Testosterone Use and Abuse

Methodological Aspects in
Forensic Toxicology and
Clinical Diagnostics

Yvonne Lood

Linköping University Medical Dissertation No. 1762

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Clinical Diagnostics

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When you talk, you are only repeating
what you already know. But if you listen,
you may learn something new.

Dalai Lama

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ABSTRACT

Abuse of anabolic androgenic steroids (AAS) is widespread in society and is today a major public health problem, associated with mental and somatic adverse effects and risk behavior, such as use of other illicit drugs and criminality. Testosterone, the most important endogenous male androgen, is therapeutically used in replacement therapy but is also extensively used as a doping agent. Traditionally, testosterone abuse is detected in urine in forensic cases and in serum in clinical diagnosis and monitoring, and free bioavailable serum testosterone is calculated by formulas. Salivary testosterone is however an attractive biomarker, as testosterone in saliva is supposed to reflect free testosterone in serum.

The aim of this thesis was to investigate the abuse of AAS from a forensic perspective, particularly focusing on testosterone and methodological problems and potential alternative matrices for measurements of testosterone in forensic and clinical assessments.

In the first study the toxicological findings in individuals suspected of doping offences, registered in the Swedish national forensic toxicology database were investigated (paper I). In paper II, testosterone levels in serum, saliva, and urine in clinical patients during replacement therapy with testosterone undecanoate (Nebido®) were studied. Further, the sensitivity of the current procedure for detection of testosterone abuse was investigated by method comparison using isotope ratio measurement (paper III) and a quantitative LC-MS/MS method for testosterone in serum and saliva was developed and presented (paper IV).

It was found that testosterone was most frequently detected in the forensic cases and co-abuse of narcotics was common among AAS abusers. Methodological problems in detection of testosterone abuse using the present procedures was identified, indicating a need for new analytical strategies. A sensitive and highly specific LC-MS/MS method was developed for determination of testosterone in serum and saliva, which was shown suitable for analysis of forensic and clinical samples. Salivary testosterone was shown to correlate well with free serum testosterone in both male and female, and a sensitive marker in testosterone therapy, especially in females. In conclusion, it was found that saliva might have a potential as an alternative matrix for detection of illicit administration of testosterone and for diagnosis and monitoring of androgenic status.

SVENSK SAMMANFATTNING

Missbruket av anabola androgena steroider (AAS) är idag utbrett i samhället och är ett betydande folkhälsoproblem, associerat med fysisk och psykisk ohälsa och riskbeteende, såsom bruk av andra illegala droger och kriminalitet. Testosteron, det viktigaste manliga könshormonet används medicinskt vid klinisk substitutionsbehandling, men missbrukas även omfattande som dopningsmedel. Traditionellt detekteras missbruk av testosteron i urin i forensiska fall och i serum i klinisk diagnostik och monitorering och fritt biotillgängligt testosteron beräknas utifrån olika formler. Salivtestosteron är emellertid en attraktiv biomarkör, då testosteron i saliv anses spegla den fria fraktionen testosteron i serum.

Denna avhandling syftade till att studera missbruket av anabola androgena steroider utifrån ett forensiskt perspektiv, speciellt med fokus på testosteron och metodologiska problem och möjligheten att använda alternativa biomarkörer för detektion och mätning av testosteron i forensiska och kliniska frågeställningar.

I det första delarbetet studerades de toxikologiska fynden hos individer misstänkta för brott mot den svenska dopinglagen, registrerade i Sveriges nationella databas för forensisk toxikologi. I delarbete II studerades nivåerna av testosteron i serum, saliv och urin hos patienter vid substitutionsbehandling med testosteronundekanoat (Nebido®). Vidare studerades känsligheten för detektion av missbruk av testosteron med befintlig metod genom jämförelser med analyser med isotop ratio (delarbete III) och en kvantitativ LC-MS/MS metod för testosteron i serum och saliv utvecklades och presenterades (delarbete IV).

Testosteron detekterades frekvent i de forensiska fallen, och ett blandmissbruk av AAS och narkotiska preparat var vanligt förekommande. Metodologiska problem identifierades med den nuvarande proceduren för detektion av testosteronmissbruk, vilket indikerar ett behov av nya analytiska strategier. En känslig och högst specifik LC-MS/MS metod för bestämning av testosteron i serum och saliv utvecklades, vilken visade sig lämplig för analys av forensiska och kliniska prover. Salivtestosteron korrelerade med fritt testosteron i serum hos både män och kvinnor, och visade sig vara en känslig markör vid testosteronbehandling, speciellt hos kvinnor. Slutsatsen är att saliv kan ha potential som en alternativ matris för detektion av missbruk av testosteron och för diagnostisering och monitorering av androgent status.

LIST OF PAPERS

This thesis is based on following papers, which from here on are referred to in the text by their Roman numerals.

- I. **Lood Y**, Eklund A, Garle M, Ahlner J. Anabolic androgenic steroids in police cases in Sweden 1999-2009. *Forensic Sci Int* 2012;219:199-204.
- II. **Lood Y**, Aardal-Eriksson E, Webe C, Ahlner J, Ekman B, Wahlberg J. Relationship between testosterone in serum, saliva and urine during treatment with intramuscular testosterone undecanoate in gender dysphoria and male hypogonadism. *Andrology* 2018;6:86-93.
- III. **Lood Y**, Aardal E, Gustavsson S, Prasolov I, Josefsson M, Ahlner J. False negative results in testosterone doping in forensic cases: sensitivity of the urinary detection criteria T/E and T/LH. Submitted for publication in *Drug Test Anal*.
- IV. **Lood Y**, Aardal E, Ahlner J, Ärlemalm A, Carlsson B, Ekman B, Wahlberg J, Josefsson M. Determination of testosterone in serum and saliva by liquid chromatography-tandem mass spectrometry: an accurate and sensitive method applied on clinical and forensic samples. *J Pharm Biomed Anal*. 2021;195:113823.

ABBREVIATIONS

AAS	anabolic androgenic steroids
CV	coefficient of variation
DHEA	dehydroepiandrosterone
EI	electron ionization
ESI	electrospray ionization
FAI	free androgen index
FSH	follicle stimulating hormone
GC-MS	gas chromatography-mass spectrometry
GD	gender dysphoria
HG	hypogonadism
i.m.	intramuscular
IRMS	isotope ratio mass spectrometry
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LH	luteinizing hormone
LOQ	limit of quantification
MRM	multiple reaction monitoring
MSTFA	N-methyl-N-(trimethylsilyl)trifluoroacetamide
NPV	negative predictive value
PPV	positive predictive value
SHBG	sex hormone-binding globulin
SIM	selected ion monitoring
S/N	signal to noise
T/E	testosterone/epitestosterone
TM	transgender male
TU	testosterone undecanoate
UGT	uridine diphospho-glucuronosyl transferase
WADA	World Anti-Doping Agency

INTRODUCTION

Anabolic androgenic steroids

General introduction

Testosterone is the main male hormone with promoting effects on muscle growth, protein synthesis, erythropoiesis and skeletal growth (i.e. anabolic effects), and responsible for the development and maintenance of secondary sexual characteristics, libido and spermatogenesis (i.e. androgenic effects).¹ The medical indication for testosterone is replacement therapy in pathological androgen deficiency or in gender dysphoria (female to male). Despite the absence of new indications, there have been a major increase in testosterone prescribing in most countries during the last years. This systematic over-prescribing of testosterone is apparently mainly for off-label use including male ageing.²

Anabolic androgenic steroids (AAS) are synthetic derivatives of testosterone that exhibit similar anabolic and androgenic effects.³ Abuse of AAS was historically confined to the elite sports but has diffused from the doping in sports into the general society during the 1980s and is today a major public health issue. Long-term abuse of AAS is associated with several adverse physical and psychological effects, increased mortality and criminality.⁴⁻⁶ Non-therapeutic use of AAS is prohibited in Sweden by the Act Prohibiting Certain Doping Substances (1991:1969).⁷ AAS abuse is carried out by self-administration of often supra-physiological doses, usually obtained illicitly, for non-medical purposes. AAS are readily obtainable illegally via selling sites on the Internet and black markets and legally in countries where they are not prohibited.⁸ The doping law in Sweden is quite unique in an international perspective and in accordance with this legislation, the use of AAS is denominated as abuse. The Swedish police perform forensic doping investigations in cases of suspected doping offences.

There are no reliable data available on the prevalence of AAS use in Sweden, but at least 10,000 active users and even up to 100,000 individuals have been estimated to use or have used AAS annually, among a population of 10 million.^{9, 10} A regional study reported that 3.2% of 16

and 17 years old male adolescents had used AAS, but none of the females.¹¹ Global lifetime prevalence rate of AAS abuse for males was in a meta-analysis shown to be 6.4% and 1.6% for females.¹² It was found that AAS abuse has become particularly prevalent in the general population in Scandinavia, the United States, Brazil and the British Commonwealth countries, but is rare in countries such as China, Korea and Japan, a pattern that reflects cultural differences and attitudes towards male muscularity.¹³ Nowadays, young men in the Western societies are growing up with images of muscular male bodies, from e.g., television, movies, advertisements, and action toys in childhood.¹⁴

Reliable bioanalytical methods for determination of testosterone are of utmost importance in forensic as well as in clinical applications. The analytical results in the forensic investigations will be used in a legal proceeding, and the results are in clinical practice used for medical purpose in diagnosis of androgen disorders as well as monitoring testosterone therapy and patient status, (e.g., prostate cancer). In this thesis, the characteristics of the abuse of AAS were investigated from a forensic perspective. The bioanalytical methods and procedures were focused on testosterone detection and measurement to improve analytical approaches in forensic toxicology and laboratory medicine.

The history of AAS

It has been known for centuries that castration of men leads to loss of virility and fertility and loss of secondary male sex characteristics, but first in 1849 Arnold Adolph Berthold observed that testes transplanted from roosters to capons restored androgenic functions.¹⁵ He concluded that the testicles secrete a substance into the bloodstream that affects behavioral and sexual characteristics.¹⁶ In 1889 Charles Edouard Brown-Séquard injected extracts from the testicles of dogs and guinea pigs and demonstrated the effects on himself.¹⁷ At the end of 1889, the news about these substances were spread all over the western world and were sold as an “Elixir of Life”.¹⁸ Early in the 20th century, testosterone was used as therapy for male homosexuals, and in the United States, at least 11 homosexual men received transplants of testicular tissue extracted from heterosexual men.¹⁶ Testosterone was first synthesized in 1935 by Adolf Butenandt and Leopold Ruzicka who were rewarded the Nobel Prize in Chemistry in 1939.^{19, 20} Synthetic testosterone products were early used to treat hypogonadism in men, and since the 1940s testosterone has been used off-label for treatment of various conditions, such as anemia,

depression, melancholia, menorrhagia in women and wasting conditions (e.g., burns, surgery and radiation therapy).²¹

During the 1950s and 1960s use of AAS started to spread among athletes, both men and women, especially in strength-intensive sports, such as weightlifting. In one of the largest pharmaceutical experiments in history, several thousand athletes were during the 1960s and for more than three decades treated with androgens in the German Democratic republic (GDR) promoted by the government.²² A study of the best male Swedish athletes in different sports in 1973 found that one third of 144 athletes had been using AAS, particularly throwers.²³ It was not until 1974, that AAS was included in the list of banned substances by the International Olympic Committee (IOC). In order to promote anti-doping activities, the World Anti-Doping Agency (WADA) was created in 1999 and the World Anti-Doping Code was implemented in 2004 to harmonize the rules in all sports all over the world.^{24, 25} However, legal and illegal use of these drugs have gained in popularity and have spread outside competitive sports.

Biochemistry

Steroidogenesis

Testosterone in males is derived from cholesterol through pregnenolone and synthesized in the Leydig cells located in the testicular interstitium (Fig. 1). Approximately 95% of circulating testosterone in men is produced by the Leydig cells and the remaining part is derived from the adrenal gland. The testes in a normal man secretes about 6-7 mg testosterone daily. In contrast, testosterone in females is mainly produced in the ovaries and adrenal glands, but in about 10 times lesser amount. The Leydig cell is the only cell expressing all enzymes essential for conversion of cholesterol to testosterone.²⁶ After testicular secretion, testosterone is disposed along four major pathways, one direct pathway where testosterone binds to and activates the androgen receptor of skeletal muscles and one pathway where a small proportion of testosterone is converted to the more potent androgen DHT by type 2 5 α -reductase enzyme, characteristically expressed in the prostate but also at lower levels in skin (hair follicles) and the liver. The pathway characteristic for bone and brain converts testosterone to estradiol by the enzyme aromatase and the inactivation pathway with oxidation and conjugation of testosterone to inactive metabolites occur in the liver.²⁷ Testosterone production follows a diurnal rhythm, with a peak concentration in the morning between 7 and 10 am, with declining

concentration during the day, and rising again at night during sleep.²⁸ Due to the diurnal variation, it has been suggested that measurement of testosterone should be performed in samples collected in the morning after overnight fasting. It has been reported that food intake can lower circulating testosterone levels up to 30% compared to fasting conditions.²⁹ Despite this evidence, most samples for testosterone analysis are today not taken in a fasting state, as the reference ranges used in clinical practice are not based on fasting values.

Epitestosterone is a naturally occurring 17 α -hydroxy epimer of testosterone. The excretion of epitestosterone glucuronide and sulfates in human was first reported in the 1960s.³⁰ Epitestosterone is produced by the testis, but has no biological activity. The mechanisms of synthesis and action of epitestosterone are still not well characterized. Even if no clear results have been published about the potential precursor of epitestosterone, 5-androstene-3 β , 17 α -diol (Ae-17 α -diol) has been suggested to be the main precursor.³¹ Epitestosterone is neither a metabolite nor a precursor of testosterone.³² The production of epitestosterone is only 3% of that of testosterone, but the clearance rate is about 30% of that of testosterone.³³ The nearly constant ratio of urinary testosterone to epitestosterone (T/E) of approximately 1 made it attractive as a reference substance in detection of exogenous administered testosterone.³⁴

Figure 1. Steroid pathways in Leydig cells.

Regulation of testosterone synthesis

The circulating levels of testosterone in males are regulated by the hypothalamic-pituitary-gonadal (HPG) axis, via a negative feedback loop (Fig. 2). Gonadotropin-releasing hormone (GnRH), released from the hypothalamus in a pulsatile manner, stimulates the synthesis and secretion of the gonadotropins luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary gland. LH is a dimeric glycoprotein containing an α - and β -subunit that binds to specific receptors in the Leydig cells in the testicles to induce synthesis and release of testosterone.¹ LH stimulates the synthesis of testosterone in both sexes, and FSH along with intra-testicular testosterone and DHT plays an important role in spermatogenesis in male.

LH is mainly regulated by testosterone, DHT and estradiol acting on hypothalamus and pituitary via negative feedback. The negative feedback on FSH is affected by inhibin, a gonadal hormone produced by the Sertoli cells in the testicles, which control the secretion of FSH.³⁵ It is well known that administration of exogenous AAS leads to suppression of the male HPG axis via negative feedback. The recovery to normal levels of testosterone, after ending abuse, may take months and even years, with a risk of manifest hypogonadism.³⁶

Hypothalamic-pituitary gonadal axis

Figure 2. Regulation of Leydig cell steroidogenesis by luteinizing hormone and a sensitive and rapid negative-feedback loop. Gonadotropin releasing hormone (GnRH) stimulates synthesis and secretion of both luteinizing hormone (LH) and follicle-stimulating hormone (FSH).

Synthetic derivatives of testosterone

Testosterone ingested orally in its unmodified form has no significant effect due to the first-pass effect of the liver.³⁷ To circumvent this, synthetic AAS with modifications of the testosterone molecule have been designed in attempt to reduce the rate of metabolism, maximize the anabolic effect, and minimize the undesired androgenic side effects (Fig. 3). There are three main classifications of androgen analogs. Class A modifications are esterification of the 17 β -hydroxyl group with any of the several carboxylic acid groups (e.g., testosterone cypionate). The longer carbon chains increase the lipophilic properties that makes the molecule more soluble in lipid vehicles. Intramuscular (i.m.) injection of testosterone esters result in a gradual release from the oily solution in which they are administered, thereby slowing the absorption of testosterone. Class B analogs have been alkylated at the 17 α -hydroxy position, such as methyltestosterone. Class C are produced by modification of the A, B or C ring, e.g., introduction of a double bond between C-1 and C-2 (boldenone), attachment of a methyl group at C-1 or C-2 (mesterolone) or attachment of pyrazol to the A ring through C-2 and C-3 (stanozolol) (Fig. 4). Alkylated analogs and those with modified ring structure are relatively resistant to hepatic metabolism and

are therefore available for oral use.³⁷ Even if attempts to find a pure anabolic steroid have not succeeded, varying affinities to the androgen receptor have been shown, e.g., stanozolol and nandrolone have predominantly anabolic effects in skeletal muscles.^{37,38} Non-reducible AAS (e.g. oxandrolone) have less androgenic side-effects such as acne, baldness, and prostatic hypertrophy, due to lower binding affinity for the androgen receptor.³⁹

Figure 3. Chemical structure of testosterone (4-androsten-17 β -ol-3-one)

Figure 4. Chemical structures of some synthetic anabolic androgenic steroids

Use and abuse of AAS

Androgenic disorders

The primary clinical use of testosterone is replacement therapy for pathological androgen deficiency.⁴⁰⁻⁴² This includes male hypogonadism (HG), a clinical syndrome that refers to a decrease in testosterone synthesis. Testosterone deficiency can either results from primary testicular disorder (hypergonadotropic) or occur secondary to hypothalamic-pituitary dysfunction (hypogonadotropic), or as a combination of both the defects. The symptoms of androgen deficiency include decreased libido, impaired erectile function, muscle weakness, depressed mood, and increased adiposity.

The clinical diagnosis of hypogonadism is based on consistent symptoms and signs of androgen deficiency in combination with a subnormal serum testosterone concentration.⁴³ According to current international guidelines, a serum testosterone level <8 nmol/L suggests deficiency, while a level above 12 nmol/L is considered normal.^{44, 45} LH and FSH can be measured to distinguish between hypergonadotropic and hypogonadotropic HG. In testicular dysfunction is LH elevated, indicating hypergonadotropic HG, while in hypogonadotropic HG, LH and FSH levels are normal together with a low testosterone concentration. The principal goal of testosterone therapy is to restore testosterone levels to the normal male range. Testosterone is available in three different formulations for clinical replacement therapy, depot injections, transdermal gels, and oral preparations. Long-acting i.m. injections of testosterone undecanoate (TU) (Nebido®) 1000 mg every 12th week is the most commonly used treatment regimen. Transdermal preparations have the advantage to mimic the normal physiological diurnal rhythm, thus representing the most physiological form of substitution. This preparation is applied to the skin on arms, shoulders, or thighs once a day in doses of 50-100 mg (1%) testosterone. Oral TU is not normally used in clinical therapy, due to the low and varying bioavailability necessitating administration three times a day. Monitoring of long-term testosterone replacement therapy should according to the Endocrine Society Guidelines include clinical evaluation of the patient's health status and measurements of total serum testosterone, haematocrit and prostate-specific antigen (PSA) at 3 to 6 months and at 12 months and annually after initiating testosterone therapy.⁴⁴

There has been a dramatic increase in the prescription of testosterone in most countries over more than a decade.² Testosterone prescription in

Sweden has increased threefold over 13 years (2006-2019), rising from 6600 to 17,100 individual male patients 40 years or older, and the number of dispensed prescriptions rose from 24,000 to 73,000 over the same period.⁴⁶ The prescription of transdermal products (gel, patch) was found to be remarkably high in Sweden compared to other countries world-wide. It has been suggested that the observed increase in testosterone prescribing appears to be for older men with age-related functional androgen deficiency (andropause).² Overuse of testosterone in healthy, older men with non-specific symptoms, such as decreased energy and sexual interest, may lead to adverse cardiovascular effects.^{47, 48}

Testosterone is also used off-label in cross-sex hormone treatment in biological females with gender dysphoria (GD).⁴⁹⁻⁵¹ GD is defined as the feeling of discomfort in individuals whose gender identity differs from their sex assigned at birth. Instead of the term transsexualism previously used, the current classification system of the American Psychiatric Association uses the term GD in diagnosis in incongruence between an individual's experienced gender and the assigned sex.⁵² The number of persons with a GD diagnosis have increased in Sweden during the last five years. The increase has been most pronounced among children and adolescents aged 13-17, especially among individuals assigned female at birth.⁵³ The diagnostic criteria include: persistent incongruence between gender identity and external sexual anatomy at birth, and the absence of a confounding mental disorder or other abnormality.⁵⁴ The treatment for individuals diagnosed with GD include psychotherapy, cross-sex hormone treatment and sex reassignment surgery if the patient desires.⁵⁵ Hormonal treatment is used to reduce the biological sex hormone levels and to replace endogenous sex hormone levels consistent with the individual's gender identity for development of the secondary sex characteristics. The physical changes induced by testosterone replacement therapy in females-to-males (transgender males (TM)), include increased muscle mass and decreased fat mass, deepening of the voice, increased facial and body hair, and increased sexual desire. The testosterone treatment in TM uses the same principles as the replacement therapy in HG male. The Clinical Practice Guidelines for treatment of gender dysphoric persons, suggest that total serum testosterone should be monitored every third months during the first year of hormone therapy and then once or twice yearly.⁵⁴

Abuse of AAS

The Swedish law prohibiting non-therapeutic use of AAS is quite unique compared to other countries world-wide. Of the five Nordic countries, e.g., the use of doping substances is only regulated in Sweden and Norway. The law covers certain doping substances that are criminalized; synthetic anabolic steroids, testosterone and its derivatives, growth hormones, and chemical substances which enhance the production or release of testosterone and its derivatives or of growth hormone. ⁷

The main reason for using AAS is the desire to increase muscle mass and strength to enhance athletic performance. Today, the great majority of the illicit AAS users in general society are individuals who take these drugs simply to become “big”, or to improve personal appearance for other reasons. ^{4,5,56} Other motives reported by males in a study at an out-patient clinic specialized in treatment of addiction in adults, were to become more aggressive/braver, to alleviate insecurity or low self-esteem or in preparation of committing a crime. ⁵⁷

AAS are used in complex programs of so called “cycling, stacking and pyramiding”. ⁵⁸ Cycles of 6-12 weeks are often used with complete abstinence in-between in the attempt to minimize side-effects. However, continuous use is also frequent. Several types of AAS used simultaneously, so called stacking is a common strategy based on the expectations to achieve a synergistic effect. In pyramiding, a low initial dose of AAS is administered which is gradually increased, often 5-100 times the therapeutic doses, and towards the end of the cycle tapered off. ⁵ The rationale for this abuse pattern is the expectations to avoid withdrawal symptoms, caused by decreased endogenous testosterone production, due to inhibition of the HPG axis.

Administration of AAS

AAS are available in a wide range of various preparations, including oral, injectable, and transdermal preparations. The most commonly used form of testosterone administration is i.m. injections of testosterone esters. Various testosterone esters are available on the illegal market, such as testosterone acetate, propionate, enanthate, benzoate, phenylpropionate, isocaproate, cypionate, decanoate, and undecanoate. The testosterone ester detection window is varying, depending on the length of the carbon side-chain. Testosterone esters administered as a depot injection, diffuses slowly into the bloodstream and even if the cleavage process by esterase enzymes starts immediately, the testosterone ester is still detectable in

blood. The elimination of i.m. testosterone esters is suggested to be absorption rate-limited depending on the length of the ester side chain, testosterone enanthate showed a half-life of 4.5 days compared to 29.5 days for testosterone undecanoate.⁵⁹

Multisubstance use

Studies have reported that the abuse of AAS is often combined with the misuse and abuse of other drugs, such as cannabis, amphetamine, heroin, cocaine, benzodiazepines and alcohol.⁶⁰⁻⁶³ The reasons given by AAS users for combining AAS with use of other drugs were to enhance the effects of AAS or to counteract the side-effects of AAS use, e.g., amphetamine was used to increase endurance and burn fat, opioids to decrease pain from training and cannabis and benzodiazepines to improve their sleep.⁶⁴ Furthermore, a mixed drug abuse was commonly observed in autopsied AAS users, who also were found to be more often involved in violent death (i.e. homicide and suicide) than users of other drugs, suggesting a particular high risk for AAS users to get involved in violence or to develop depressive symptoms.⁶⁵

Side effects of AAS abuse

Non-therapeutic use of AAS is associated with a wide spectrum of adverse somatic and psychiatric effects. The frequency and severity of side effects is quite variable, depending on several factors such as type of drug, dosage, duration of use and the sensitivity and response of the individuals.

The most common side-effects are acne vulgaris, characteristically distributed in the face, shoulders, chest and back, as well as oily skin, striae distensae, hirsutism, and alopecia.⁶⁶ Moreover, AAS abuse is associated with gynecomastia caused by aromatization of androgens to estrogens and testicular atrophy with reduced sperm count, due to disruption of the normal production of hormones in the body.^{67, 68} Liver toxicity has been described in AAS abusers, especially the orally active 17-alkylated analogs are connected with hepatotoxic effects, due to the slower clearance in the liver.⁶⁹ AAS can induce serious liver disorders, such as subcellular changes of hepatocytes, impaired excretion function, cholestasis, peliosis hepatis, and carcinomas.^{70, 71}

It has been reported that AAS abuse have toxic effects on the cardiovascular system, especially long-term abuse increases the risk of incidence of cardiovascular morbidity and mortality, such as coronary atherosclerosis,

hypertension, myocardial necrosis, left ventricular hypertrophy, thromboembolism, arrhythmia, acute myocardial infarction and sudden cardiac death.⁷²⁻⁷⁶ Several studies have demonstrated that self-administered AAS induce deleterious alterations in blood lipid profiles, with an elevation of low-density lipoprotein (LDL) and a decrease of high-density lipoprotein (HDL), together with a reduction in the levels of apolipoprotein A1 (Apo A1), which increases the risk of coronary heart disease.⁷⁷⁻⁸⁰ These lipid abnormalities have been shown to occur rapidly also in moderate abuse of AAS.⁸¹ Furthermore, high levels of testosterone can cause polycythemia.^{82, 83} The mechanism by which this occur remains incompletely understood. It has been shown that testosterone induced increase in hemoglobin and hematocrit is associated with increased erythropoietin and reduced hepcidin levels. It was proposed that testosterone stimulates erythropoiesis by increased erythropoietin secretion and recalibration of the set point of erythropoietin in relation to hemoglobin and by increasing iron utilization for erythropoiesis.⁸⁴

Moreover, AAS abuse has been shown to be associated with mental health problems, such as anxiety, moods swings, depression, aggression, suicide and violent behavior.^{4-6, 85-87} These side-effects appear to be idiosyncratic, maybe explained by other factors, such as use of narcotics, social background and diagnosis of personality disorder.⁸⁸ Recent evidence also suggests that supraphysiological doses of AAS may cause neurotoxicity and might be a risk factor for dementia.⁸⁹

Approximately 30% of AAS users develop a dependence syndrome, characterized by withdrawal symptoms and continued AAS use for years despite adverse side effects and social consequences.^{90, 91} It has been reported that males with AAS dependence, unlike non-dependent AAS users, has shown distinctive pattern of comorbid psychopathology, overlapping with that of other forms of substance dependence, particularly strong association with opioid dependence.⁶¹ This was supported by a previous study that showed association between AAS dependence and executive dysfunction.⁹² Furthermore, it has been shown that male dependent AAS users appear to have thinner cortex in widespread areas of the brain, specifically in pre-frontal areas involved in inhibitory control and emotional regulation, compared with non-dependent AAS users.⁹³ AAS dependence differs from classical drug addiction, in the way that AAS are not used to achieve an immediate “reward” of acute intoxication. Therefore, the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (*DSM-V*) substance dependence criteria are difficult to apply to AAS. There have been suggestions that the existing *DSM* criteria could be adapted for

diagnosing AAS dependence with only small interpretive changes ⁹⁴ and that the diagnosis of AAS dependence can be made reliable and valid. ⁹¹

Analytical techniques

Mass spectrometry in combination with gas chromatography or liquid chromatography (i.e., GC-MS, GC-MS/MS and LC-MS/MS) are today routinely used techniques for detection of steroids in humans applied in forensic toxicological investigations and in doping tests in sports. In clinical laboratories, immunoassays are widely used for measurement of plasma/serum concentrations of steroid hormones. However, these methods have proven to be less specific and less accurate, especially at low concentrations. This has been observed in androgen deficient men, women and children, showing interference by cross-reactivity with structurally related steroid hormones or synthetic derivatives. ⁹⁵⁻¹⁰⁰ Mass spectrometry methods are today considered gold standard because of their high accuracy, specificity and sensitivity and the ability of measurements of a great variety of compounds across a wide range of concentrations.

Immunoassays

Immunoassay methods are widely used in clinical laboratories for measurement of steroid hormones in plasma/serum such as cortisol, estradiol, progesterone, and testosterone. There are several reasons for using immunoassays in clinical laboratories, including fully automated assay procedures, availability of different types of commercial reagents from several vendors and the ability of high through-put testing on large analyze platforms. Immunoassay methods are bioanalytical methods based on a binding reaction between an antigen (analyte) and a highly specific antibody. ¹⁰¹ A variety of immunoassays are available for quantitative analysis. The most commonly used types are competitive and non-competitive (sandwich) methods. ¹⁰² The competitive immunoassays are based on the competition between the antigen and a constant amount of a labeled antigen for a limited amount of specific antibody. The non-competitive immunoassays use at least a pair of antibodies towards the antigen of interest. The capture antibody, highly specific for the antigen is occupied by the antigen in the added sample. The second antibody added binds to a different site (epitope) on the antigen that is “sandwiched”. Labels commonly used for detection antibody include enzymes (ELISA,

EIA), radioactive isotopes (RIA), luminescence marker (LIA, CLIA, ICMA), and electrochemiluminescence marker (ECLIA). The antibodies can be either polyclonal or monoclonal. Today, monoclonal antibodies are mostly used, due to their higher specificity against the analyte. The response signal is achieved by measuring the label activity in the bound or free fraction. In competitive immunoassay, the signal is inversely proportional to the concentration of the analyte in the sample, while in the sandwich methods, the signal is direct proportional to the concentration of the analyte in the sample. Competitive immunoassays were used for determination of testosterone in serum (ECLIA) and saliva (EIA) in paper II and III. Electrochemiluminescence immunoassay (ECLIA), a two-step competitive assay is illustrated in Fig. 5.

Figure 5. Test principle of two-step electrochemiluminescence assay (ECLIA)

Gas chromatography mass spectrometry (GC-MS)

Steroids have been investigated using GC-MS as early as the 1930s and in the 1960s the developed techniques were advanced enough for investigation of steroid metabolism and urinary steroid profiles were defined.^{103, 104} The sample work-up for GC-MS analysis includes conjugate hydrolysis and derivatization of the steroids to increase volatility and stability to produce optimum sensitivity and chromatographic resolution. Despite the complexity of sample preparation, GC-MS remains the most powerful discovery tool for determining the steroid metabolome and is widely used in steroid chemistry. In summary, the liquid analytes are vaporized in the GC injector and travelled through the heated column by an inert gas (such as helium). Separation of the analytes is based on relative solubility in the liquid phase coated on the inside of the chromatographic column and the vapor pressures of the analytes. The effluent from the GC

column passes from ambient pressure into a vacuum region in the mass spectrometer. The MS-inlet consists of a heated ion source, where high energy electrons strike the neutral analyte molecules, causing ionization and fragmentation. Electron ionization (EI) is the oldest and most commonly used technique for ionization. The charged particles are repelled and attracted by charged lenses into the mass analyser consisting of four parallel metal rods (quadrupole), where they by alterations of radiofrequency and direct current are separated by their mass-to-charge ratio (m/z) before entering the detector.

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

LC-MS/MS is today considered state of the art technology for quantitative determinations of drugs and metabolites in biological fluids, because tandem mass spectrometry is highly selective and thus effectively eliminate interferences by endogenous impurities. LC-MS/MS is an analytical technique that combines the separation power of LC with the mass analysis capabilities of MS. The LC system consists of an autosampler for injection of samples, high pressure pumps for continuously constant flow of the mobile phase and a chromatographic column (stationary phase), where the separation takes place. The analytes injected from a prepared sample are pumped through a stationary phase by an aqueous mobile phase and retained at different degrees depending on their chemical affinity and interactions between the mobile and stationary phase and are eluted sequentially. In reversed phase chromatography, most commonly used, the stationary phase consists of particles with a nonpolar surface (e.g., C18 bonded silica) and the mobile phase consists of a polar solution, usually a mixture of water and polar organic solvent (e.g., methanol, acetonitrile).

Several types of interfaces/ion sources can be used to transform the eluted liquid phase into the gas phase before the analytes are subjected to MS analysis. The most common interfaces currently used, are electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI). The liquid eluate from the LC is pumped through a capillary at high voltage and is nebulized at the tip of the capillary to form a fine spray of charged droplets. The droplets are further evaporated by heated and dry nitrogen gas before the analytes, ionized by the high voltage, are transferred into the high vacuum of the MS.

The tandem quadrupole mass spectrometer consists of two quadrupole mass analyzers separated by a collision cell (Fig. 6). By changing the

voltages and the radiofrequencies applied over the four parallel metal rods in the two quadrupoles, specific m/z values of precursor/product ion pairs can be selected and allowed travelling through the analyzer. The analyte ion of interest is selected by the first quadrupole, and then allowed to collide with an inert gas flow in the collision cell causing fragment ions. These fragments also called product ions obtained are preselected to pass through the second quadrupole to finally reach the detector. Due to the highly selective measurements with high sensitivity this instrument setting, called multiple reaction monitoring (MRM) is most commonly used for quantitative analysis by LC-MS/MS.

Figure 6. A tandem quadrupole mass spectrometer. Mass filter Q1 and Q3 can independently be set to stepping the voltages. The collision cell, Q2 contains low pressure of inert gas of argon or nitrogen to produce ion fragmentation by Collision Induced Dissociation (CID). (Illustration by Svante Vikingsson, former colleague at the National Board of Medicine)

Gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS)

GC-C-IRMS is a highly specialised technique used for detection of testosterone abuse in sport, determined by measuring the relative ratio of stable isotopes of carbon ($^{13}\text{C}/^{12}\text{C}$) in individual compounds in a sample mixture. The compounds in the prepared sample are separated by the GC

before the carbon containing compounds are passing through a combustion reactor (maintained at 940°C) where they are oxidatively combusted, followed by reduction and removal of water. The analyte gas formed (CO₂) are then ionized using EI before detection.

Carbon isotope ratios (CIRs) are expressed as $\delta^{13}\text{C}$ values traced on the Vienna Pee Dee Belemnite (VPBD) standard according to the equation: $\delta^{13}\text{C} = (\text{R}_{\text{sample}} / \text{R}_{\text{standard}}) - 1$ where R_{sample} is the measured $^{13}\text{C}/^{12}\text{C}$ isotope ratio for the sample and $\text{R}_{\text{standard}}$ is the measured $^{13}\text{C}/^{12}\text{C}$ isotope ratio for a defined standard. ¹⁰⁵ $\Delta\delta^{13}\text{C}$ values were established to compensate for biological variability depending on the diet and thresholds were introduced by WADA as a doping control measure. ¹⁰⁶ $\Delta\delta^{13}\text{C}$ are defined as the difference between the $\delta^{13}\text{C}$ of an endogenous reference compound (ERC) and a target compound (TC):

$$\Delta\delta^{13}\text{C} \text{ ‰} = \Delta\delta^{13}\text{C}_{\text{ERC}} - \Delta\delta^{13}\text{C}_{\text{TC}}$$

Anti-doping strategies

Since approximately 30 years the misuse of endogenous AAS in elite sports is detected via alterations in the urinary steroid profile. ¹⁰⁷ The main parameters initially analyzed in the WADA accredited laboratories are the concentrations and ratios of the glucuronidated testosterone metabolites androsterone, etiocholanolone, 5 α -androsterone-3 α ,17 β -diol, 5 β -androsterone-3 α ,17 β -diol and the glucuronidated epitestosterone. Administration of testosterone leads to abnormal steroid profiles, with the T/E ratio as the best elucidated and investigated parameter. With growing knowledge about factors influencing the steroid profile, for example genetic, pharmaceutical, pathological and analytical aspects, the strategy has since the implementation of the Athlete Biological Passport (ABP) changed from the use of population-based reference limits to individual reference ranges. ^{108, 109} The steroidal module of the ABP aims to detect doping with endogenous substances by longitudinal monitoring of several biomarkers. ¹¹⁰ In addition to the ABP, a suspect urine sample has to undergo a confirmation analysis by IRMS to determine the carbon isotope composition of targeted androgens. ¹¹¹ IRMS can differentiate between natural and synthetic endogenous steroids by the ratios of ^{13}C and ^{12}C because synthetic testosterone is supposed to have a ^{13}C abundance different from that of natural endogenous human steroids. The carbon isotope signature of endogenously produced testosterone depends on the diet, reflecting an average of all the carbon vegetal and animal material eaten by the individual, while synthetic testosterone is usually synthesized

from a single plant species material (phytosterols). Plant tissues reflect differences in isotopic composition of the carbon fixed in photosynthesis. The plant species commonly used is soy, which exhibits lower ^{13}C content as compared to human produced testosterone.¹¹²

Determination of AAS in biological matrices

Exogenous AAS

In forensic toxicology investigations and in anti-doping testing, detection of AAS and their metabolites is mainly performed in urine. Although urine in general is a very suitable matrix for determination of several substances with long detection windows, there are some issues particularly in testosterone testing. The non-polar parent AAS compound is often metabolized in the body prior to elimination and excretion in urine. The metabolic reactions include phase I and phase II metabolism aiming to convert the compounds into less potent, more polar and water-soluble metabolites. Phase I reactions involve hydroxylation, oxidation, and reduction by CYP450 enzymes, dehydrogenases, 5α -reductases and 5β -reductases. Phase II reactions, conjugations, are the main metabolic pathway for androgens as less than 3% of the total amount is excreted unconjugated in urine. Testosterone is mainly excreted as conjugates after glucuronidation with glucuronic acid, a reaction catalyzed by uridine diphospho-glucuronosyl transferase (UGT).

Urinary testosterone

Detection of exogenous testosterone is based on the determination of testosterone glucuronide/epitestosterone glucuronide ratio (T/E) by GC-MS. A T/E ≥ 12 together with a ratio of testosterone/luteinizing hormone (T/LH) > 400 nmol/IU is considered as a sign of illegal administration of testosterone in forensic investigations. In samples with elevated T/E ratio, the T/LH ratio is used to confirm exogenous administration of testosterone. The use of the urinary T/LH ratio was first suggested by Brooks et al. in 1979¹¹³ and has in further studies been reported to be useful together with the T/E ratio to detect testosterone doping in male.^{34, 114} This methodology is unfortunately not directly applicable to females, because oral contraceptive therapy suppresses LH secretion. However, in doping tests controlled by WADA a T/E ratio > 4 is considered suspicious and is

forwarded to an IRMS confirmation analysis.¹¹¹ This is however a complex and expensive technique and is not used in forensic doping investigations. Urine testing is a challenge due to inter-individual variations in testosterone excretion, caused by genetic differences. Individuals with a deletion polymorphism in UGT2B17 has been found to have no or negligible testosterone excretion.¹¹⁵ The deletion genotype was seven times more common in Koreans (67.0%) than in Swedish people (9.3%).¹¹⁶ The T/E and T/LH ratios for detecting testosterone doping is unfortunately not reliable in individuals with natural low T/E values due to this polymorphism.

Serum testosterone

The circulating testosterone is to approximately 97-98% bound to plasma proteins. In male is 44% and in females 66% of testosterone bound with high affinity to sex hormone-binding globulin (SHBG) and the remaining major part is with much lower affinity bound to human serum albumin, leaving only 1-2% as free circulating testosterone.¹¹⁷ The free hormone hypothesis, which has been questioned, states that only unbound testosterone is biologically active in target tissues. An alternative hypothesis is that free testosterone and weakly albumin-bound testosterone both contribute to androgen effects.¹¹⁷ The sum of the free testosterone and weakly bound testosterone is referred to as the bioavailable testosterone. It has also been suggested that SHBG bound testosterone can act on prostate and testicles.¹¹⁸ Although various procedures have been described for the measurement of free testosterone, e.g., ultrafiltration, equilibrium dialysis and ammonium sulphate precipitation, these methods are too complex and time consuming for routine use in clinical laboratories.

Free- and bioavailable testosterone can be calculated by mass action binding algorithms, and the measured values of total serum testosterone, SHBG and albumin.¹¹⁹⁻¹²¹ The Vermeulen equation has been the most widely applied. In a previous study comparing five algorithms, large differences were found between the results of the calculations.¹²² Furthermore, it was shown that commonly used formulae overestimate free testosterone in male relative to equilibrium dialysis measurement.¹²³ The accuracy of the calculations largely depends on the methods used for measurement, SHBG concentrations, choice of affinity constant and other factors, such as age, gender, somatic diseases and medication.¹²⁴ The mathematical models used for calculation of free circulating testosterone

assume that all SHBG molecules react similarly immunologically and that the two binding-sites on the SHBG homodimer have identical binding properties.¹²⁵ Recently it was shown that the binding of testosterone to SHBG is a more complex process including multi-step interactions and that this new model better correlate to equilibrium dialysis in both men and women.¹²⁶ The calculated estimates of free testosterone have limitations and it is an on-going debate about the validity of the methods to calculate free testosterone.¹²⁷ “Free androgen index” (FAI) is sometimes used as a measurement of estimated free testosterone for which testosterone is simply divided by SHBG. However, FAI is no longer recommended, as it is not valid in men. An implied assumption of the FAI was that the binding capacity of SHBG should greatly exceed the concentration of its ligand testosterone.¹²⁸ A recent study reported that FAI is not a reliable indicator of free testosterone in women when the SHBG concentration is low and could give misleading information in the assessment of hyperandrogenism.¹²⁹

Salivary testosterone

Saliva is attractive as a diagnostic matrix because salivary steroid levels are supposed to reflect the free circulating levels in plasma as only free neutral lipid-soluble and unconjugated molecules such as steroids are able to pass through the acinar cells of the salivary glands into the saliva.¹³⁰ The majority of the oral fluid is produced by three pairs of salivary glands (parotid, submandibular and sublingual) with a small contribution from the buccal glands which line the mouth. Saliva also contains a small amount of gingival crevicular fluid that leaks out from the tooth-gum margin.^{131, 132} The transfer of free biomolecules from the circulating blood through the cell membrane to oral fluids occurs through different mechanisms, such as passive diffusion or active transport, depending upon the physiochemical properties of the molecule, such as molecular weight, protein binding and charge. The most common route for small (<500 Da) lipophilic and unconjugated molecules such as testosterone, is by rapid passive diffusion, and as such the concentration of the testosterone in saliva is independent of the rate of saliva flow.^{131, 133}

However, there are several factors that can influence the process of sample collection. The largest confounder of salivary concentrations of drugs is blood leakage into the oral mucosa as a result of microinjuries following e.g., tooth brushing. The risk of contamination of the saliva with blood is of particular importance when measuring testosterone, because serum

testosterone concentrations are approximately 50 times higher than testosterone levels in saliva.¹³⁴ Increased testosterone levels were observed immediately after tooth brushing and remained elevated for 30 minutes.¹³⁵ To reduce the risk of blood leakage into saliva, it is recommended to avoid tooth brushing or use of dental floss for at least 1 h prior to sample collection. In testosterone testing, samples should be visually inspected for blood contamination or verified by detection of the amount of hemoglobin.¹³⁶ In addition, quantitative testing results can be affected by food and drink intake and chewing (chewing gum) which should be avoided 1-2 h prior to saliva collection. An earlier in vitro study found that testosterone was more actively metabolized in submandibular glands compared to parotid glands in male subjects.¹³⁷ The main metabolites formed by the action of 17 β -hydroxysteroid dehydrogenase and 5 α -steroid reductase, were androstenedione and DHT. It was however concluded that this is not an important source of error in testosterone measurement, due to the rapid passage by passive diffusion.

There are several devices available for sample collection of saliva. The most common sample collection methods used for measurement of steroid hormones have over time been cotton or synthetic swabs and collection of whole saliva by passive drool. Testosterone concentrations were found to be lower when using synthetic Salivettes® and were higher when using cotton Salivettes®, compared to the collection of whole saliva by passive drool.¹³⁸ It has been suggested that synthetic swabs absorb testosterone and that the results with cotton swabs might be due to interfering substances present in the cotton.^{139, 140} Collection of whole saliva by passive drool is therefore considered the most reliable method for testosterone measurement.

AIMS

The overall aim of this thesis was to investigate the abuse of AAS, in particular testosterone, from a forensic perspective and to develop better analytical methods to determine testosterone in different biological matrices in order to improve the detection and interpretation ability in forensic investigations and diagnostics in clinical assessments.

The specific aims of the respective papers were:

- I) To investigate and describe the abuse of AAS in forensic cases in Sweden, the prevalence of use, age and gender, type of AAS, concentration levels and the co-abuse of AAS and other illicit and licit drugs
- II) To study the relationship between testosterone in serum, saliva, and urine during testosterone replacement therapy in male hypogonadism and gender dysphoria
- III) To study the sensitivity of the urinary detection criteria T/E and T/LH and the possibility of false negative testosterone results in forensic cases
- IV) To develop an LC-MS/MS method, with high specificity, accuracy, and sensitivity, to determine testosterone in serum and saliva in order to be applied on clinical and forensic samples

MATERIAL AND METHODS

In this section, the subjects and methods used in the papers included in this thesis, are presented. All studies were approved by the Regional Ethical Board in Linköping, Sweden, and the Swedish Ethical Review Authority.

Subjects, sampling, and procedures

Paper I

The national forensic toxicology database (ToxBase) was used to identify suspected forensic doping cases, and the analytical toxicology results, during the years 1999-2009 (n=6362). The samples were collected by the police units in Sweden at suspected doping offences, most often in connection with other drug-related crimes as well, such as petty drug offences, driving under the influence of drugs (DUID) and violent crimes. A doping screening was only performed at a specific request by the police. Additionally, urine samples collected by the Swedish correctional institutions (n=5779) were included. The data consisted of prevalence of AAS abuse, type and concentration levels of confirmed AAS, age and sex. Additional information on the AAS-positive cases were retrieved and evaluated to assess the presence of other illicit and licit drugs in combination with AAS (co-abuse), such as narcotics, pharmaceuticals, and ethanol. The drug abuse pattern of the AAS users were compared to the drug abuse pattern of drug abusers not screened for AAS (n=148,585).

Paper II

Forty outpatients at the Department of Endocrinology, University hospital, Linköping, Sweden were recruited to the study. Twenty-three males, 18-68 years, diagnosed with primary or secondary HG, seventeen GD (TM), 18-55 years, and a reference group of 32 healthy males were investigated. There were two dropouts in the GD group. Data were collected between March 2010 and March 2014.

The patients were treated with long-acting TU, (Nebido®) 1000 mg i.m. injections administered every 12th week. Blood, saliva, and urine samples were taken prior to administration of TU and 4, 7, 14 and 28 days after the first as well as after the last injection after one year. All patients followed the same protocol, except for the patients that initiated their therapy at the study start (naïve) (13 HG and 10 GD (TM)), who were given a second injection six weeks after the first injection and thereafter at 12 weeks intervals. Sampling for saliva, blood and urine was performed before 10 am for all individuals. Saliva was collected prior to blood sampling. The first morning urine was collected.

Paper III

Subjects with both a serum and urine sample collected by the police authority in Sweden were consecutively selected from authentic forensic routine cases suspected of doping offence during 2017 and 2018. Of the total number of 1509 cases of requested doping screening, 258 cases were finally included in the study. Out of these, samples from 58 males between 19-53 years old (median age 32), with a T/E more than 4 and less than 40 were further investigated. The threshold T/E >4 was set, as this is the threshold used in anti-doping tests in sports. The suspected doping offenders were simultaneously suspected and investigated for other drug-related crimes as well, such as impaired driving, petty drug offences and violent crimes. The origin of testosterone and its metabolites were confirmed by means of GC-C-IRMS. One subject with a T/E value of 4.7 was excluded due to inconclusive IRMS analysis caused by insufficient urine volume.

Paper IV

An ESI-LC-MS/MS method for determination of testosterone in serum and saliva was developed and validated at the Department of Clinical Pharmacology at the University Hospital in Linköping. The strategy was to utilize a sensitive mass spectrometric method that could be applied on both clinical and forensic samples, using a uniform sample preparation. The system consisted of an Acquity Ultra Performance Liquid Chromatography I-Class-Plus system coupled to a Xevo TQ-XS mass spectrometer (Waters Corporation, Milford, MA, USA). Separation of the analytes were achieved on an HSS-T3 C18 column (2.1 x 50 mm, particle size 1.8 µm) protected by a guard column BEH C18 VanGuard™ (2.1 x 5 mm, particle size 1.7 µm) (Waters Milford, MA, USA) at 50°C. Mass detection was performed with

ESI operating in positive ion mode. Analytes were monitored by MRM and quantified using $^{13}\text{C}_3$ -testosterone as internal standard. The method was optimized for the transitions $289.2 > 97.1$ and $289.2 > 109.2$ for testosterone and $292.2 > 100.2$ for the internal standard $^{13}\text{C}_3$ -testosterone. The cone voltage was 20 and 40 V and the collision energy was 20 eV for both testosterone transitions and 26 eV for $^{13}\text{C}_3$ -testosterone. Argon was used as collision gas.

Method validation was performed in according to procedures recommended for forensic toxicological analyses described in a publication by Peters et al.¹⁴¹ and the guidelines used at the accredited Swedish national forensic laboratory. The following parameters were evaluated: selectivity, calibration model (linearity), limit of quantitation (LOQ), limit of detection (LOD), accuracy, precision (repeatability, intermediate precision), matrix effects and recovery. Selectivity was evaluated using authentic serum and saliva samples, but as testosterone is naturally present in body fluids the approach suggested by Botelho et al.¹⁴² was used. The mean ratio of the peak areas of quantitation ion/confirmation ion (QI/CI) of testosterone in the authentic serum and saliva samples were compared against those obtained in serum and saliva calibrators. Interferences were assumed if the QI/CI ratio differed more than 20%. Different structural analogs of testosterone were also measured to ensure chromatographic separation.

Calibration model and linearity was evaluated in both human serum (DC Mass Spect Gold) and MilliQ-water. A mean value deviation of <10% of the nominal value for each level was considered the measuring range of the method. To determine the LOQ, human serum (BioIVT), spiked with testosterone concentrations below the lower calibration level, were analyzed in five replicates at each level. The concentration in serum was considered to be acceptable when precision (CV) $\leq 25\%$ and accuracy $\pm 25\%$, were met. LOQ for testosterone in saliva was determined by analysis of five replicates of authentic human saliva samples, using the signal to noise ratio (S/N) > 10 as criteria for LOQ. Estimation of the LOD in serum and saliva were based on the $S/N > 3$.

Accuracy was defined as the relative difference between the measured concentration and the theoretical value and a deviation less than 20% was considered acceptable. Repeatability and reproducibility were calculated as the relative standard deviation (RSD) expressed as percentages. A maximum deviation of 15% was accepted for all levels.

Matrix effects and recovery were estimated according to Matuszewski et al.,¹⁴³ together with the recommendation by Hess et al.¹⁴⁴ to complement the

approach of three sets of samples with a fourth set of blank matrix samples for endogenous substances. Recovery and matrix effects were calculated using the following formulas by Hess et al.¹⁴⁴ (A = absolute peak area of target peak of analyte)

$$\text{recovery [\%]} = \frac{A_{\text{extended matrix}} - A_{\text{blank matrix}}}{A_{\text{extended extract}} - A_{\text{blank matrix}}}$$

$$\text{matrix effects [\%]} = \frac{A_{\text{extended extract}} - A_{\text{blank matrix}}}{A_{\text{control}}}$$

The assay was applied to serum and saliva samples from ten voluntary healthy male and female subjects (41-65 years: mean 53 years), eleven out-clinical patients (27-63 years: mean 40 years) with androgen disorders, at the Department of Endocrinology at the University Hospital, Linköping, Sweden, and serum samples from sixteen males and two females suspected of doping offence (19-48 years: mean 29 years), at the National Board of Forensic Medicine, Linköping, Sweden. The 11 patients were; females diagnosed with Turner syndrome (n=5), Congenital Adrenal Hypoplasia (n=1) and pituitary insufficiency (n=1) and males diagnosed with primary HG (n=3), and GD (MT) (n=1). Saliva and venous blood were collected at the clinic between 8-10 am. The blood samples in the forensic investigations were collected by the police at the time of the crime.

Methods

In paper I, III and IV other illicit and licit drugs were identified in the routine management of the authentic forensic cases. Suspected doping offenders are most often suspected and investigated for other drug-related crimes as well, such as impaired driving, illicit use of drugs e.g., narcotics and pharmaceuticals and violence related crimes. In drug abuse testing at the Swedish national forensic toxicology laboratory, an initial screening of specimens is performed by enzyme immunoassay to indicate the presence of drugs. Seven classes of drugs are routinely tested for, amphetamines, cannabis, cocaine and metabolites, opiates, tramadol, buprenorphine, and benzodiazepines. The positive screening results are confirmed by a secondary analysis by mass spectrometry. Blood and urine are analyzed for

other drugs on request by the police, e.g., ethanol, GHB and new psychoactive substances (NPS).

Urinary analyses

The doping screening in urine (paper I, II and III) was performed by GC-MS and verified by GC-MS and LC-MS/MS. Since 2008, AAS are determined at the department of forensic toxicology in Linköping. During 1999-2004 the AAS analyses were performed by the Doping Control Laboratory, United Medix Laboratories Ltd in Helsinki, Finland, and during 2005-2007 the AAS analyses were performed by the Doping Control Laboratory at Karolinska University Hospital in Sweden. Screening and verification of AAS were performed by GC-MS at the Doping Control Laboratory in Finland as well as at the Doping Control Laboratory in Sweden, who later also introduced LC-MS/MS. An LOQ of 2-20 ng/ml urine was used by the Doping Laboratory in Finland and an LOQ of 10 ng/ml urine for all AAS and the metabolites was used at the Doping laboratory in Sweden and at the forensic toxicology laboratory.

Detection of testosterone abuse was based on the urinary T/E ratio, calculated by the GC-MS peak areas (m/z 432). A T/E ≥ 10 was considered positive at the Doping Laboratories in Finland and Sweden and at the forensic toxicology laboratory a T/E ≥ 12 was considered positive. In case of exceeded T/E ratio at the Doping laboratory in Sweden and at the forensic toxicology laboratory, the urines were further investigated by measuring LH in urine and the T/LH ratio, using a T/LH threshold of 350 and 400 nmol/IU, respectively. LH in urine was determined by sandwich immunoassay at the Division of Clinical Chemistry, Linköping University Hospital, Sweden (paper I) and at the Department of Clinical Chemistry, Northern Älvsborg County Hospital (NÄL), Trollhättan, Sweden using Immulite 2000XPi (Siemens, Munich, Germany) (paper III).

The GC-C-IRMS analysis and steroid profile measured in paper III were performed at the Swedish Doping Control Laboratory in Stockholm in accordance to requirements of TDIRMS2019¹⁰⁶ and TDEAAS2018.¹¹¹ The isotopic contents were determined using IRMS Delta V Plus (Thermo Fisher Scientific, Bremen, Germany) coupled to Agilent 7890A GC-System (Agilent Technologies, Santa-Clara, CA, USA). The steroid profile in urine included androsterone (A), etiocholanolone (Etio), 5 α -androstane-3 α ,17 β -diol (5 α Adiol), 5 β -androstane-3 α ,17 β -diol (5 β Adiol), testosterone (T) and epitestosterone (E) and the ratios; T/E, A/T, A/Etio, 5 α Adiol/5 β Adiol and 5 α Adiol/E.

Serum analyses

Venous blood in paper II and IV was collected in clot activator tubes (Vacuette®, Greiner, Hettich, Sweden) and centrifuged at 2500 *g* for 5 min before serum was separated from blood. In paper II, serum was analyzed in daily routine at the laboratory, while in paper IV serum was aliquoted and stored at -80°C until batch analysis.

Measurement of serum testosterone, SHBG and albumin, in paper II, III and IV, were performed by immunoassays at the Division of Clinical Chemistry at Linköping University Hospital, Sweden. Total serum testosterone was determined with the Roche Elecsys® testosterone II assay using a high-affinity sheep MAB with electrochemiluminescence detection on a cobas e602 (Roche, Basel, Switzerland). SHBG was determined using cobas e602 and P-albumin by turbidimetry on ADVIA 1800 (Siemens, Munich, Germany) (paper II) and by Roche cobas c701 (paper IV). Free serum testosterone was calculated using the Vermeulen equation, based on the measurement of total serum testosterone, SHBG and albumin.¹¹⁹ Serum and salivary testosterone in paper IV were quantified by the LC-MS/MS method.

Salivary analyses

In paper II, saliva was collected using Salimetrics® oral swabs and Salimetrics® storage tubes, while in paper IV, saliva samples for testosterone analyses were collected by passive drool using Salimetrics® saliva collection aid as recommended by Büttler et al.¹³⁸

Salivary testosterone was in paper II determined with the Salimetrics® expanded range enzyme immunoassay kit (Salimetrics LLC, State College, PA, USA) applied on a Tecan Freedom Evolyzer (Tecan AG, Männedorf, Switzerland). Salivary testosterone was in paper IV determined by the described LC-MS/MS method. To prevent blood contamination of the saliva samples, the participants were requested to avoid tooth brushing, and to avoid food or fluid intake or to use of tobacco for at least 1 hour prior to sample collection. Saliva was checked for hemoglobin (Hb) with Hemocue Plasma/Hb Low (Hemocue AB, Ängelholm, Sweden) and Hb > 2 g/L was set as cut-off value for rejecting the sample (paper II). The saliva samples were in paper II stored at -20°C until assayed in batch and in paper IV stored at -80°C until analysis, when thawed at room temperature and centrifuged at 1500 *g* for 10 min.

Statistics

The statistical analyses in paper II were performed using between-groups comparisons by independent samples *t*-test and paired *t*-test. Correlations were evaluated using Pearson's coefficient. Results are given in mean $\pm$ SD, and 95% CI. Statistical significance was considered at $\leq 5\%$ level ($p \leq 0.05$). All statistical calculations in paper II were performed using IBM® SPSS® Statistics version 23. The statistical analyses in paper III were carried out using sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV), where the results of the confirmation test, IRMS, was seen as the true state (i.e., whether or not the subject had abused testosterone). All concentrations measured below the limit of quantification (LOQ) were set at LOQ of the method. For these statistics, 95% confidence intervals were calculated using percentile with 2000 bootstrap samples. All statistical calculations in paper III were performed using IBM® SPSS® Statistics version 26. Testosterone concentrations are given in pg/mL or nmol/L (1 pg/mL = 3.467 nmol/L).

RESULTS

Paper I - Anabolic androgenic steroids in police cases in Sweden 1999-2009

In this study the abuse of AAS in Swedish forensic cases suspected of drug-related crimes was investigated. The number of suspected doping offences markedly increased after the law came into force in 1999, especially during the years since 2006. Of the total number of 6362 suspected doping offences, 31.5% were tested positive for one or more exogenous AAS. The majority of the abusers were primarily young men 26.2 ± 6.2 years old (15-53 years), and only 15 (0.8%) were females at a mean age of 29.6 ± 6.5 years old (18-42 years). Of the 5779 doping screenings of the inmates at the correctional institutions during the same period, 11.5% were tested positive, a number that decreased from 19% (1999) to 6.6% (2009).

The most commonly detected AAS were nandrolone (62%), testosterone (36%) followed by methandienone, stanozolol, boldenone, trenbolone and drostanolone. A follow-up search in the database for toxicological results during the years 2010 to 2018 showed that testosterone was the most commonly detected AAS (unpublished data) (Fig. 7). Drostanolone and trenbolone were also more frequently detected compared to the previous period.

Figure 7. Anabolic androgenic steroids detected in the urine samples in the forensic doping cases 1999-2009 and 2010-2018.

Fifty-six percent of the positive cases were found to have used more than one type of AAS. In the majority of the cases one or two AAS were used simultaneously, but even up to eight different AAS were detected. High concentrations were measured both for unchanged steroids (2000-10,000 ng/ml) and their metabolites (>30,000 ng/ml).

Sixty percent of the AAS users also used other illicit and licit substances. The most commonly co-abused substances were cannabis (36.6%), amphetamines (including methamphetamine and MDMA) (36.6%), benzodiazepines (28.2%), cocaine (18.1%), opiates (morphine and codeine) (6.4%), 6-acetylmorphine (heroin) were present in 12% of the opiate cases. Ephedrine and GHB were both found in 5.7% of the cases. Pharmaceuticals such as analgesics (tramadol, buprenorphine, dextropropoxyphene, oxycodone, methadone, phenazone), antidepressants, hypnotics, anti-estrogens, and muscle relaxants were detected in 7.2% of the cases. The drug abuse pattern of the AAS abusers corresponded to the abuse pattern of the other drug addicts (AAS-non-measured). Amphetamine and cannabis were most frequently detected in both groups, but to a wider extent noted in the non-AAS group. Opiates were more common within the non-AAS group, while cocaine and benzodiazepines were more frequently detected in AAS abusers (Fig. 8).

Figure 8. Distribution of illicit and licit drugs in AAS users, compared to drug addicts in cases concerning petty drug offences.

Paper II – Relationship between testosterone in serum, saliva and urine during treatment with intramuscular testosterone undecanoate in gender dysphoria and male hypogonadism

The aim of this study was to investigate the relationship between testosterone in different matrices to gain knowledge about the concentration levels achieved during conventional clinical replacement therapy with TU. Salivary testosterone correlated with statistical significance to total and calculated free testosterone in both controls and patients ($r = 0.74$, $p < 0.001$ in both). Weak correlation was noted between salivary- and urinary testosterone ($r = 0.40$, $p < 0.001$). The correlation

between salivary- and serum free testosterone was stronger in the GD (TM) ($r = 0.82$) group than in the HG group ($r = 0.61$) ($p < 0.001$ in all cases).

Serum testosterone levels were at baseline significantly lower in male HG and GD (TM) compared to the control group. Extremely high baseline salivary testosterone (1.91-2.53 nmol/L) were noted in three of the GD (TM) and one HG male, which might be explained by self-administration of testosterone gel previously prescribed.

After 12 months TU therapy, the GD (TM) group showed significantly higher mean salivary testosterone values (0.77 ± 0.35 nmol/L) compared to the HG male (0.53 ± 0.23 nmol/L) and the controls (0.46 ± 0.15 nmol/L). However, no statistically significant differences in either serum testosterone or urinary testosterone values were noted between the groups. Ninety-five percent of the subjects had a total serum testosterone concentration within the range of a normal male after 12 months replacement therapy. Lower trough values of total serum, salivary, and urinary testosterone were noted in the subjects in the GD (TM) group that were oophorectomized compared to the subjects with remaining ovaries.

Markedly elevated salivary testosterone concentrations were observed in the GD (TM) patients 7-14 days after the TU injection, with a maximum value of 7.20 nmol/L (Fig. 9). The mean maximum testosterone concentrations measured in all patients after the first and last TU injections after 12 months: saliva 1.85 ± 1.38 nmol/L, serum 42 ± 22 nmol/L and urine 47 ± 37 nmol/mmol creatinine. There were no significant differences in salivary, serum or urinary testosterone maximum levels between the groups or between the maximum levels measured after the first TU injection and the injection after 12 months. Significantly higher mean ratio of maximum salivary testosterone per kg body weight was noted in the GD (TM) group compared to the HG males ($p = 0.03$). Salivary and total serum testosterone were negatively correlated to weight, BMI, and waist at maximum levels 7-14 days after injection for all patients ($p < 0.001$), but not at trough values after 12 months TU treatment.

In short, this study showed that testosterone in saliva correlated well with total and free serum testosterone in both patients and controls. The trough values in saliva were significantly higher in the GD (TM) group compared to the HG male and the control group. Extremely high testosterone in saliva were noted in the some of the GD (TM) patients 7-14 days post-injection.

Figure 9. Salivary testosterone concentrations measured in HG males and GD (TM) 7-14 days after i.m. injection of 1000 mg testosterone undecanoate (peak values) and after 12 months treatment (trough values) and in the control group (controls).

Paper III – False negative results in testosterone doping in forensic cases: sensitivity of the urinary detection criteria T/E and T/LH

In this study the sensitivity and specificity of the current criteria used at the Swedish national forensic laboratory in detection of testosterone abuse in suspected forensic doping cases was investigated. Twenty-five of the 57 subjects analyzed had a T/E value 4-12 and 32 subjects had a T/E 12-40. Twenty-six (46%) of the subjects were considered testosterone abusers using the current criteria T/E ≥ 12 in combination with T/LH > 400 nmol/IU. On the other hand, the analysis by IRMS confirmed 47 (82%) positive subjects, thus 21 (37%) subjects were considered false negatives using the forensic criteria. The negative predictive value (NPV) was limited to 32% (95% CI: 16-50) with a sensitivity of 55%. No false positive subjects were found (Table 1).

Using a threshold of T/E > 9 as a single criterion, the positivity rate increased to 39 (68%) together with a decreased proportion of false negatives to 8 (26%), a higher NPV of 56% (95% CI: 31-80) and a clear increase in sensitivity to 83%.

Table 1. Cross-tabulations over the results for the current and alternative criteria and the confirmation results by IRMS.

Test result	T/E ≥ 12 and T/LH > 400 nmol/IU			T/E > 9		
	Confirmation test (IRMS)			Confirmation test (IRMS)		
	Positive	Negative	Total	Positive	Negative	Total
Positive	26 (TP)	0 (FP)	26	39 (TP)	0 (FP)	39
Negative	21 (FN)	10 (TN)	31	8 (FN)	10 (TN)	18
Total	47	10	57	47	10	57
NPV	32% (95% CI: 16-50)			56% (95% CI: 31-80)		
PPV	100% -			100% -		
Sensitivity	55% (95% CI: 41-70)			83% (95% CI: 72-93)		
Specificity	100% -			100% -		

TP: true positives, FP: false positives, FN: false negatives, TN: true negatives

The T/LH values were found widespread over the T/E range 4-40 and the majority of the false negatives were found at T/E ratios below 12 (Fig. 10).

Figure 10. A scatterplot showing the T/E and T/LH (log nmol/IU) of the confirmed negative, false negative and positive subjects. The dotted lines show the thresholds of $T/E \geq 12$ and $T/LH > 400$ nmol/IU. The shaded area shows the subjects that tested negative using a threshold of $T/E > 9$.

The urinary steroid profile concentrations and ratios used in doping analyses in sports overlapped between the groups, except for the ratio $5\alpha\text{Adiol}/E > 10$ that in combination with the cut-off $T/E > 9$ resulted in an NPV of 56%. One individual that tested negative with IRMS showed an abnormal steroid profile.

Co-abuse of other AAS (36%) and other illicit drugs (60%), such as narcotics and pharmaceuticals, in addition to testosterone were observed in the confirmed positive subjects. Other exogenous AAS (60%) and other illicit drugs (80%) were also found in the confirmed negative subjects. The most commonly detected AAS overall were nandrolone (38%), drostanolone 38%), trenbolone (29%), methandienone (17%), stanozolol (17%), boldenone (13%) and mesterolone (4%). Twenty-three percent of the subjects were positive for testosterone only. The most commonly co-used narcotics found were cannabis, amphetamine, cocaine, and benzodiazepines.

In this study it was concluded that there are a great number of false negative test results in detection of testosterone abuse using the current ratios $T/E \geq 12$ in combination with $T/LH > 400$ nmol/IU. The weak sensitivity of 55% (NPV 32%) indicates a need to introduce new analytical strategies to increase sensitivity in detection of testosterone abuse in forensic doping investigations.

Paper IV – Determination of testosterone in serum and saliva by liquid chromatography-tandem mass spectrometry: an accurate and sensitive method applied on clinical and forensic samples

The aim of this study was to develop and validate an accurate and sensitive LC-MS/MS method for determination of testosterone in serum and saliva. A uniform protocol was developed for sample pretreatment and quantitation procedure using 200 μ L sample volume and solid-phase extraction (SPE) on a 96-well plate after protein precipitation with methanol and dilution with water. The human blank serum, used throughout the study, DC Mass Spect Gold and BioIVT, charcoal-stripped and filtered serum, showed measurable amounts of endogenous testosterone present, 2-4 pg/mL (DC Mass Spect Gold), depending on the batch, and 1.5 pg/mL (BioIVT). The testosterone levels measured in the human blank serum DC Mass Spect Gold was in accordance with the findings in a recent study.¹⁴⁵ The method was linear between 2-1000 pg/mL. LOQ was 4.0 pg/mL and LOD 2.0 pg/mL for serum and saliva. The calibration model with weighting $1/x^2$ showed good linearity ($r^2 > 0.998$) with a mean accuracy value within 90-110%. The accuracy was well within 90-110% for all QCs in serum and saliva. The precision CV for testosterone was <6.2% in serum and <9.2% in saliva (Table 2).

Table 2. Validation data. Within-run and between-day precision and accuracy for testosterone in serum and saliva.

Testosterone Concentration (pg/mL)	Within-run (n=5)			Between-day (n=10)		
	Mean $\pm$ SD (pg/mL)	CV (%)	Accuracy (%)	Mean $\pm$ SD (pg/mL)	CV (%)	Accuracy (%)
Serum						
10	10.7 $\pm$ 0.68	6.3	107	10.1 $\pm$ 0.63	6.2	101
750	763 $\pm$ 57	7.4	102	742 $\pm$ 40	5.5	99
Saliva						
5.4	5.38 $\pm$ 0.17	3.1	100	5.12 $\pm$ 0.35	6.9	95
65	65.2 $\pm$ 0.62	1.0	100	60.7 $\pm$ 4.0	6.7	93
252	252 $\pm$ 5.5	5.4	100	238 $\pm$ 21.8	9.2	95

Total recoveries in serum were 61% (146 pg/mL) and 57% (332 pg/mL), and in saliva 99% (23 pg/mL) and 107% (186 pg/mL). The matrix effects calculated from the mean peak areas were 98% and 128% in serum and 83% and 75% in saliva, at 20 and 200 pg/mL, respectively. Endogenous compounds in saliva co-eluting with testosterone slightly suppress the ionization, which is compensated by the nearly identical suppression of the peak area of the internal standard $^{13}\text{C}_3$ -testosterone of 79% and 73%. A value >100% in serum indicates ionization enhancement, which was compensated for by the enhancement of the internal standard by 118% and 127%.

In healthy male and female, serum testosterone was in the range 3016-4844 pg/mL and 125-398 pg/mL, respectively, and salivary testosterone 25-59 pg/mL and 2.0-17 pg/mL, respectively. About four times higher mean concentration of testosterone in serum was found in the forensic cases with detected exogenous administration of testosterone ($T/E \geq 12$), compared to the healthy male references. A serum testosterone concentration <202 pg/mL (<0.7 nmol/L), which is the LOQ in routine assays in many clinical laboratories and the threshold level in castrated prostate cancer patients, was measured in nine of the subjects, six clinical patients and three healthy females. In general, inter-individual variations were greater in the clinical material as compared to healthy volunteers, especially in saliva, as expected with this heterogeneous group.

The method was successfully applied to the analysis of human serum and saliva from clinical patients with various androgen disorders and healthy adults, as well as to serum samples from forensic doping investigations. The application showed the ability to cover a wide range of concentrations in multiple discipline purposes, from detection of the very low pg/mL testosterone concentration in the diagnosis and management of androgen disorders to the high supraphysiological $\mu\text{g/mL}$ concentrations found in the samples in cases of suspected testosterone abuse.

DISCUSSION

Several aspects of doping analysis are of interest in many related fields, e.g., forensic toxicology, clinical laboratory medicine, endocrinology, and sport drug testing, all of which benefit from co-operation and interaction. Besides the methodological similarities and problems, some findings and developments in one field may also be of interest to the others.

The aims of the present thesis have been to investigate the abuse of AAS, the relationship between testosterone levels in different matrices during physiological replacement therapy and to develop accurate methods for detection of testosterone. In the following paragraph, the main findings and methodological considerations will be discussed.

Abuse of AAS

The main finding in paper I was the frequently detected illicit use of AAS in the forensic cases, strongly indicating that AAS abuse is a serious public health issue. The number of doping investigations requested by the police units have markedly increased since 2006. The most reasonable interpretation of this finding is that doping in society has increased. On the other hand, increased knowledge and awareness of the doping problem may have influenced the police to an increased activity against doping. Nevertheless, the police rarely request a doping screening analysis compared to other drug offences. Less than 2% of the total number of the forensic cases were investigated for abuse of AAS. The annual number of doping controls at the prisons increased threefold from 2006, while the number of AAS positives remained at the same level. The reason for the percentage reduction in AAS abuse among the prisoners may be explained by more general testing, restricted access to weight-training at the institutions or maybe more effective drug controls.

The findings in the forensic cases (paper I) showed the current prevalence of AAS abuse, while most previous studies reported the life-time prevalence of AAS use based on questionnaires. Life-time prevalence should naturally be higher than current prevalence, as it covers a wider time period and AAS

use from one single occasion to repeated use over several years. Furthermore, most questionnaires have failed to clarify the difference between illicit AAS, nutritional supplements or other legal available products and have generated false-positive responses for “steroid” use.¹³ Comparing to current prevalence, life-time prevalence cannot be validated against objective measurements such as urine testing.¹⁴⁶ The study in paper I gives an important picture of the situation but does not provide any information about the abuse pattern. It could be a regular addiction or a one-time occurrence, which was not explored in this study.

The legislation against the use of AAS in Sweden is quite unique compared to other countries worldwide, which makes it difficult to relate the findings to previous research. A meta-analysis of the life-time prevalence of non-medical use of AAS in the five Nordic countries found that Sweden has the highest prevalence rate of AAS use 4.4% followed by Norway 2.4%, Finland 0.8%, Iceland 0.7% and, Denmark 0.5%.¹⁴⁷ The abuse of AAS in the society often remains undetectable, for several reasons. The great majority of AAS users are ordinary male gym clients, who remains undetected.¹³ For example, in one recent international study, 78.4% of 500 male AAS-using respondents were found to be non-competitive bodybuilders or nonathletes.⁵⁶ Furthermore, AAS use rarely lead to need for emergency medical care as other drug emergencies, e.g., opioid overdoses or alcohol intoxication. When in need of medical care for long-term side-effects, the users rarely disclose their AAS use to the clinicians.

The most commonly detected AAS was nandrolone and testosterone, which was in accordance with previous findings.⁶⁰ These steroids are commonly administered as intramuscular injections in form of esters having a long detection window, especially the 19-norandrosterone metabolite to nandrolone, that has been shown to be detectable for up to 9 months in urine after one single dose of nandrolone decanoate.¹⁴⁸ Interestingly, testosterone was much more commonly detected than nandrolone in the forensic cases during the following nine years after the study (paper I), but the reason for this change is unclear.

The co-abuse of AAS and other drugs of abuse found in 60% was in accordance with previous reports.^{60, 149} This high number of multi-substance abuse highly increases the risk of severe health problems. The reason for the concomitant use of other drugs might be to counteract the side-effects of the AAS use or to enhance the effect of AAS.⁶⁴ Furthermore, several studies have discussed AAS as a gateway to other drugs of abuse.^{56,62}

Testosterone in biological fluids

Testosterone concentrations in urine is not appropriate in terms of making correct interpretations and statements of unknown dosage and administration times of exogenous testosterone in forensic cases. The frequently detected abuse of testosterone found in paper I, prompted further study of testosterone in different biological fluids, to increase the knowledge of the testosterone levels achieved following physiological testosterone replacement therapy in clinical patients. Moreover, could testosterone measurement in saliva give additional valuable information and be a useful tool in forensic and clinical assessments?

The main finding in paper II was that salivary testosterone correlated well with total serum and calculated free serum testosterone in both male and female patients during i.m. TU therapy. The correlations between salivary testosterone and total as well as calculated free serum testosterone were in agreement with the findings in previous studies of salivary and serum testosterone in normal men and women using LC-MS/MS and the Vermeulen equation ¹⁵⁰ as well as in hypogonadal men using immunoassays and the Vermeulen equation. ¹⁵¹ However, for females, a weak correlation between salivary testosterone and free testosterone has been reported. ¹⁵² The discrepancy may be caused by several factors, such as differences in methodology (i.e., immunoassays, mass spectrometry), direct measurement of free testosterone or calculated free testosterone (based on measured serum testosterone, SHBG and albumin and the choice of algorithm), study population and concentration levels measured. For example, it has been demonstrated that salivary testosterone is not influenced by variations in serum SHBG. ¹⁵³ Salivary testosterone has the advantage of being a direct measurement of free testosterone in serum, while calculated free testosterone is based on a number of parameters.

The GD (TM) patients showed higher salivary testosterone levels after 12 months treatment with i.m. injections of TU compared to the male HG patients, whereas the levels in serum and urine were not affected in the same way. The injections lead to extremely high peak salivary and serum testosterone concentrations in three of the females seven days after the injection. Supra-physiological salivary testosterone levels were also reported in a previous study, where samples were taken immediately post-injection of testosterone-esters in treatment of GD (TM) adolescents. ¹⁵⁴ These extreme concentrations may affect the well-being of the patients. Sharp increases of salivary testosterone shortly after dose have been attributed to the fact that 98% of serum testosterone is bound to SHBG and albumin. Administration of testosterone will result in a high amount of free

testosterone in serum, causing a disruption of the equilibrium between testosterone and the binding proteins.¹³⁴ The free fraction of testosterone in serum and the resulting transfer of free testosterone into saliva might therefore be more elevated. As salivary testosterone concentrations probably mirror the serum free testosterone concentrations, salivary testosterone might better reflect the biological activity.

The majority of the treated patients showed post-injection testosterone concentrations in serum and saliva clearly above the upper physiological ranges of a normal male. In urine, significantly higher testosterone concentrations, up to 10,000 ng/mL, were found at testosterone abuse in the forensic cases, compared to the testosterone levels in the patients during replacement TU therapy in paper II (Fig. 11). This confirms that supra-physiological doses are administered at testosterone abuse.

Figure 11. Urinary testosterone levels in forensic doping cases compared to patients during treatment with i.m. injection of testosterone undecanoate.

This study showed that salivary testosterone has potential as a biomarker in clinical assessment, adding information for optimal dosing of long-acting testosterone injections. Salivary testosterone might also be an alternative and more sensitive marker than urine in detection of non-medical use of testosterone in forensic cases.

Detection of testosterone doping in forensic cases

Detection and confirmation of the abuse of endogenous AAS, such as testosterone is much more difficult and challenging than detection of other synthetic exogenous AAS. Reliable analytical results are of great importance in the field of forensic toxicology, and the methods need to be highly specific to avoid unjustified legal consequences for the individual. In the absence of a gold standard method (IRMS) for confirmation of exogenous testosterone, there are difficulties in setting strict criteria, without a risk of obtaining false positive test results.

In paper III, a high number of false negative testosterone results was found using the current criteria $T/E \geq 12$ in combination with $T/LH > 400$ nmol/IU. An NPV of 32% indicates that when the test shows a negative result, there is 68% chance of a false negative test. These numbers confirmed the limitation of the method used today to reveal testosterone abuse in forensic cases. The sensitivity could possibly be increased by lowering the cut-off value of T/E from 12 to 9 regardless of the T/LH value, but further decrease of T/E below 9, would result in loss of specificity and an increased risk of false positive results. The measured LH in urine and the ratio T/LH used today for confirmation of testosterone abuse in case of a $T/E \geq 12$, did not prove to add additional value. It has earlier been suggested that T/LH ratio exceeds 340 in case of testosterone doping.³⁴ It has also been suggested to use a specific gravity adjusted LH cut-off of 4 IU/L as a screening, particularly in UGT2B17 individuals with natural low T/E and T/LH ratios, as it was shown that LH values below 4 was uncommon in normal subjects.¹¹⁴ However, unlike doping in sports, other exogenous AAS are often used in forensic cases, which may suppress LH secretion by the negative feed-back regulation on the HPTG axis and this effect could last for several months after AAS withdrawal.¹⁵⁵

The strongest marker for detection of testosterone abuse in forensic cases was found to be the T/E ratio (paper III), but further confirmation analysis is still required to unequivocally prove an elevated T/E ratio to an exogenous administration and not to an exceptionally high endogenous concentration. However, introduction of the gold standard IRMS technique to the forensic toxicology laboratory is today unrealistic due to the costs and complexity. Alternative matrices, such as serum and saliva may provide an alternative solution. One alternative way to unequivocally detect testosterone abuse is a direct detection method to identify the intact testosterone ester in serum, as testosterone esters are not naturally produced by the human body. This has been shown in a previous pilot study at the forensic toxicology laboratory, by determination of a broad range of

testosterone esters in serum using LC-MS/MS.¹⁵⁶ Depending on the ester chain length, the release of active testosterone can be sustained from days to months. It was recently shown that the ester TU in serum could be detected by GC-MS/MS for two or three months after i.m. injection of a single dose of 1000 mg testosterone (Nebido®).¹⁵⁷

Analytical techniques for testosterone measurement in humans

A highly sensitive and specific LC-MS/MS method was (paper IV) developed for quantification of testosterone in serum and saliva over wide concentration ranges for use in forensic and clinical assays and for future research projects. Accurate detection of endogenous substances such as testosterone, is of utmost importance in both forensic cases and in clinical diagnosis. The ability to improve analytical testing methods relies on the expert knowledge and experience, the application of novel information regarding target analytes, alternative sample matrices and the access to sensitive analytical instruments. A strength in the work on the present thesis was the broad co-operation with other disciplines and experts. For example, the confirmation analyses (paper III) were carried out using the advanced analytical IRMS technique at the Swedish Doping Control Laboratory in Stockholm and a highly sensitive state of the art LC-MS/MS system was available at the department of Clinical Pharmacology, Linköping University Hospital for development of a quantitative method for testosterone in serum and saliva (paper IV).

Possible benefits of testosterone measurements

Finally, I would like to discuss the potential use of salivary testosterone as a biomarker in forensic and clinical applications. Today, detection and quantification of testosterone in these disciplines are performed in serum and urine at diverged concentration levels. In forensics, the aim of the analysis is to detect and confirm an abuse of testosterone, often measured at supra-physiological levels, while in clinical practice physiological or trace amounts of testosterone are quantified for diagnosis, treatment, or prevention of diseases. In other words, the goal in the clinic is good health for the patients. As salivary testosterone concentrations mirror the serum free testosterone concentrations, salivary testosterone may reflect the

physiological state and biological activity on a cellular level more closely than total serum testosterone.

One of the biggest advantages of salivary testosterone testing compared to urine or serum is the easy, non-invasive sample collection, which can be performed with minimal training by caregivers and by the patients themselves at home allowing repeated sampling and monitoring in clinical practice. In forensic cases, saliva can be collected under direct supervision without compromising privacy and with decreased risk of sample manipulation e.g., dilution or sample substitution.

To be able to discriminate between normal endogenous values in saliva and elevated concentrations caused by testosterone administration, population-based reference thresholds or ratios with a stable denominator (like epitestosterone in urine) need to be identified. In a recent study for detection of transdermal application of testosterone the salivary T/DHEA showed to be a sensitive parameter and DHEA an excellent denominator.¹⁵⁸ Although the potential of salivary testosterone as a marker has been discussed, it has still not been implemented in routine clinical practice or in forensic investigations. Even if saliva has several advantages compared to serum and urine, there are some preanalytical limitations in connection with the sampling procedure that have to be considered, such as the choice of sampling technique, risk for blood contamination, and deviations due to close food and drink intake.

Based on both previous studies and personal observations at the collecting of saliva samples in paper IV, it should be emphasized that producing sufficient volume of saliva for analysis may vary for different individuals. This could be dependent on the time of the day, medications (dry mouth), method of sampling and psychological status (e.g., afraid or stressed). Dry mouth can develop as an adverse effect of certain medications, e.g., diuretics, neuroleptics, antidepressants, and opioids, and is also associated with several diseases, such as diabetes, rheumatoid arthritis and Sjögren's syndrome.¹⁵⁹ Thus, the major difference between oral fluid and serum is the various composition of the saliva, while venous blood is a more homogenous matrix.

In this thesis the abuse of AAS and in particular testosterone was from a forensic perspective investigated to identify the problems associated with the abuse. Methodological challenges in detection of exogenous administration of testosterone (i.e., false negative test results) have been demonstrated. What would be the best future strategy to improve the analytical sensitivity in forensic doping tests? It seems possible to adjust

the current criteria to achieve some improvements, however it might be even better to find new markers or to introduce alternative matrices for detection of testosterone abuse?

In clinical practice, considerably lower testosterone concentrations, especially in saliva, are expected to be quantified, which requires more sensitive methods than immunoassays, such as the mass spectrometry method developed in this thesis. As there seems to be a need for individual follow-up of testosterone levels during testosterone therapy, due to the supraphysiological peak levels measured in paper II, saliva might be a potentially suitable and more sensitive matrix.

CONCLUSIONS

The work presented in this thesis has led to the conclusions that testosterone is one of the most frequently detected AAS in forensic cases and the high concentrations measured indicate use of supra-physiological doses. It has furthermore supported previous studies demonstrating that abuse of AAS is associated with multiple drug abuse. In addition, the present used methods for detection of testosterone abuse in forensic doping cases, have been shown resulting in a great number of false negative test results, indicating a need for future new analytical strategies. Salivary testosterone has been proved to correlate well with serum free testosterone in both male and female and might have a potential as an alternative matrix for detection of testosterone abuse and for diagnosis and monitoring of androgenic status. A sensitive and highly specific LC-MS/MS method was successfully developed for determination of testosterone in serum and saliva suitable for forensic and clinical assessments.

FUTURE PERSPECTIVES

The abuse of AAS was frequently found in the forensic doping cases, often in combination with other legal and illegal substances. This reinforces the view that abuse of AAS is a health and social problem. To further increase knowledge of AAS abuse, it would be of great interest to evaluate the forensic toxicology findings during the following period 2010-2020 in an extended study by using different Swedish national databases. Swedish citizens have a unique ten-digit personal identification number and individual-level data may be collected from the Swedish Prescribed Drug Register (SPDR), the National Patient Register and the Database for official crime statistics at the National Council for Crime Prevention (Brå) register of persons found guilty of offences. These registers could add information on to which extent the drugs detected are prescribed to the individual, whether the individual have received hormonal treatment or have received hospital care related to AAS use and to which extent the individual has been convicted for a crime and with what penalties.

There are several issues in the analyses of testosterone in urine and the ability to discriminate endogenous testosterone from exogenous intake of testosterone. One way to avoid these challenges would be direct detection of testosterone esters in serum. A pilot study was made by LC-MS/MS (unpublished data). This would be an unambiguous proof of illegal administration of testosterone, independently on endogenous testosterone levels and genetic variations.^{156, 157} It would also be of clinical interest to evaluate whether the serum levels of the ester TU have a potential role in monitoring androgen therapy.

Supra-physiological levels of testosterone in saliva were observed in some patients receiving clinical testosterone treatment, especially among the females with GD. These results indicate a need for further studies of the variations in androgen response and metabolism, due to e.g., genetic differences, gender, age, and weight. It is of clinical importance to individualize the dosing of testosterone to achieve effective and safe testosterone substitution treatment.

To implement the new LC-MS/MS method as a reference method for determination of testosterone in forensic and clinical applications, there is a need to establish reference intervals for testosterone in serum and saliva in our population. Saliva has become more and more interesting as a matrix for measurement of testosterone. Further investigations of the relationship between testosterone in serum and testosterone in saliva determined by mass spectrometry, and between testosterone in saliva and direct measured free testosterone in serum, might show testosterone in saliva as a reliable indicator of the free fraction of testosterone in serum.

Such a study was part of the original scientific plan, but had to be postponed, due to the global coronavirus pandemic (Covid-19) during 2020. It was no longer possible to collect samples in the population, due to restrictions to prevent the spread of the infection.

By the development of a highly sensitive and accurate LC-MS/MS method, future research studies in different areas have become possible to perform, which would be of great interest and importance. In forensic toxicology, testosterone in serum or saliva might be an alternative to urine analyses for detection of illegal intake of testosterone and might also give additional information for interpretation of the analytical result.

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