be due to variations in *UGT2B17* genotypes amongst individuals.

According to WADA guidelines, if the ratio of concentrations of testosterone to epitestosterone glucuronide in urine is greater than 4, then drug doping is suspected [41]. Since deletion polymorphism in the gene coding for the *UGT2B17* enzyme affects the urinary level of testosterone, the accuracy of the T/E ratio test is challenged. A study carried out on a heterogeneous group of healthy volunteers with different *UGT2B17* genotypes (*ins/ins*, *ins/del* and *del/del*) reported that administration of exogenous testosterone to individuals lacking the *UGT2B17* gene did not yield T/E values above the population based threshold of 4 for all individuals [18]. This is because the testosterone glucuronide excretion rate in individuals with *del/del* genotype was found to be significantly less than those carrying the *ins/del* and *ins/ins* genotype. No significant effect on epitestosterone excretion was observed. The excretion of unconjugated steroid was found to be a minor elimination pathway even in individuals devoid of the gene. This indicates the possibility of an increase in serum levels of biologically active steroids. It has been reported previously that individuals of Asian ethnicity excrete less testosterone which complements the surveillance that the *del/del* genotype is more frequent amongst the Asian population [18]. Many researchers have observed similar difficulty in testing testosterone abuse pertaining to the *UGT2B17* deletion, and have suggested making use of genotype based cut-off levels to overcome inter-individual and inter-ethnic variations [42].

These results indicate that a deletion polymorphism in the gene coding is associated with urinary levels of steroid glucuronide conjugates. Owing to an impaired glucuronidation pathway, deactivation and elimination of steroids is reduced. This may result in elevated serum levels of active steroids which can be harmful over a long period of time. Thus, specific groups of individuals who are devoid of the *UGT2B17* gene are more susceptible to adverse health conditions due to chronic exposure to elevated doses of steroids, compared to individuals having the gene.

Concomitant use of drugs

UGTs not only contribute to AAS glucuronidation but they also act as conjugating enzymes for various other pharmaceutical drugs [35,43]. It has been reported that athletes use AASs in combination with other medications which may enhance the AAS effects and decrease the side effects associated with such performance-enhancing drugs. Co-administration of steroids along with other UGT substrates may lead to competitive inhibition of steroid glucuronidation. Sten et al. discovered that commonly used over-the-counter (OTC) non steroidal anti-inflammatory drugs (NSAIDs) like diclofenac and ibuprofen inhibited the testosterone glucuronidation activity of *UGT2B17*, *UGT2B15* and some other UGTs that have previously shown low but detectable activity [43]. Compared to *UGT2B17*, *UGT2B15* was found to be more sensitive to both the NSAIDs, particularly ibuprofen. Since, *UGT2B17* shares 96% homology with *UGT2B15*, in a *UGT2B17* deficient individual, *UGT2B15* may be the major contributor to glucuronidation. However, use of steroids together with NSAIDs will competitively inhibit the steroid glucuronidation activity of *UGT2B15*. The inhibitory effect was also found to be dependent on the *UGT2B17* genotype. Regular use of such painkillers is common amongst athletes in order to overcome the pain associated with extensive exercise regimes [44]. Thus, athletes devoid of the *UGT2B17* gene are susceptible to having complications in steroid elimination, as well as to other medications administered simultaneously. Also, such drug combinations can have direct deleterious effects on renal health.

The occurrence of renal disorders among AAS users

Herlitz and colleagues reported a study on a cohort of bodybuilders (white and hispanic) and observed that long term use of anabolic steroids combined with high protein intake was associated with proteinuria and secondary focal segmental glomerulosclerosis (FSGS). The pattern of glomerular injury observed, involves scarring of the glomerules and is mediated by elevated glomerular filtration rate (hyperfiltration), glomerular pressure and other adaptive structural-functional responses within the kidneys [29]. One of the bodybuilders with a history of occasional urinary tract infection progressed to end stage renal disease (ESRD) after prolonged use of AASs. It has been reported that non obese individuals with increased body mass index (BMI) owing to elevated muscle mass are susceptible to developing secondary FSGS [29,45]. This indicates the possibility of developing serious renal conditions after prolonged use of steroids by the gene deficient individuals. Many researchers have also reported other adverse renal conditions such as acute kidney injury (AKI), nephropathy, diffuse type 1 membranoproliferative glomerulonephritis and also renal fail-

ure owing to long term use of AASs [30,31]. In all these case studies, improvements in renal conditions have been observed in patients who discontinued the use of anabolic steroids. Also, acute renal failure has been reported along with cholestatic liver damage after long term use of AASs [32-34]. Disorders in the liver, the major site for detoxification of steroids and other xenobiotics, can hinder its performance and hence worsen the renal conditions due to elevated level of circulating toxins.

Presentation of the hypothesis

We hypothesize that with chronic and/or excessive use of AAS, individuals with a deletion polymorphism in the *UGT2B17* gene (*del/del*) carry an increased risk of developing renal disorders owing to an increase in body mass index and possible direct toxic effects of steroids on the kidneys. Inadequate elimination of the biologically active steroids will lead to elevated serum levels and cause surplus increase in muscle mass. An increase in body mass index may cause renal injuries due to sustained elevated glomerular pressure and flow rate.

The fate of anabolic steroids in UGT2B17 deficient individuals

Once in the body, plasma protein binding and enzymatic conversions of AASs regulate the availability of free and active steroids at local target sites. Depending on the chemical structure, steroids are deactivated primarily in the liver by phase I (e.g., oxidation, reduction, or hydroxylation) and phase II (glucuronide or sulphate conjugation) metabolic reactions [46]. Glucuronidation by UGTs is considered to be the major deactivation and elimination pathway for steroids and their phase I metabolites. The UGTs act by catalyzing the transfer of glucuronosyl group from the uridine 5'-diphosphoglucuronic acid to the steroid molecule. The resulting glucuronide drug conjugates are less toxic, more polar, and hydrophilic in nature and get easily excreted from the body in urine. The 3 α- and 17 β-OH positions of C-19 steroids and their metabolites are the major sites involved in glucuronide conjugation [35].

UGT2B17 catalyses glucuronidation at both oxygens whereas *UGT2B15* acts only at the 17 β-OH position, hence it is less efficient than *UGT2B17* in the elimination of steroids. It can be argued that in individuals devoid of the *UGT2B17* gene, other UGTs like 1A1, 1A3, 1A4, 1A8, 1A9, 1A10, 2B4, 2B7, and 2B15 may contribute to steroid glucuronidation [43,47]. But, less than normal production of steroid glucuronides in individuals devoid of the *UGT2B17*gene indicates inadequate efficiency of other UGTs in eliminating steroids. Alternatively, it may be expected that in order to compensate for impaired glucuronidation activity, steroids may be excreted as sulphate conjugates. However, it was determined by Borts and Bowers that individuals with *del/del UGT2B17* genotypes did not produce more than normal amounts of steroid sulphate [48,49]. Also, the excretion of unconjugated steroids in urine is considered to be a minor elimination pathway, even in *del/del* individuals [18]. Thus, it can be postulated that lipophilic, unconjugated steroids in the systemic circulation may get distributed into body tissues, get incorporated into the hair by endogenous route or get excreted in sweat [50,51]. Drugs excreted in sweat can further get absorbed by hair via endogenous-exogenous pathways.

Since glucuronidation is the major pathway for deactivation and elimination of steroids, *UGT2B17* deficiency may increase the serum level of biologically active steroids and their metabolites. Thus, it can be postulated that in *UGT2B17* deficient individuals, chronic exposure to AAS can result in high BMI and circulating levels of proteins, relative to individuals with a functional enzyme. Increase in body mass can force the kidneys to elevate the glomerular filtration rate (hyperfiltration) and glomerular pressure. Adaptive responses to sustained hyperfiltration and elevated glomerular capillary pressures can lead to the development of renal injuries like scarring of glomerules, which may be associated with proteinuria due to elevated circulating proteins. Hence, we postulate that *UGT2B17* gene homogeneous polymorphism (*del/del*) increases the chance of developing renal disorders with prolonged use of AAS due to increase in BMI and direct toxic effect of steroids on kidneys.

Testing the hypothesis

As the hypothesis is based on the dysergistic effect of the *UGT2B17* polymorphism and long term exposure to AAS, a single clinical trial approach does not present a feasible way to generate evidence to support or refute the hypothesis. Therefore an alternative, multi-component approach is proposed with three distinct components: i) delineation of single dose pharmacokinetics, ii) animal model studies, and iii) genotype testing in humans suffering from renal failure.

To delineate single dose pharmacokinetics for commonly abused AAS, a population based study can be carried out which involves the administration of a single dose of a [^{13}C] labelled AASs to healthy volunteers with different *UGT2B17* genotypes. Routine blood tests, biofluid analyses, urinalysis and hair analyses [50,51] will ascertain the deviation in the pharmacokinetics of steroids owing to the prevalence of different *UGT2B17* genotypes. Thus, the circulating levels of active steroids (plus phase I metabolites) and alternative metabolic pathways of steroids in individuals with impaired glucuronidation activity can be determined.

An animal study involving chronic administration of AASs to mice or rats with different *UGT2B17* genotypes,

accompanied with routine examination of renal function will help in scrutinizing the relationship between development of renal disorders and deletion polymorphism in the gene. In addition to affording AAS induced renal impairment investigations, animal models will facilitate controlled comparative pharmacokinetic studies of short and long term AAS exposure in mice. In addition, these data will allow comparison to the pharmacokinetics from the human single dose study.

For clinical observations, patients with renal impairment after long term use of anabolic steroids should be analysed for genotype using real time PCR with allelic discrimination to determine the prevalence of *UGT2B17* deletion genotypes (*del/del, ins/del* and *ins/ins*). Studies would also involve the evaluation of other UGTs which have been reported to play a role in AAS glucuronidation to accurately estimate the impact of each phenotype [52]. Thus, prospective or retrospective genotype analysis of tissues from patients undergoing clinical treatments for renal impairment with admitted steroid abuse are recommended. In addition, depending on the pharmacokinetic results from study i), analysis of hair samples could confirm cases of self-reported chronic AAS abuse.

Implications of the hypothesis

A system that is primarily based on detection-based deterrence and sanctioning may be easily perceived as a barrier to overcome if a competitive edge can be secured by using performance-enhancing substances or methods. Thus, the current repressive approach to anti-doping inevitably leads to finding ways in detection evasion, with health concerns being secondary. In this 'nothing or all' system, the harm reduction approach that characterizes many drug prevention programmes, cannot be accommodated.

Following the paper by Schulze and colleagues [18] on the effect of *UGT2B17* gene deletion polymorphism on doping tests, it is likely that this discovery is turned into practice and abused by athletes determined to use AAS. In this scenario, athletes believe that they take the known risk associated with AAS use, but not the risk of being caught. However, if the hypothesis is correct, these athletes expose themselves to an increased risk in developing renal disorders as a consequence of their *UGT2B17* gene deletion polymorphism.

The harm reduction approach to doping, not yet official but increasingly advocated among doping researchers [13,53], would benefit greatly from an empirical verification of the assumption presented in this paper. The potential danger present in this connection, a controlled clinical trial with volunteers is not feasible but primary care physicians and nephrologists are in a favourable position to advance our knowledge in this area.

Although it has been recognized by now that education based anti-doping prevention is a challenging task [54], having objective information is the *sine qua non* of all efforts relying on informed choice. This is not to advocate the less effective loss-framed messages [55] or fear appeals [56] in preventing AAS use, but rather to draw attention to the fact that inter-individual variations in metabolizing steroids could lead to serious health detriments in individuals even at doses below the problematic supraphysiological doses.

Groups that may benefit from this research are not only AAS users who deliberately take advantage of their unique genetic make-up to evade doping testing, hence will face increased risk of developing renal complications, but include other AAS users, such as recreational users and bodybuilders unaware of the risk, who may inadvertently subject themselves to risks of developing kidney disease. Beyond the athletic arena, administration of AAS should be avoided for patients with nephritic symptoms as it may worsen renal conditions. AAS have been found to be beneficial in improving conditions of individuals with muscle wasting disorders. Researchers have observed improvement in lean body mass of individuals with HIV and chronic obstructive pulmonary disease (COPD) after administration of AAS [27]. Thus, AAS, if not misused, can have therapeutic applications. However, patients that are devoid of the gene will be more vulnerable to serious health consequences. As pharmacokinetic investigations are a key part of the proposed hypothesis testing studies, additional information will be generated on inter-individual differences which may have implications beyond the hypothesis. In such situations, alternative treatment should be employed or a genotype based safe dose should be identified.

List of abbreviations

AASs: Anabolic androgenic steroids; AKI: Acute kidney injury; BMI: Body mass index; COPD: Chronic obstructive pulmonary disease; ESRD: End stage renal disease; FSGS: Focal segmental glomerulosclerosis; HDL: High density lipoprotein; HIV: Human immunodeficiency virus; LDL: Low density lipoprotein; T/E: Testosterone to epitestosterone ratio; UGT2B15: Uridine diphosphate-glucuronosyltransferase 2B15; UGT2B17: Uridine diphosphate-glucuronosyltransferase 2B17; WADA: World Anti-Doping Agency

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AP conceived the study. All authors have contributed equally to formulating the hypothesis. ND, AP and DPN have drafted the paper. All authors have read and approved the final version of the manuscript.

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SUBSTANCE ABUSE TREATMENT, PREVENTION, AND POLICY

METHODOLOGY

Open Access

New non-randomised model to assess the prevalence of discriminating behaviour: a pilot study on mephedrone

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Abstract

Background: An advantage of randomised response and non-randomised models investigating sensitive issues arises from the characteristic that individual answers about discriminating behaviour cannot be linked to the individuals. This study proposed a new fuzzy response model coined *'Single Sample Count'* (SSC) to estimate prevalence of discriminating or embarrassing behaviour in epidemiologic studies.

Methods: The SSC was tested and compared to the established Forced Response (FR) model estimating Mephedrone use. Estimations from both SSC and FR were then corroborated with qualitative hair screening data. Volunteers (n = 318, mean age = 22.69 ± 5.87, 59.1% male) in a rural area in north Wales and a metropolitan area in England completed a questionnaire containing the SSC and FR in alternating order, and four questions canvassing opinions and beliefs regarding Mephedrone. Hair samples were screened for Mephedrone using a qualitative Liquid Chromatography-Mass Spectrometry method.

Results: The SSC algorithm improves upon the existing item count techniques by utilizing known population distributions and embeds the sensitive question among four unrelated innocuous questions with binomial distribution. Respondents are only asked to indicate *how many* without revealing *which* ones are true. The two probability models yielded similar estimates with the FR being between 2.6% - 15.0%; whereas the new SSC ranged between 0% - 10%. The six positive hair samples indicated that the prevalence rate in the sample was at least 4%. The close proximity of these estimates provides evidence to support the validity of the new SSC model. Using simulations, the recommended sample sizes as the function of the statistical power and expected prevalence rate were calculated.

Conclusion: The main advantages of the SSC over other indirect methods are: simple administration, completion and calculation, maximum use of the data and good face validity for all respondents. Owing to the key feature that respondents are not required to answer the sensitive question directly, coupled with the absence of forced response or obvious self-protective response strategy, the SSC has the potential to cut across self-protective barriers more effectively than other estimation models. This elegantly simple, quick and effective method can be successfully employed in public health research investigating compromising behaviours.

Keywords: random response technique, non-random model, Mephedrone, survey, illicit substances, epidemiology

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Background

Outcome based evaluation of interventions, which play a central role in public health prevention, need to show the effect the policy or intervention makes at the public level. Whilst a plethora of literature focuses on evaluating various social marketing campaigns that tackle public health and safety issues such as drug use, health compromising lifestyle choices, unprotected or risky sexual behaviour, or unsafe driving practices, tend to rely on self reports, regardless of whether or not they were conducted in laboratory or field settings [1-3]. The issues that may hinder an evaluation of any health promotion [4] are further complicated by the influence of social desirability that may cast doubt over the validity of self-reported information when to the study topic relates to socially sensitive behaviour [5]. In addition to public health concerns where obtaining accurate information on drug use is vital in establishing the need for and to evaluate preventive measures or intervention strategies, policy makers in public service utilities and law enforcement agencies also require the most accurate estimates of the problematic behavioural choices as possible in order to make informed choices.

The need to obtain the maximum intelligence on health related behaviours stems from the necessity to develop and deploy optimal intervention measures to counteract consistent failures to attain acceptable levels of behaviour across a wide range of health practices. These range from adherence to medication, resistance to addiction, avoidance of exploration of social drug use through to uptake of illegal and health damaging performance enhancement agents. The immense health, financial and social consequences of enhancing these health related behaviours has led to decades of investigation into improved approaches to obtain accurate data on sensitive personal behaviours.

Investigating the epidemiology of socially sensitive or transgressive behaviours such as illicit drug use, unhealthy weight management practices, risky behaviour, cheating, doping or non-adherence to prescribed medication or treatment, is hindered by respondents evasively answering questions about sensitive behaviours [6]. A recent research programme provides further evidence for self-protective strategic responding, even under anonymous answer conditions [7-9]. Consequently, much effort has been made to develop reliable methods to collect valid epidemiological data in these sensitive behavioural domains.

Approaches range from techniques such as the Bogus Pipeline [10] to providing incentives for honest answers such as the Bayesian Truth Serum (BTS) [11]. Whilst the Bogus Pipeline has been used for decades and accumulated reasonable evidence that the BPL shifts self-reports toward veracity [12], the BTS approach is relatively new and in need for further refinements [13,14]. Based on empirical evidence, Barrage and Lee [13] also suggest that to be effective, respondents may need to have a positive experience with and trust in the BTS method, which can lead to respondents learning how to maximise their incentives and therefore their answers might be biased towards maximum income at the expense of telling the truth. Although these methods possess the potential to overcome to an extent, self-protective response bias by either evoking fear of exposure of lying or providing financial gain for truthfulness, their feasibility in self-administered epidemiological scale studies appears to be compromised. An alternative approach has made notable progress in collecting data on sensitive behaviours through the development of indirect methods using randomisation or deliberate uncertainty to provide respondent protection over and above ensuring anonymity [6].

The concept behind randomised response models (collectively termed RRT) rests on introducing a randomising element to the survey question by using some device (e.g. by rolling a dice, flipping a coin or picking a card) which determines how the respondent should answer [15]. Since the researcher has no control over this randomising device, answers cannot be directly traced back to any particular individual, which in turn heightens the respondents' sense of increased protection. A common characteristic of RRTs is that to obtain useful data on the sensitive question, the technique requires respondents to answer directly, in some form, the sensitive question. By contrast, non-random models (NRM) do not require a direct answer as they rely on implicit uncertainty rendering impossible the link between an individual and the sensitive behaviour. Whilst NRMs build on combining the sensitive question with unrelated innocuous questions, some RRTs also incorporate innocuous questions where the population prevalence may or may not be known. When population prevalence needs to be established, it requires an independent sample randomly selected from the same population.

Randomised response models

The RRT aims to elicit sensitive, embarrassing or compromising information that may portray respondents unfavourably. The common characteristic of the RRT is that sensitive behaviour estimation can only be made at the aggregated population level. The method is based on the principle introduced by Warner [16] using a spinner as a randomising device to gauge the proportion of the sample with a compromising behaviour. The method assumed that any person in a sample is either characterised by the behaviour (group A) or is not (group B). The respondents, hidden from the interviewer, were asked to use the spinner which either landed on group

A or on group B and answer with a simple 'yes' or 'no' depending on whether the spinner pointed to the group he/she belonged to. Whilst the outcome of the spinner exercise for each individual was not known to the interviewer, hence protecting the individual, the chance that a spinner points to group A or B was known (p and $1-p$). Thus compared to the observed pattern of 'yes' and 'no' answers Warner was able to determine the proportion of respondents in the sample admitting the sensitive behaviour.

Subsequent adaptations of the RRT have covered a wide range of sensitive issues along with numerous attempts to refine the approach [15]. Among the wide array of models, the Forced Alternative/Response model, used only when the sensitive question is presented [16], has been found to be one of the most efficient variants of Warner's original conception [17]. Recently the RRT method has been expanded to multi-item scales and tested with male date rape attitude [18] and alcohol abuse [19]. The extension of the RRT to multi-item scales allows its application to psychological measures such as attitudes toward sensitive issues. This approach can be expanded to areas where honest responding might be compromised by self-protective lying, for example illegal substance dependence, domestic violence, disordered eating or cheating and doping use in sport.

Non-randomised methods

Research has shown that whilst respondents understand the reason behind the use of the RRT approach in surveys, they generally find it obtrusive and favour simpler approaches [20]. Contrary to the RRT, non-random models present a more straightforward approach that provides protection by asking the number or combination of behaviours respondents are engaged in rather than asking about each behaviour in turn.

The non-randomised model (NRM) has received increased attention lately. A recent review [21] showed that NRMs appear to successfully address many of the limitations typically associated with RRTs such as the need for a randomisation device which often requires interviewers; forcing participants to say 'yes' to an embarrassing question when their honest answer would be the opposite or requiring a direct answer to the same question. Contrary to the RRT, in the NRM every participant is required to answer the research question in an evasive way. The fact that a response is required to the research question can help participants to feel that they have made a contribution by volunteering to take part in the research whereas with many RRT variations, a significant proportion of respondents are simply instructed to ignore the research question and just say 'yes' or 'no'. Owing to this characteristic, NRMs can also be more efficient with comparable or even increased privacy protection levels.

Alternative approaches have been progressively developed which preclude the need for the randomising device. These include an item count method [22], later termed the 'item count technique' [19] and later the 'unmatched count technique' [23]. In a similar concept to the unrelated question (UQ) method [16,24], item counts (IC) utilise a simple response task whilst embedding the sensitive question in a list of innocuous questions. In place of the randomising device the experimental group receives all questions with instruction and are asked to indicate only the number of affirmative answers. As a control sample is required to establish the population prevalence of the innocuous questions, respondents are randomly assigned to one of two groups (experimental and control), where the control sample receives the identical list of questions minus the sensitive question. The mean number of 'yes' responses are compared between the two groups. Assuming that the innocuous behaviour is equally manifest in both groups, the difference between the observed proportion of 'yes' answers must be due to the presence of the sensitive question in one of the groups and not the other.

Using prior knowledge of the population prevalence for an innocuous question, has led to the development of a number of competing techniques over the past five years. In these models, the innocuous question is outside the researcher's control, independent of the research question but the population prevalence is already established such as birth month or season, geographical location for the person or a family member. The Triangular Model (TM) and the Crosswise Model (CWM) use a combination of a sensitive and an innocuous question with known population prevalence [25]. The question and answer options are then placed in a 2 × 2 contingency table where two 'quadrants' relate to the innocuous questions are with known population prevalence (e.g. 3/12 and 9/12 if someone's birth month is used as the innocuous question). The other two quadrants represent the binomial response options to the sensitive question. In the TM respondents are asked to indicate whether they belong to the No-No quadrant or any of the other three quadrants (Yes-No, Yes-Yes or No-Yes). The CWM asks people to indicate whether they belong to any of the mixed categories (Yes-No and No-Yes) which only reveals that *one of the two* statements is true but *which* one remains hidden. Similarly, the Hidden Sensitivity (HS) model for two sensitive questions with binary outcomes using one quadrant such as season for birthday or geographic location (e.g. South/West/North/East, East/West side of a river or any criteria that creates meaningful and useable groups)

[26]. In this technique two response pathways are provided. Respondents are required to either answer truthfully or are forced to an option for the non-sensitive question (e.g. about birth date or place of living) based on their answers to the two sensitive behaviours. The drawback of this technique is that only those who belong to the category of not having a sensitive behaviour (0,0) are asked to answer the innocuous question honestly, whereas others (0,1; 1,0; 1,1) are forced to select an answer for the innocuous question based on their sensitive behaviour. Therefore, people admitting to a sensitive behaviour (or both) are protected by the true answers of those who do not have a sensitive behaviour to declare. The advantage of the HS model over the Triangular or Crosswise models is that HS allows two sensitive questions to be simultaneously investigated [27].

Other models such as the Unmatched Count Technique (UCT) [28] or the Cross-Based Method (CBM) and the Double Cross-based Method (DCBM) [29] work with unknown population prevalence. The common characteristic of these models is that an independent sample randomly drawn from the same population is required to establish the prevalence rate for the innocuous questions in order to estimate the prevalence rate for the sensitive question. The UCT [28] contains two parallel questionnaires with several innocuous questions but only one version of the questionnaire features the sensitive questions. The total number of endorsed answers is calculated for each version independently, and then compared. The difference between the two sample means indicates the proportion of the respondents who endorsed the sensitive question.

Currently, studies comparing the performance of the item count method to other NRM or RRT models, or direct self-reports, are inconclusive. Coutts and Jann [28] found that the UCT outperformed the RRT counterparts in assessing many sensitive behavioural domains. By contrast, Tsuchiya et al. [30], using a web-based survey, compared the item counts to direct self reports and concluded the item count technique yielded lower numbers of endorsed behaviour. However, Tsuchiya's [30] list of behaviours contained items to which over-reporting can reasonably be expected (e.g. donating blood), which might have skewed upwards the total numbers of reported behaviours in direct self-reports. Where differences were found between self-reports and item counts (using CBM and shoplifting) the differences were explained by the sample demographic. The largest difference was found among the middle-aged, domiciled in urban areas and highly-educated (e.g. in or completed tertiary education) female respondents [30].

Practical issues

Constraints of each approach were associated with whether or not the population prevalence used for the non-sensitive questions was known. When this information is not available, the research requires an independent sample of significant size to establish this, parallel to collecting a sample to answer the research question about some sensitive issue. Furthermore, the chosen probability that requires respondents to answer truthfully determines the proportion of the sample that is directly useable to answer the research question. Finally, the actual prevalence rate of the target behaviour also has an effect on the minimum required sample size.

Investigating the efficiency of the RRT, Lensvelt-Mulders et al. [17] compared five RRT methods and found the Forced Response method and a special from of the Unrelated Question design the most efficient requiring about 2.2 times the sample size required of a direct self-report method. Sample sizes for the Crosswise model were estimated for a number of combinations of power and population prevalence [31] where estimates for minimum required sample sizes ranged between 2.5 and 19.3 times the sample size required for direct questioning surveys. Based on these simulations, the Crosswise model's efficiency compared favourably to Warner's [32] model.

An alternative way to think about efficiency is to consider the proportion of the population sample solely used to provide an estimate of the population prevalence for the non-sensitive questions. This 'waste', which accompanies most models, is the acceptable efficiency cost of providing the added anonymity. The proportion of the sample inefficiency ranges between 25% and 75%, depending on the research design. Consequently, in order to achieve a sample size with sufficient statistical power for meaningful analysis there is a requirement for more extensive data collection than in a typical survey.

Aims

The recent change in legal status (in the UK) of the drug Mephedrone provided an opportunity to explore a novel approach to data collection on a sensitive issue. Mephedrone is a central nervous system stimulant that produces effects similar to amphetamines. It produces a euphoric effect, and has been reported to increase empathy, stimulation and mental clarity, but can lead to adverse effects such as nasal irritation, tachycardia and restlessness [33]. Although limiting in scope (i.e. we asked about the use of one specific drug), Mephedrone was a topical choice at the time of the study's conception as it had been reclassified as a Schedule 1 Class B drug on April 16th 2010 [34], making it unlawful to possess, produce, and/or distribute without licence and carrying a five year prison sentence for possession and up to 14 years for producing, selling or distributing. The ban generated considerable debate, with some expressing discontent about the hastened reaction and the

generic ban [35] along with a concern that the ban may not stop Mephedrone use, but could make the demand and supply clandestine, leading to unintended consequences from the addition of toxic excipients (through "cutting" or chemical by-products) and thus present an even greater danger to health [36]. In spite of the new legislation, internet retailers appear to have continued to sell products under different brand names that contain, albeit unlabelled, Mephedrone-like substances [37]. This case is a good illustration of the situation when the change in regulation could (and should) have been supported with at least an estimation of what proportion of the population uses Mephedrone and is at risk.

Recent inter-disciplinary approaches to estimating doping prevalence in sporting sub-populations has led to advances in estimation through improved efficiencies [38]. The current study aimed to develop and test a new research tool for use at the epidemiological scale. To achieve this aim, a fuzzy response model, Single Sample Count (SSC), was proposed.

Methods

The study utilised a mixed design questionnaire method with chemical analysis of hair samples collected from the questionnaire respondents. This approach has been successfully employed in research investigating social cognitive factors in prohibited performance enhancing and illicit drug use [7,8].

To establish validity and reliability, the SSC was compared to an established RRT model, the Forced Response (FR), estimating Mephedrone use in a three-month period preceding the data collection. Estimations from both SSC and FR were then corroborated with qualitative hair analysis. Ethical approval was obtained from the two HEIs' Research Ethics Committees. Data were collected in two sites: a rural area in north Wales (51.3% of the surveys; 92.8% of the hair samples) and a metropolitan area in England (48.7%) from 318 volunteers (mean age 22.69 ± 5.87, 59.1% male). Of the 153 hair samples, 95 (61.7%) were donated by males. The majority of the data (91.5% of the questionnaires and 92.2% of the hair samples) were collected in May-June 2010, capturing the period in which Mephedrone has become a controlled substance in the UK. The remaining samples were collected up to February 2011.

Measures

Along with the newly developed SSC, the questionnaire consisted of an established RRT, the Forced Response model [16], incorporated into the questionnaire in alternating order to mitigate any potential learning or priming effect, and always separated by four single questions evaluating the respondents' understandings and social projection of Mephedrone among a student population. To establish prevalence of recent use, the sensitive question asked respondents to indicate whether they have used Mephedrone in the last three months. One-hundred and fifty-three (48.43%) of the questionnaire respondents were asked to provide a hair sample for Mephedrone analysis to determine the drug's use over approximately three months prior to the study survey.

Forced Response model

The FR method has been shown to be one of the most efficient designs [15,17] and was consequently considered suitable as a validation tool for the new method. This variation of the FR [39] requires a pair of ordinary D6 dice. Respondents were instructed to shake the die in an opaque container in order to hide the score from all other observers and then to answer the following question 'Have you used Mephedrone in the previous three months? (Yes/No)' according to the outcome. If the combined score from the two dice is

- 2 - 4 = ignore the question and tick the 'Yes'
- 11, 12 = ignore the question and tick 'No'
- 5-10 = answer the question truthfully by ticking either 'Yes' or 'No'

As for scores 5-10 there are more variations (27/36) for suitable dice outcome than for scores 2-4 (6/36) or 11 and 12 (3/36), theoretically 75% of the respondents were instructed, by chance, to answer the target research question honestly.

Additional questions

In addition, three questions were included to gauge directly reported opinion, belief about health hazards and social projection. The questions were:

- In your opinion, should Mephedrone be a controlled substance? (Yes/No)
- What percentage of students in the UK do you think use Mephedrone (0% = nobody, 100% = everybody)? (Yes/No)
- On a scale of 1 (not harmful at all) to 10 (very harmful), how harmful do you think Mephedrone is for your health?

These questions were also used to establish that the two samples collected at different locations differed significantly.

Analyses

Statistical analyses

Prevalence rates for the last three months were estimated using model specific formulae (detailed below). Testing sample means against the pre-set value was performed using single sample t-tests. The 95%CI for the binomial distribution was calculated using the Wilson

interval. Simulations for establishing the required sample size for the SSC model were performed using varying levels of prevalence rates. Statistical analyses were performed using PASW 18.0, R and Minitab.

Hair analysis

Hair samples were screened for the presence of Mephedrone using a qualitative method developed in-house, Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS). Sample preparation included enzymatic digestion to preserve the drug and liquid-liquid extraction as detailed below. All solvents/chemicals apart from Mephedrone and Mephedrone -d3 were of analytical or general purpose reagent grade and purchased from Sigma-Aldrich UK Ltd (Gillingham, Dorset, UK). Mephedrone and Mephedrone-d3 were purchased from LGC Ltd, (Teddington, Middlesex, UK). Mephedrone-d3, the triply deuterated form is used as a standard reference for mass spectrometric measurements.

Hair digestion Hair (50 mg) was cut into fine segments and Cleland's Reagent (100 mg) was added followed by the addition of the enzyme Proteinase K (15 mg). Internal standard Mephedrone-d3 (100 μL) with 5 ng total concentration was added to the mixture and finally incubated with Tris buffer (1 mL) for 2 hours at 37.5°C with constant stirring.

Liquid Liquid Extraction (LLE) The digested hair solution was then placed in a centrifuge tube for Liquid-liquid extraction with hexane (3 mL). The contents of the tube were mixed using a vortex mixer and centrifuged for 5 min at 1750 × g. The top layer was decanted using Pasteur pipettes and placed in a glass test tube. The extracted samples were dried completely with nitrogen gas and reconstituted with 100 μL acetonitrile.

Qualitative analysis Qualitative analysis was carried out using a Thermoscientific liquid chromatography - tandem mass spectrometry (LC-MS/MS) system (Fisher Scientific UK Ltd, Loughborough, Leicestershire, UK). Three microlitres of reconstituted sample solution were injected into an Agilent SB-C18 column (Agilent Technologies UK Ltd, Wokingham, Berkshire, UK), (maintained at 45°C) for analysis. Acetonitrile (with 0.1% v/v formic acid) and water were used as mobile phase solvents. Total flow rate through the column was 200 μL/min. The LC mobile phase gradient composition is detailed in Table 1.

The mass spectrometer was operated in selective reaction monitoring (SRM) mode to confirm the presence of Mephedrone. One precursor > two product ion transitions for Mephedrone (m/z = 178.1 > 160.1, 145.1) and Mephedrone-d3 (m/z = 181.2 > 163.2, 148.2) were monitored for qualitative analysis. The retention times for Mephedrone and Mephedrone-d3 were found to be 1.68 and 1.92 minutes, respectively. The calibration curve of Mephedrone was found to be linear in the range 1 ng/mL to 80 ng/mL (Lower limit of detection 0.5 ng/mL). Qualitative analysis of 154 hair samples was carried out using this calibration curve. Blank (control) hair without any Mephedrone was analysed to detect any artefact peaks that might elute at the same retention time or have similar isobaric transitions and thus lead to false results. However, no such interferences were observed. Thus, retention time and the most abundant SRM transitions were used to qualitatively determine the presence of Mephedrone.

Sampling

Respondents completed either version of the question in randomly allocated order, separated by three questions soliciting responses to social projection and opinions to the target drug. Participants were recruited at universities and social spaces such as clubs and sport grounds outside the higher education institutions in the UK.

Respondents were approached by a data collector (two in total, one in each study region). The participation was voluntary. Participants who provided hair samples received a small monetary compensation (value of £5) for any inconvenience incurred in completing the survey. A hair sample was requested from each respondent upon completion of the questionnaire survey. Approximately half of the respondents provided usable hair samples. The exclusion criteria included treated (e.g. dyed or permed) or too short hair (less than 3 cm). Over 80% of the hair samples were dark in colour. The different sample sizes are owing to 70 volunteers receiving a 4-question (four innocuous questions only) version for the Single Sample Count (data not shown).

Table 1 LC-MS Methods for Mephedrone-d3

LC run time (min)	Acetonitrile in presence of (0.1% Formic acid)	Water (%)
0	60	40
3	100	0
4	100	0
5	60	40
10	60	40

Retention time (min)	Lower Limit of Detection (ng)	Flow rate (μL/min)	Injection volume (μL)	Column Temperature (°C)
1.92	0.5	200	3.0	45

Results

Results from the survey

Using the full dataset (n = 318), no gender*region interaction effect was observed in social projection (F(1,310) = 1.547, p = 0.211; partial eta^2 = 0.004) or in perceived harm (F(1,308) = 1.242, p = 0.266; partial eta^2 = 0.005). Participants in the metropolitan area gave significantly higher estimates for others using Mephedrone (F(1,310) = 16.90, p < 0.001) but no difference was evidenced by gender (F(1,310) = 0.506, p = 0.478; Cohen's d = 0.100). The main effect for gender and region in perceived harm was significant (F(1,308) = 5.237, p = 0.023; F (1,308) = 5.000, p = 0.026, respectively). The slight discrepancies in sample sizes are due to missing values. Means and standard deviations by area and gender are shown in Table 2. The opinion regarding the legal status of Mephedrone overwhelmingly favoured control (81.7%), independent of area (Fisher's Exact Test = 2.104, p = 0.370) but not of gender (Fisher's Exact Test = 7.731, p = 0.011), with the preference for non-control of Mephedrone being higher amongst males (21.8%), compared to 11.6% amongst females.

Higher estimation of prevalence by participants in the metropolitan area is likely to be due to them holding different descriptive norms arising from the person's social context. Declared drug use among the active population (16-59) in England and Wales is consistently around twice as high in males than females and higher prevalence rates have been documented for urban compared to rural areas in last year's usage; with a similar but slightly more ambiguous trend for the 16-24 age group [40-42]. Biased social projection is one of the most intriguing areas in social cognition research. On the one hand, it suggests that the repeatedly observed association between self-reported behaviour or personality characteristics is explained by an egocentric bias (i.e. finding comfort in false consensus) [43], which is in keeping with the Bayesian approach [11]. On the other hand, particularly regarding the chosen sensitive and/or transgressive behaviours, it is suggested that the distorted perception of what eventually leads to a behavioural choice is congruent with this perception [44,45]. Conversely, recent research provides evidence showing that the prediction of population prevalence relates to the behaviour or characteristics the respondents wish to project about themselves, but not the actual behaviour [46,47].

Table 2 Social projection (0: nobody - 100%: everybody) and perceived harm (1: not harmful at all - 10: very harmful)

		Area		
		Rural	Metropolitan	ALL
Social projection	Male	28.00 ± 23.690	35.51 ± 23.231	31.45 ± 23.717
	Female	26.56 ± 20.780	40.68 ± 22.898	33.79 ± 22.926
	ALL	27.45 ± 22.572	37.74 ± 23.155	
Health risk	Male	5.87 ± 2.415	6.71 ± 1.912	6.26 ± 2.139
	Female	6.73 ± 1.968	7.01 ± 2.303	6.87 ± 2.139
	ALL	6.20 ± 2.286	6.84 ± 2.083	

Age was significantly negatively related, with the prevalence estimate (Spearman's r = -.150, p = 0.01) suggesting that younger people consider Mephedrone to be more prevalent. This is in line with the notion that Mephedrone is a drug for the young [33]. The correlation between age and the belief that Mephedrone was harmful was positive and significant (Spearman's r = .190, p = 0.001). As regional differences were not significant, the data from the two collection sites was combined and treated as one unified sample for future analyses.

Estimation using the Forced Response model

Subsequent to completing the questionnaire, the prevalence rate for Mephedrone use, using the formula suggested by Tourengeau & Yan [6] was calculated as follows:

$$\widehat{p} = \frac{\lambda - \pi_1}{\pi_2}$$

where:

π_1 = probability that the respondent is forced to say 'yes'

π_2 = probability that the respondent is forced to answer a sensitive question honestly

λ = observed percent that responded 'yes'

From the dice instructions, we see that π_1 = 1 out of 6 and π_2 = 3 out of 4. There were 74 'yes' responses out of 318 total, thus

$$\widehat{p} = \frac{74/318 - 1/6}{3/4} = 0.0881$$

The estimated prevalence rate for Mephedrone is 8.81%. The variance and standard error of this estimator are calculated as:

$$Var(\widehat{p}) = \frac{\widehat{\lambda}(1-\widehat{\lambda})}{n(\pi_2)^2}$$

$$Var(\widehat{p}) = \frac{74/318(1 - 74/318)}{318(3/4)^2} = 0.000998$$

$$SE = \sqrt{0.000998} = 0.034159$$

A 95% CI for the prevalence rate of Mephedrone would be the estimated prevalence rate ± the product of the $z_{\alpha/2}$ value and the standard error: 1.96 × 0.034159 = 0.061925, yielding the 95% CI of 0.026175 and 0.150025. Thus, the prevalence rate as determined by the Forced Response model with a standard error of 0.034159 and a 95% confidence interval of (0.02611, 0.14999) is estimated to be between 2.6% and 15.0%.

Hair analysis

Among the available 154 hair samples, the presence of Mephedrone was found in six samples giving a 3.9% positive rate. As the quantity of substance potentially used and time of exposure is not known, it is plausible that the actual positive rate is higher than 3.9%. It is likely that the hair analysis would only capture 3 months preceding drug use and could not detect a single exposure, nor any use that might have taken place in the immediate two weeks preceding the sample collection during which the hair is still in the scalp. Thus this period is considered as a 'blind period' for hair analysis.

Combining these positive samples with known use from the questionnaire where respondents accidentally give away this information by either answering each question on the Single Sample Count/Unmatched list five or answered each question on the same individually, the prevalence rate rises to 5.7% (9/157). Two of the nine known positive cases overlap between analytical and questionnaire results.

The simplified SSC algorithm

The fuzzy response SSC model is a new method and uses known population prevalence to estimate the proportion of affirmative answers to the sensitive question. As such, it is a simplified and more economical version of the Unmatched List Count using only one (experimental) sample. In order to avoid the need for a control sample (which inevitably leads to 50% loss of the sample), we embedded the target sensitive question into a set of four questions with 50-50 probability and benchmarked the sum of the number of observed 'yes' responses against the expected sum of the number of 'yes' responses for the four questions.

The benchmark questions were:

- My birthday is in the first 6 months (January - June) of the year.
- My house number is an even number.
- The last digit of my phone number is even
- My mother's birthday falls between July and December

The probability of a 'yes' answer to each of the four questions is therefore 50%, the expected average (sum of the number of 'yes' responses divided by the total number of responses) is two. Any upward deviation from this benchmark figure is the estimated proportion of 'yes' answers to the target question.

The target research question was:

- I have taken Mephedrone at least once in the previous three months

Respondents were instructed to indicate only the total number of their affirmative answers to the five questions without revealing which ones.

Based on the nature of the four non-sensitive questions, it was assumed that the population distribution for each question follows a binomial distribution, thus the distribution of the total number of 'yes' responses for non-sensitive questions is B(4*k, 0.5) where k is the sample size. In other word, the probability of an honest 'yes' response to each of the four non-sensitive questions is 50%. Assuming that there are equal numbers of 'yes' and 'no' responses to each of these four non-sensitive questions, it is possible to calculate the expected value of responses for the baseline non-sensitive questions:

$$E[response] = 4 \times [0.5(1)] = 2$$

Thus, if the probability distributions are exactly the same for all non-sensitive questions individually (assumed to be 0.5 in this case), the mean response for the four non-sensitive questions is expected to equal two, thus obtaining a mean response value greater than two is the indication of the estimated prevalence rate for the sensitive question. The prevalence rate estimation is calculated as:

$$d = (\lambda/n) - 2$$

where d is the estimated population distribution of the 'yes' answers to the sensitive question, λ is the observed number of 'yes' answers; and n is the sample size. The observed probability distribution of the number of 'yes' answers is shown in Table 3.

The three-month prevalence rate and 95%CI for Mephedrone use, using the SSC method, was calculated as follows:

The observed number of 'yes' answers is derived from the sum of two random variables with distribution of B (4*237, 0.5) and B(237, d), where d is the population distribution of the sensitive key question and 237 was the number of respondents in the sample. The observed number of 'yes' answers in the sample was 469.

Whilst the distribution of the sum of these two random variables is unknown, we can make use of the normal approximation for a binomial distribution. A rule of thumb is that the normal approximation is applicable if

Table 3 Observed probability distribution of X = the number of 'yes' answers

X	Observed P(X)
0	0.063
1	0.270
2	0.376
3	0.215
4	0.068
5	0.008

$np > 5$ and $n*(1-p) > 5$, $d > 0.021$ and $d < 0.979$, where n and p are the distribution of the two binomial parameters. The normal approximation is derived as mean = np and variance = $n*p*(1-p)$. Thus B(4*237, 0.5) is approximately the same as N(2*237, 237) and B(237, d) is N(237*d, 237*d*(1-d)). Since the maximum likelihood approximation of the mean of the normal distribution is the sample mean, 237*(d+2) = 469, hence d = -0.021097. Note that the estimated d is negative, since the observed number of 'yes' responses (469) is less than the expected number of 'yes' responses for the non-sensitive questions (474). This does *not* mean that the prevalence rate for Mephedrone is negative, only that the random fluctuations in the sample were too large and mask the expected upward bias in the number of observed 'yes' responses. We can nevertheless calculate the 95%CI for d, which is 469 ± $Z(0.95)*\sqrt{(237*(1+d*(1-d)))}$, where $Z(0.95)$ = 1.959964. Thus 95%CI is d ± 0.12731334 = -0.021097 ± 0.12731334 = 0, 0.099634. Therefore the estimated prevalence rate for Mephedrone use is between 0 and 10.0%.

T-test statistics indicated that the mean score (1.9789, 95%CI 1.85, 2.11) obtained on the SSC did not differ significantly from 2, thus there was no evidence that the prevalence rate for Mephedrone use in the population would differ significantly from zero (t(236) = -0.3113, p = 0.7558, Cohen's d = 0.041). This non-significant test result can be explained by the relatively small sample size. Notably, the sample prevalence was estimated to be between 0 and 10%.

The above calculation holds if the probability distribution of answers to each baseline question is equal (e.g. 50/50 in all 4 cases), thus we can assume that the sum of the binomial distributions is also binomial. However, the sum of the binomials is not necessarily binomial if the probabilities vary among the questions. Therefore, in such cases the normal approximation is calculated individually for each question before the probabilities from the baseline questions are added together, as we know that the sum of the normal distributions also follows normal distribution.

SSC algorithm taking the divergence from the 50/50 distribution into consideration

In order to test whether the estimation from the simplified SSC algorithm differs significantly from the estimation that takes the observed likely distribution for the 4 innocuous questions into consideration, we calculated d in a two-step process.

Firstly, we assumed that the probabilities of the innocuous binomial variables are not the same, so we estimated the probability distribution for each baseline question independently. In order to calculate the probabilities of the 4 innocuous binomial questions, we used the following datasets. For distribution of house and phone numbers, we used 7,500,000 UK residential data (usable dataset for house numbers: n = 6,859,957 and for phone numbers: n = 6,895,960) purchased from a commercial provider, whereas for birthdays, we used anonym datasets from two UK universities (n = 495,870 and n = 11,157). For the subsequent analysis, we used the large UK university dataset (n = 495,870) for birthdays. Details are presented in Table 4.

House numbers (including apartment/flat number in the absence of house number) were split as 3,405,322 even (p = 0.4964057) and 3,454,635 (p = 0.5035943) odd numbers. 0.5 (t = -18.828, df = 6859956, p-value < 2.2e-16, 95% CI: 0.4960316, 0.4967799). Among the listed phone numbers, the last digit of the phone number was an even number in 3,429,497 cases (p = 0.4973197) with 3,466,463 last digits being an odd number (p = 0.5026803). The probability of a birthday falling on the first half of the year was p = 0.5004075 (247,447 cases) vs. 248,423 (p = 0.499016) birthdays registered for the second half of the year. Single sample t-test statistic testing H_0: p = 0.5 for the 4 innocuous questions are as follows.

1. *My birthday is in the first 6 months* (January - June) of the year (t = -1.386, df = 495869, p = 0.1657; with estimated probability of 0.4990159 (95% CI = 0.4976242, 0.5004075)
2. *My house number is an even number* (t = -18.6633, df = 6952970, p < 0.001; with estimated probability of 0.49646115 (95% CI = 0.4960895, 0.4968328)
3. *The last digit of my phone number is even* (t = -14.077, df = 6895959, p < 0.001); with estimated probability of 0.4973197 (95% CI = 0.496946, 0.4976929)
4. *My mother's birthday falls between July and December* (t = 1.386, df = 495869, p = 0.165); with estimated probability of 0.5009841 (95% CI: 0.4995925, 0.5023758)

Table 4 Birthday distributions

	Frequency count	Probability	Frequency count	Probability
Birthday on/in[a]				
odd/even days	245,269	0.509872	235,771	0.490128
first half (up to and including the 15th)/second half of the month	239,157	0.497167	241,883	0.502833
first half/second half of the year	232,666	0.483673	248,374	0.516327
odd/even numbered months	242,683	0.504497	238,357	0.495503
Birthday on/in[b]				
odd/even days	253,438	0.511098	242,432	0.488902
first half (up to and including the 15th)/second half of the month	247,927	0.499984	247,943	0.500016
first half/second half of the year	247,447	0.499016	248,423	0.500984
Odd/even numbered months	251,226	0.506637	244,644	0.493363
Birthday on/in[c]				
odd/even days	5,739	0.514386	5,418	0.4856144
first half (up to and including the 15th)/second half of the month	5,562	0.498521	5,595	0.501479
first half/second half of the year	5,606	0.502465	5,551	0.497535
Odd/even numbered months	5,731	0.513669	5,426	0.486331

[a]US life insurance application data (n = 481,040)
[b]UK university registration data (n = 495,870)
[c]UK university registration data (n = 11,157)

Therefore, we used these empirically derived probabilities to approximate normal distribution.

The number of 'yes' answers for the

1st question is binomial, B(k, 0.4990159) → N (k*0.4990159, k*0.4990159*0.5009841)

2nd question is binomial, B(k, 0.4964611) → N (k*0.4964611, k*0.4964611*0.5035389)

3rd question is binomial, B(k, 0.4973197) → N (k*0.4973197, k*0.4973197*0.5026803)

4th question is binomial, B(k, 0.5009841) → N (k*0.5009841, k*0.4990159*0.5009841)

Sensitive question is binomial, B(k, d) → N(k*d, k*d*(1-d))

Therefore, by adding these approximations together, the distribution of the 'yes' answers are

$$N\left(k^{*}\left(1.9937808+d\right),\ k^{*}\left(0.999978355+d^{*}\left(1-d\right)\right)\right)$$

The Mephedrone dataset contained 469 'yes' answers from 237 respondents, therefore k = 237, and 237*(1.9937808+d) = 469, thus d = -0.0148779. The 95%CIs for the number of 'yes' answers with the above estimated mean and variance are439.0453 and 498.9547, thus d is between -0.1412 and 0.1115. Consequently, d (the estimated prevalence of Mephedrone use) is, indeed, between 0% and 11%, which is in keeping with the estimation we received using the simple algorithm with assumed p = 0.5 for 'yes' answers in all baseline non-sensitive questions. Therefore, applying the principles of Occam's razor, the simple algorithm should prevail.

Triangulating the SSC with the FR and hair analysis

The single most useful aspect of the hair analysis was to provide evidence that the sample prevalence of Mephedrone use was higher than zero. Figure 1 shows the combination of information available from the sample on Mephedrone use including an objective chemical analysis based on the presence of the drug in hair, accidental exposure via direct self-reports and two estimates representing two different indirect models. Combining these prevalence rates and estimates, we can conclude that the prevalence of Mephedrone use in the sample ranges between 5.7% and 15.0%. The two models yielded similar estimates with the FR up to 15% and the new SSC up to 10%. The close proximity of these estimates provides evidence that supports the validity of the new SSC model.

Implementation

Practical issues relating to the indirect estimation methods are i) the chance of exposure, ii) minimum and optimal sample sizes required to achieve a desirable power, iii) efficiency and iv) potential to eliminate or detect noncompliance. This section discusses these in the context of implementing the SSC approach.

Potential exposure

One notable drawback of the Single Sample Count model (as well as for the Unmatched List) is the scenario in which a respondent happens to have 'yes'

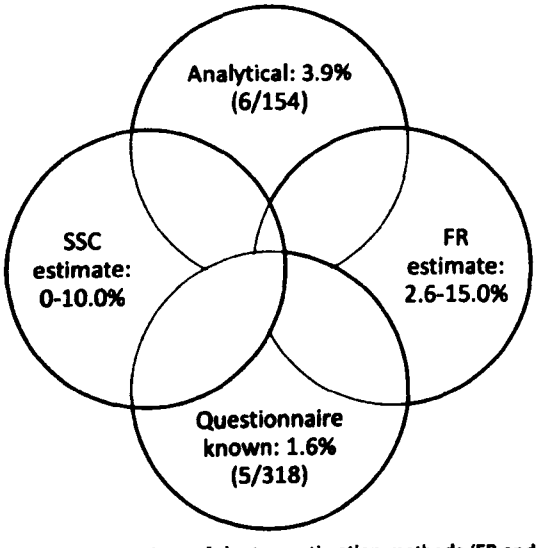

Figure 1 Comparison of the two estimation methods (FR and SSC) hair sample results and limited information on Mephedrone use directly available from self-reports.

answers to all innocuous questions and a 'yes' answers to the sensitive question. In this case, the respondent, if he/she answers truthfully, would reveal the information about the compromising behaviour. Note that the level of exposure in this situation becomes equivalent of the risk of exposure in an anonymous direct self-report.

This potential exposure situation can be mitigated by either increasing the number of innocuous questions (thus reducing the probability that such a scenario occurs (Table 5), or by offering an option of a new set of questions. Naturally, this latter option requires a bank of innocuous questions and only works in face-to-face interview settings or computer-assisted self-administration. Selecting the number of questions should take into consideration not only the probability but also the cognitive demand on respondents.

Table 5 The percentage of respondents potentially required to answer in a revealing way as the function of model design and prevalence rate of the sensitive question

Design	Innocuous	Sensitive[a]						
		5%	10%	15%	20%	30%	40%	50%
1 + 1	50.00	2.50	5.00	7.50	10.00	15.00	20.00	25.00
2 + 1	25.00	1.25	2.50	3.75	5.00	7.50	10.00	12.50
3 + 1	12.50	0.62	1.25	1.87	2.50	3.75	5.00	6.25
4 + 1	6.25	0.31	0.62	0.94	1.25	1.87	2.50	3.12
5 + 1	3.12	0.16	0.31	0.48	0.62	0.94	1.25	1.56
6 + 1	1.56	0.08	0.16	0.23	0.31	0.47	0.62	0.78
7 + 1	0.78	0.04	0.08	0.12	0.16	0.23	0.31	0.39
8 + 1	0.39	0.02	0.04	0.06	0.08	0.12	0.16	0.19

[a] for illustration we assume that the compromising behaviour is proportionally distributed

In cases where d is large, the potential exposure might be significantly high enough to consider alternative approaches. One example would be where answer options either combine 0 and 5 or allow respondent with answer '5' to select any answer options (0-4). Comparing the distribution of a hypothetical honest answer scenario with d = 0.2 prevalence rate for the sensitive question to the two proposed solutions using Kolmogorov-Smirnov's maximum divergence of the cumulative distribution function, no statistically meaningful preference was found between the two options (KS = 0.0125 for '0& 5'; and KS = 0.0125 for 'any option'). Using Root Mean Square (RMS) indicated a slight preference towards the 'any other' option (RMS = 0.0027 vs. RMS = 0.0035 for the '0 & 5'). Probabilities for the three scenarios are presented in Table 6. Simulations with 1 million data responses also showed very similar distributions (Figure 2). For simplicity, we assumed a 0.5 probability for each innocuous question. Given these results and taking practical issues into consideration, the combined 0 and 5 answer option is suggested for its relative simplicity. As one might expect, this solution to the '5-yes' problem affects the complexity of the computation to derive the estimated probability for the target sensitive question.

Required minimum sample size

Owing to the relatively small sample sizes, estimates using either the Forced Responses or the Single Sample Count method yielded negative values, making the lower bound of the 95%CI set to zero. The sample size required for the SSC model is chiefly determined by the sample size required to obtain a mean value for the four non-sensitive baseline questions to be as close as possible to two. Figure 3 shows that the bin width did not change significantly unless a significant increase in n is in place. Table 7 gives the exact values for the lower and upper 95%CI for selected sample sizes. From the practical point of view, the gain from increasing the sample by 500 is negligible compared to the potential cost of generating 500 samples. For comparison, 95%CIs are also calculated for 5 and 6 baseline questions, where the same logic applies as in the 4+1 model.

Power analysis

The first four (non-sensitive) questions will be distributed B(n = 4, p = 0.5) while the 5th question (sensitive question) will follow the Bernoulli distribution with a success probability of p, where d is the unknown prevalence rate. Let X = number of 'yes' answers out of the 5 questions, using the 4+1 SSC design.

Table 6 Probability of answer distributions if i) questions are honestly answered, ii) 0 and 5 answers are combined and iii) respondents are instructed to select any response option; *d* = probability of doping

	Questions are honestly answered	0 and 5 answers are combined	Any other response options are selected
0	1/16 - d/16	1/16	1/16 - d/20
1	1/4 - 3d/16	1/4 - 3d/16	1/4 - 7d/40
2	3/8 - d/8	3/8 - d/8	3/8 - 9d/80
3	1/4 + d/8	1/4 + d/8	1/4 - 11d/80
4	1/16 + 3d/16	1/16 + 3/16d	1/16 + d/5
5	d/16		

Testing H_0: $\mu = 2.0$ vs. H_1: $\mu > 2.0$ with $\alpha = 0.05$, the test statistic is calculated as:

$$t = \frac{\bar{x} - 2.0}{s/\sqrt{n}}$$

Solve this equation for n:

$$n = \left(\frac{t * s}{\bar{x} - 2.0}\right)^2 = \left(\frac{t * s}{\Delta}\right)^2$$

With $\alpha = 0.05$, the critical value t would be equal to 1.645. Substituting $t = 1.645$, n becomes a function of the effect size, Δ and standard deviation of X, s. We can calculate s from simulations using different prevalence levels. We will use $d = 0.05, 0.10, 0.15, \ldots 0.50$. This in turn will allow us to calculate the standard deviation of X, s.

From Table 8 below, it can be shown that when p = 0.15, s can estimated though 10,000 simulations to be 1.063. From this, recommended sample sizes can be developed. For example, to detect a significant effect size, $\Delta = 0.04$ with $\alpha = 0.05$ and $d = 0.10$, Table 8 shows that the simulated standard deviation would be s = 1.043 and the recommended sample size would be n = 1,839.

Mapping the information from Table 8 to Figure 3 and Table 7, it is easy to see the minimum required sample size is a direct function of the achieved bin width of the baseline questions. In practical terms, the required sample size ensures that the sample mean for the SSC is above the 95%CI for the baseline questions. The same logic applies for the models where the sensitive question is embedded in five or even the six

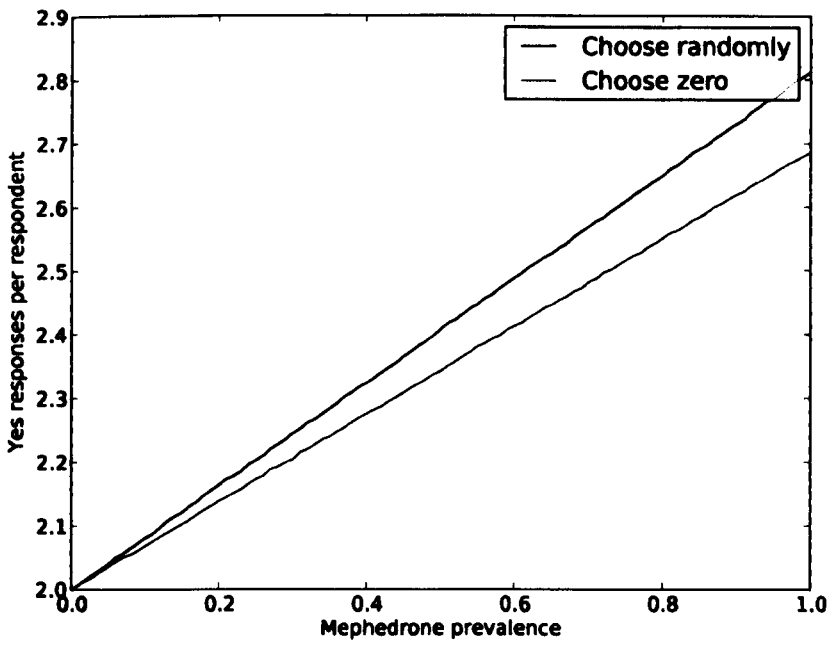

Figure 2 Comparison of the simulated probability distributions under two scenarios: A) '0 & 5' combined and B) 'any other option' to avoid exposure (n = 1,000,000).

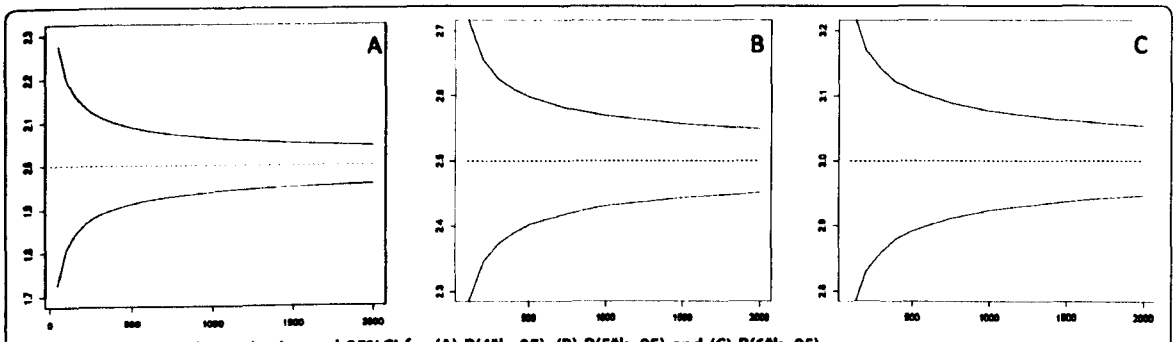

Figure 3 Function of sample size and 95%CI for (A) B(4*k, 05), (B) B(5*k, 05) and (C) B(6*k, 05).

innocuous questions. Table 9 displays the sample size values for the 5+1 SSC model.

Comparing the 4+1 model to the 5+1 model, the price that must be paid for the reduced chance of exposure is a slight increase in the required sample size. More importantly, however, this is in addition to the increased cognitive load on respondents which should be taken into consideration when designing the questionnaire.

In comparison, the minimum sample size for the FR is presented in Table 10 for SE {0.01, 0.02, 0.03, 0.04}, where we calculate a minimum sample size to form a confidence interval with varying levels of confidence. The conservative estimate of the minimum necessary sample size was calculated as follows:

π_1 = P(forced to say yes)

π_2 = P(forced to answer honestly)

λ observed percent that responded 'yes'

$$\widehat{p} = \frac{\lambda - \pi_1}{\pi_2}$$

$$Var(\widehat{p}) = \frac{\widehat{\lambda}(1-\widehat{\lambda})}{n(\pi_2)^2}$$

Solve for n:

$$n = \frac{\lambda(1-\lambda)}{Var(\widehat{p})(\pi_2)^2}$$

Maximize this equation by using $\lambda = 0.5$ and $\pi_2 = 3/4$. Setting $\lambda = 0.5$ creates the maximum variance possible thus result in a conservative estimate establishing the necessary sample size assuming the worst case scenario.

Using a 95% confidence interval, we get:

$$\widehat{p} \pm 1.96SE(\widehat{p})$$

Now use a standard error = {0.05, 0.04, 0.03, 0.02, 0.01}. Values are presented in Table 10.

For example, to obtain a 95% CI with width of 9.8 percentage points, n = 178 is required. To obtain a 95% CI with width of 1.96 percentage points, the required sample size rises to n = 4,445. This is comparable to the sample size required for the SSC to obtain a sufficiently narrow 95% CI for the innocuous questions (Table 7), which in turn, is very reassuring for the SSC as the FR model has been shown to be one of the most efficient model in terms of sample size with some 2.2 times of the direct question equivalent [17]. For example, Table 7 shows that with n = 400, the SE is 0.050 (n = 336 for

Table 7 95%CI intervals for 4, 5 and 6 baseline question models when n = 100, 200, 300, 400, 500, 750, 1500 and 2000

Sample size	4 baseline questions B(4*k, 05)		5 baseline questions B(5*k, 05)		6 baseline questions B(6*k, 05)	
	Lower	Upper	Lower	Upper	Lower	Upper
100	1.800	2.200	2.280	2.720	2.760	3.240
200	1.860	2.140	2.345	2.655	2.830	3.170
300	1.887	2.113	2.373	2.627	2.860	3.140
400	1.903	2.098	2.390	2.610	2.880	3.120
500	1.912	2.088	2.402	2.598	2.892	3.108
750	1.928	2.072	2.420	2.580	2.912	3.088
1000	1.938	2.062	2.431	2.569	2.924	3.076
1500	1.949	2.051	2.443	2.557	2.938	3.062
2000	1.956	2.044	2.451	2.549	2.947	3.054

Table 8 Minimum sample sizes as the function of difference (denoted by Δ) for the 4 baseline question SSC model

	Prevalence Rate									
	0.05	0.1	0.15	0.2	0.25	0.3	0.35	0.4	0.45	0.5
	Standard Deviation (s)									
	1.022	1.043	1.063	1.080	1.092	1.104	1.112	1.118	1.118	1.121
Effect Size (Δ)	Minimum Sample Size (n)									
0.01	28247	29421	30566	31563	32251	32975	33479	33799	33823	33993
0.02	7062	7355	7641	7891	8063	8244	8370	8450	8456	8498
0.03	3139	3269	3396	3507	3583	3664	3720	3755	3758	3777
0.04	1765	1839	1910	1973	2016	2061	2092	2112	2114	2125
0.05	1130	1177	1223	1263	1290	1319	1339	1352	1353	1360
0.1	282	294	306	316	323	330	335	338	338	340
0.15	126	131	136	140	143	147	149	150	150	151
0.2	71	74	76	79	81	82	84	84	85	85
0.25	45	47	49	51	52	53	54	54	54	54
0.3	31	33	34	35	36	37	37	38	38	38
0.35	23	24	25	26	26	27	27	28	28	28
0.4	18	18	19	20	20	21	21	21	21	21
0.45	14	15	15	16	16	16	17	17	17	17
0.5	11	12	12	13	13	13	13	14	14	14

this scenario in Table 10). Similarly, SSC n = 500, 1000 and 2000 give SE = 0.041, 0.032 and 0.023, respectively. These sample sizes map well onto those presented in Table 10 as n = 525, 934 and 2101, respectively. This congruence only holds for the 4+1 SSC design. As the number of the innocuous questions increases, so does the minimum required sample size. For example, reading from Table 7 (and in comparison to Table 10), we see that the sample required for SE ~ 0.03 is around 1,000 for the FR model and for the 4+1 SSC model, but reaching 1,500 for the 5+1 SSC with a further increase for the 6+1 SSC models. Thus the increase in sample size is the consequence of the increased security provided to respondents. Similarly, reducing the proportion in the FR model where honest answer is required results in increased security as well as in increased sample size. Notably, however, the large sample approximations of the proposed SSC method, along with other randomised response and non-random models, will provide reasonably close coverage for larger sample sizes, but may

Table 9 Minimum sample sizes as the function of difference (denoted by Δ) for the 5 baseline question SSC model

	Prevalence Rate									
	0.05	0.1	0.15	0.2	0.25	0.3	0.35	0.4	0.45	0.5
	Standard Deviation (s)									
	1.138	1.157	1.172	1.190	1.202	1.203	1.214	1.219	1.224	1.227
Effect Size (Δ)	Minimum Sample Size(n)									
0.01	35063	36243	37157	38294	39129	39155	39914	40224	40548	40713
0.02	8766	9061	9289	9574	9782	9789	9979	10056	10137	10178
0.03	3896	4027	4129	4255	4348	4351	4435	4469	4505	4524
0.04	2191	2265	2322	2393	2446	2447	2495	2514	2534	2545
0.05	1403	1450	1486	1532	1565	1566	1597	1609	1622	1629
0.1	351	362	372	383	391	392	399	402	405	407
0.15	156	161	165	170	174	174	177	179	180	181
0.2	88	91	93	96	98	98	100	101	101	102
0.25	56	58	59	61	63	63	64	64	65	65
0.3	39	40	41	43	43	44	44	45	45	45
0.35	29	30	30	31	32	32	33	33	33	33
0.4	22	23	23	24	24	24	25	25	25	25
0.45	17	18	18	19	19	19	20	20	20	20
0.5	14	14	15	15	16	16	16	16	16	16

Table 10 Minimum required sample size as a function of standard error (SE) with 95% confidence interval.

Standard error (SE)	Percentage points (1.96SE)	Minimum n
(0.05)	±0.0980	178
(0.04)	±0.0784	278
(0.03)	±0.0588	494
(0.02)	±0.0392	1112
(0.01)	±0.0196	4445

deviate from 95%CI for smaller sample sizes because of the discrete nature of the events.

Efficiency

Unlike other RRT/NR models, the Singe Sample Count model uses every single response in the sample to estimate the prevalence rate for the sensitive question. As the population distribution is known *a priori*, there is no need to generate an independent sample from the same population to establish population prevalence. Thus the SSC model comes with no waste of any proportion of the sample. This aspect is unique among the RRT/NRM models.

Non-compliance

The key driver for improving the random response and non-random models has been the hope that such techniques will be able to eliminate socially desirable responses. Social desirability (SD) is a known confounding factor in self reported research design, stemming from either the research tool or the person but equally resulting in dishonest responses [48,49].

Contrary to this desire, overwhelming evidence demonstrates that RRT/NRM are not cheating free [50,51]. Böckenholt et al. [51] used two separate methods, namely the Forced response and Kuk's [52] rather complicated card colour naming technique. Both demonstrated that accounting for non-compliance bias doubled the estimated prevalence. This finding is in-line with a medication non-adherence study that showed that almost half of the respondents did not follow the questionnaire instructions thus considerably distorting the prevalence rate without correcting for cheating [53]. This study used a variation of the forced response model linked to a rather low percentage when respondents have to answer honestly. The instructions were that if the respondent's father's birthday occurred in January or February then a truthful answer was requested, with a forced 'yes' for all other months. Therefore only 16.7% (2/12) of the respondents were asked to answer the sensitive question.

Self-protective no saying (SPN) is a known pattern in which respondents say 'no' without considering the instructions or truth. Considerable effort has been made to estimate the effect of dishonesty or correct for such effects [54-60]. Triggers for noncompliance could be the forced 'yes' answers in situations when respondents do not identify themselves with the discriminating behaviour; or complicated instructions which respondents are unable or unwilling to follow [51,61].

At this stage, we do not have data to ascertain what proportion of the responses on the SSC might have been affected by dishonest answering. Nonetheless, SSC does not offer an obvious self-protective response option as respondents who wish to deceive in their answers may simply chose entering zero, or any other number that is less than their true response would be. The somewhat higher estimate received using the SSC compared to the FR suggests that the SSC might be less affected by self-protective responding. Qualitative feedback received during data collection supports this assumption. Upon prompting for feedback in one group, respondents felt that they are more protected under the SSC model because as they phrased it: they *"didn't really have to answer the sensitive question"*. This is, by design, was not the case in the FR model where depending on the outcome of the dice roll, 75% of the respondents were asked to answer the sensitive question.

Potential innocuous questions

The SSC method builds on the innocuous question where the population distribution is assumed to be approximately close to 50/50. Such questions could be related to the last digits of a phone number, possibly house numbers or postcodes (even though these may vary from country to country), as well as birthdays. Selection of the most appropriate question must be informed by the research design, taking the target sample characteristics into consideration. Below, we present statistics derived from worldwide empirical data (n = 1,379), a publicly available dataset on birthdays (n = 481,040) and birthday data extracted from a UK university database (n = 495,870) to assist this process.

Empirical data were collected via Amazon Mechanical Turk in May-June 2011, with the Human Intelligence Tasks (HITs) made accessible worldwide to those with at least a 80% HITs acceptance rate [62]. The majority of the information was provided by people in India (59.2%), followed by the USA (28.4%), Canada (1.5%), Pakistan (1.1%) and the UK (1.0%). The remaining 51 countries contributed to a total of 8.8%. House numbers were odd numbers in 50.8%, whereas the last digits of the phone numbers were odd numbers in 48.6% of all records. Our results showed that more people prefer odd numbers for a lucky number (65.6%). The day of the birthday being odd occurred in 51.10% of the sample.

The publicly available birthday dataset was collected by Roy Murphy based on insurance policy applications to a Life Insurance Company between 1981 and 1994 http://www.panix.com/~murphy/bday.html and over 500 thousand birthdays captured in the internal information management system of two UK universities. The overall distribution of the birthdays in all three available datasets is remarkably similar to another database containing over 135 million records http://anybirthday.com/.

Using Roy Murphy's insurance application data, the results suggest that the 'first half vs. second half of the month' appears to give the closest split to 50/50, followed by the 'odd/even numbered month'. The analysis of two UK university population datasets of 495,870 and 11,157 birthdays provided further evidence that in the large dataset 'first half vs. second half of the month' lead to a closest split to 50/50, with the next closest distribution to 50/50 was the 'first vs. second half of the year' with the smaller dataset (n = 11,157) showing the opposite positions for the top two places. Frequency counts and probability distributions for birthdays falling on odd vs. even days and months; first vs. second half or the month and years, independently, are reported in Table 4.

Discussion

The overarching advantage of both randomised response and non-random models is that they provide greater respondent anonymity protection as question responses cannot be traced to the individual. This anonymity also removes any ethical or legal obligation from the interviewer to act upon sensitive information disclosed to them as part of the research process.

Further advantages of the SSC method are:

- The model is simple to administer, offering a self-administration option without any sense of deception.
- The SSC model reduces the complexity in instructions and places low cognitive demands upon respondents.
- Unlike the FR model, SSC asks each respondent to answer, in a fuzzy way, the (sensitive) research question and hence improves the face validity of the research tool.
- Unlike other RRT/NR models, the SSC avoids a forced 'yes' response, which can be off-putting for people whose honest answer would normally be 'no' to the sensitive question. Also, respondents are not required to answer the sensitive question directly.
- In the SSC model, no obvious self protective strategy is present (e.g. self-protective 'no' saying), thus this approach can overcome the 'self protective no' bias.

The challenges with the SSC model arise from finding a suitable set of baseline questions where the population prevalence and distribution is known to be 50-50% and adequately addressing the chance of potential exposure. There is a small but existing chance that someone encounters a situation in which the answer would be revealing. This is not only a problem for the newly proposed Single Sample Count but also affects the classic Unmatched Count technique. The potential of exposure can be mitigated by either increasing the number of baseline questions to reduce the likelihood of having affirmative answers to all baseline questions or by offering different sets of baseline questions. This latter approach requires computerised administration or personal interviews.

This study would have further benefited from an increased sample size as confidence intervals were limited to the upper bounds. Further studies are required to improve the evidence base for testing the methodological validity and reliability. The sample size was confined by a number of specific criteria. The focus of the study was restricted to the use of a single substance (Mephedrone), which on the one hand held the advantage of requiring a single screening in hair, but on the other placed limits on the study for two reasons. Firstly, the population prevalence rate of illicit substance use was low because the study restricted itself to a specific substance. As the method for detecting Mephedrone in human hair is newly developed, it is not yet known what consumed quantity of the drug signals a positive analytical result, or how natural hair colour, sex or ethnicity, for instance, might affect the deposition of the drug in human hair. However, hair analysis may provide the ultimate gold standard for validating the SSC approach for substance use. If such is the case, then careful consideration must be accorded to the research design to ensure effective synergy between social, analytical and statistical approaches. It is important that the sensitive question considers the limitations imposed by the hair analysis. For example, some drugs deposit into hair with more ease than others and stay longer. Accidental or environmental exposure may be a contributing factor in explaining the presence of a given drug in hair samples. Hair analysis is normally not suitable to detect single or very recent (i.e. last two weeks) exposure - but if research remit requires knowledge of these aspects then urinalysis may be a viable alternative. The timeframe afforded by the selected biochemical analysis must be carefully matched in the question. Exploring these issues is beyond the scope of this paper.

Secondly, whilst the hair analysis component was useful to prove that the sample prevalence is larger than zero, its labour and costs implications placed limits on the sample size. We have compensated for this

limitation with simulations to calculate the required sample sizes. The SSC model should also be tested investigating other discriminating behaviours with differing expected prevalence rates. If dishonest response patterns are known, prevalence estimation could incorporate a statistical correction component to account for this bias. Further refinement of the SSC model could include two variations where the sensitive question is positively (e.g. 'I have used drugs' or 'I do take my medications') or negatively framed (e.g. 'I have never used drugs' or 'I do not take my medications') to test whether giving confirmation, albeit indirect, of the desired or undesired behaviour has an effect on the results.

The research design could also benefit in some cases from the inclusion of a priming task to investigate whether or not the indirect approach and the additional protection afforded by the fuzzy response mode itself generates the maximum achievable admission of the discriminating behaviour. Alternatively, lie detector Implicit Associations Tests (e.g. [63,64]) could be combined with the SSC models for contrasting and comparing prevalence rates obtained via different methods from the same sample.

Conclusion

The major advantage of the Single Sample Count method over other models such as the Forced Response model is rooted in its simplicity, equal face validity for each respondent, simple calculations and maximum use of the data. This elegantly simple, quick and cost effective method can be successfully employed in public health research aiming to establish the epidemiology of potentially compromising behaviours. Notwithstanding, this approach, akin to other randomised and non-random models, is suitable to establish group level prevalence.

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Authors' contributions
AP and DPN initiated the project. AP devised the study, contributed to analyzing the data and prepared the first draft of the manuscript. PC, HT and CA collected the data and contributed to the final draft of the manuscript. TN, MS and JS analysed the survey data and provided the additional statistical information. SH, ND, DPN and JB developed the method for and conducted the hair analysis; and prepared the relevant section of the manuscript. All authors have contributed to drafting the paper; read and approved the final version of the manuscript.

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

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