UiT The Arctic University of Norway

Faculty of Health Science

Department of Pharmacy

Discovery and detection of phase-II metabolites of exogenous steroids in anti-doping analysis

Lorenz Göschl

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Acknowledgments

I started my PhD-Thesis in April 2019, not even a year before the global Covid-19 pandemic turned normality on its head. I found myself in the particular situation of completing the central part of my studies not in Norway, the university's country, but in Austria in cooperation with the company Seibersdorf Laboratories. Therefore, I live in Vienna, and in the first months, I regularly travelled to Scandinavia to attend courses, conferences, and other academic events. It was an inspiring and educational time leading to many new exciting acquaintances in the scientific community until the global pandemic changed everything. Travel to other countries was no longer possible, and so was physical access to my university. Courses, conferences, and other events could only be attended online, making it virtually impossible to get to know other students or colleagues in this environment. Nevertheless, many people accompanied me on this path, and to whom I would like to express my appreciation.

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Paper I

Development and validation of a simple online-SPE method coupled to high-resolution mass spectrometry for the analysis of stanozolol-N-glucuronides in urine samples

Lorenz Göschl, Günter Gmeiner, Valentin Enev, Nicolas Kratena, Peter Gärtner, Guro Forsdahl.

Drug Test Anal. 2020 Aug; 12(8):1031-1040. doi: 10.1002/dta.2805. Epub 2020 Jun 27.

Paper II

Stanozolol-N-glucuronide metabolites in human urine samples as suitable targets in terms of routine anti-doping analysis

Lorenz Göschl, Günter Gmeiner, Peter Gärtner, Georg Stadler, Valentin Enev, Mario Thevis, Wilhelm Schänzer, Sven Guddat, Guro Forsdahl.

Drug Test Anal. 2021 Sep; 13(9):1668-1677. doi: 10.1002/dta.3109. Epub 2021 Jun 9.

Paper III

Detection of DHCMT long-term metabolite glucuronides with LC-MSMS as an alternative approach to conventional GC-MSMS analysis

Lorenz Göschl, Günter Gmeiner, Peter Gärtner, Michael Steinacher, Guro Forsdahl.

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Summary

Anti-doping measures are an essential part of professional sports around the world. Anabolic androgenic steroids have been among the most widely used illicit substance classes since the early days of the fight against doping. The conventional method for analysing these substances is gas chromatography combined with mass spectrometry (GC-MS). This approach is sensitive and selective but also very time- and resource-consuming. The main goal of this work was to improve the detection of steroids in a time- and resource-efficient manner by shifting the analysis from GC-MS to liquid chromatography-MS (LC-MS).

In the first part of the work, we present a novel, highly functional analytical method for the analysis of steroid phase-II glucuronides in which sample preparation is reduced to a minimum. For this purpose, we developed a simple but powerful online solid-phase extraction method coupled with LC-MS. Two comprehensive validation studies demonstrated the analytical performance of this method. Initially developed for rapid confirmation analysis, this method also showed high potential for identifying and characterizing novel steroid phase-II metabolites.

The aim of the second part of the work was to identify and characterize novel phase-II metabolites of the anabolic androgenic steroids stanozolol and dehydrochloromethyltestosterone using the newly developed method. Both substances are among the most commonly used steroids in professional sports and are of great interest for anti-doping research. For stanozolol, we achieved for the first time to unequivocally confirm four different phase-II glucuronide conjugates using newly synthesized high-quality reference standards. Furthermore, using urine samples from excretion studies, we generated elimination curves for all four metabolites showing elimination windows of up to 28 days. The long excretion time makes this metabolite very interesting for the long-term detection of stanozolol. In the case of dehydrochloromethyltestosterone, we identified two different glucuronide conjugates of the important long-term metabolite M3 for the first time. Due to the lack of reference standards, we used a combination of different analytical approaches to characterize and elucidate the chemical structure of these conjugates. Since these metabolites are well suited for LC-MS analysis, we present for the first time a way to analyse metabolite M3 without requiring a laborious GC-MS approach.

Abbreviations

AIDS	Acquired immunodeficiency syndrome
AAF	Adverse fanalytical finding
AAS	Anabolic androgenic steroids
AR	Androgen receptor
APCI	Atmospheric pressure chemical ionization
API	Atmospheric pressure ionization
APPI	Atmospheric pressure photoionization
ATF	Atypical Finding
p23	Chaperone protein
Co-A	Coactivator proteins
DHCMT	Dehydrochloromethyltestosterone
DNA	Deoxyribonucleic acid
DHT	Dihydrotestosterone
DMF	Dimethylformamide
SARM	Selective androgen receptor modulators
EI	Electron impact ionization
ESI	Electron spray ionization
EQAS	External quality assurance system
FA	Formic acid
FT ICR	Fourier transform ion cyclotron resonance
FWHM	Full Width at Half Maximum
GC	Gas chromatography
Hsp90	Heat-shock protein
HCD	High-energy collision induced dissociation
HPLC	High-performance liquid chromatography
HR	High-resolution
IC	In-competition
IAAF	International Association of Athletics Federations
IOC	International Olympic Committee
FI	International Sports Federations
ISL	International Standard for Laboratories
IUPAC	International Union of Pure and Applied Chemistry
LC	Liquid chromatography
l/l	Liquid/liquid
MS	Mass spectrometry
MC	Medical Commission
MeOH	Methanol
MQ	Milli-Q purified water

MRM	Multiple reaction monitoring
NF	National Federations
NOC	National Olympic Committee
NPC	National Paralympic Committee
MSTFA	N-methyl-N-trimethylsilyl-trifluoroacetamide
NMR	Nuclear magnetic resonance spectroscopy
OOC	Out-of-competition
PRM	Parallel Reaction Monitoring
ppm	Parts per million
RF	Radio frequency
SCA	Sample Collection Authority
SIM	Selected Ion Monitoring
SRM	Selected reaction monitoring
SPE	Solid-phase extraction
TA	Testing Authority
TOF	Time-of-flight
TF	Transcription factor protein
TMIS	Trimethyliodosilane
OTMS	Trimethylsiloxy
UGT	Uridine 5'-diphospho glucuronosyltransferase
UDP-GC	Uridine-5'-diphospho-glucuronic acid
WADA	World Anti-Doping Agency
WAADS	World Association of Anti-doping Scientists

1. Introduction

1.1 Doping and Anti-Doping

1.1.1 History of Doping and Anti-Doping

The history of the abuse of doping substances is as old as the professional sport itself. The willingness to consume substances of all kinds to increase strength, growth, speed, concentration, or other qualities, seems to be a part of human nature. Even with children, the request to eat up their food in order to become "big and strong" can lead to the hoped-for success. Many people in a modern, high-performance society need their several daily doses of coffee to be able to do their work properly. Of course, these examples have nothing to do with modern professional doping, but they can give a slight impression of what might be a motivation to take performance-enhancing substances. But even early history is full of examples of early forms of doping abuse.

Organotherapy, the consumption of animal or human organs, was performed by ancient and medieval people around the world to cure diseases or increase vitality and general performance. The testicular tissues, in particular, were said to have extraordinary powers to improve strength and endurance[1]. Ancient Greek athletes, but also Roman gladiators or medieval knights, and African people used various types of alcohol, herbs, plants, mushrooms, or even cacti to increase endurance and (fighting) strength, suppress pain, or delay the onset of fatigue[2].

With the advent of modern medicine in the second half of the nineteenth century, the abuse of substances with performance-enhancing effects reached a new level. At this time, experiments with a large number of stimulants (e.g. caffeine) began, but also the large research field of anabolic hormones, such as anabolic androgenic steroids (AAS), was launched. In various, at this time popular long-distance disciplines such as swimming, running, and cycling, many substances were used to enhance performance. Drugs such as nitroglycerine, strychnine, morphine, or cocaine made their first appearance in professional sport[2]. After the first experiments in the last years of the nineteenth century with "liquide testiculaire", an extract from animal testicles, by the

well-known physiologist and neurologist Charles Edouard Brown-Sequard in Paris, the use of steroids in professional sport became really relevant later in the twentieth century.

In 1935, scientists isolated, chemically characterised, and synthesised the hormone testosterone, which laid the foundation for the misuse of this endogenous hormone as a doping substance. After the successful use of testosterone to strengthen the physique of horses in 1942, the bodybuilding scene in the western USA began to use this hormone in the early 1950s[3]. Starting with weightlifters from the Soviet Union in the mid-1950s, the use of testosterone spread over the next decades in many, primarily strength-intensive professional sports, but also in athletics and football[4]. Driven by the world wars, many new stimulants (e.g. amphetamines) were developed, some of which found their way into many different professional sports in the decades after World War 2. Although the International Association of Athletics Federations (IAAF) introduced some general rules for the use of drugs as early as 1928, these were not enforced and controlled. Thus, at that time, the use of performance-enhancing substances in professional sports was prevalent and more or less accepted[5]. But then, in 1961, at the Olympic Games in Rome, the first Summer Games to be broadcast live on television, the Danish cyclist Knud Enemark Jensen collapsed and died on camera. It turned out that he had ingested a cocktail of stimulants containing amphetamine-like drugs that probably caused the athletes' death. This tragedy and the fact that it was broadcasted live around the world were the deciding factors in the International Olympic Committee's (IOC) decision to take up the fight against drug abuse in professional sports by setting up a Medical Commission (MC). This commission established the first list of prohibited substances and the rules for testing at the Olympic Games after six years of preparation. However, AAS were not yet included in this list.

At the Mexico City Olympic Games in 1968, the first positive doping case was reported when a Swedish member of the modern pentathlon team tested positive for alcohol. In 1974, AAS were put on the prohibited list for the first time, leading to 8 positive cases at the 1976 Montreal Games[6]. After years of more or less inconsistent anti-doping analyses, the IOC decided in 1986 to introduce accreditation systems for laboratories. Three years later, in 1989, after the stanozolol scandal involving the Canadian sprinter Ben Johnson at the 1988 Soul Games, the IOC was forced to introduce out-of-competition testing. This

is especially important for the detection of steroid abuse, as these substances are usually taken during training to enhance muscle growth and shorten recovery time.

In order to harmonise international anti-doping activities, the World Anti-Doping Agency (WADA) was founded in February and established in November 1999 in Lausanne, Switzerland, by representatives of governments, intergovernmental and non-governmental organisations, the International Olympic Committee, International Sports Federations (FI), National Olympic Committees (NOC) and athletes. With the founding of WADA, the document "Lausanne Declaration on Doping in Sport" was created, in which the main objectives and plans of this organisation are written down[7]. This document is the basis for all modern anti-doping activities worldwide.

1.1.2 Current Anti-Doping activities

Nowadays, WADA is responsible for the promotion, coordination, and monitoring of the fight against doping in sport, as well as scientific research, training, and the development of anti-doping capacities[8]. One of WADA's main accomplishments was the creation of the World Anti-Doping Code, which is accepted by most sport and anti-doping organizations worldwide[9]. This key document contains a set of anti-doping rules, regulations, and policies and ensures compliance within and between sport organisations, anti-doping laboratories, national anti-doping agencies, and authorities worldwide. The Anti-Doping Code is regularly renewed and expanded, and its annexes contain all the information necessary for the transparent, efficient, and fair conduct of doping controls. One of the most important parts of the code is the WADA Prohibited List, which contains all banned substances and prohibited methods in professional sports[10]. Like all other relevant WADA documents, this list is publicly available to athletes, laboratories, and scientists. Another important document for anti-doping testing is the International Standard for Laboratories (ISL)[9]. Alongside the main standard for testing and calibration laboratories, the ISO-17025 standard, this is the basic document according to which accreditation for certified anti-doping laboratories is structured. These documents contain all the technical details on how an anti-doping laboratory must be structured and organised. As of January 2022, there are 29 accredited anti-doping laboratories worldwide[11].

Although WADA provides the basic structure of anti-doping measures, the primary responsibility for implementing anti-doping programs in a country at the national level lies with the National Anti-Doping Organizations (NADOs). The organization must be completely independent of other relevant institutions such as the National Federations (NF), National Olympic Committee (NOC), National Paralympic Committee (NPC), or any government agency responsible for sport or anti-doping[12]. The NADO's main responsibilities include adopting and implementing anti-doping rules, managing sample collection, administering test results, and planning and conducting anti-doping education activities.

1.1.2.1 WADA Prohibited List

The Anti-Doping Code describes how the Prohibited List is compiled and how its content is created. A substance or method is considered for inclusion on the list by WADA if two of the following three criteria are met[9]:

- The substance or method alone or in combination with other substances or methods shows the potential to enhance or improve athletic performance based on medical or other scientific evidence, pharmacological effect, or experience
- The use of the substance or method presents an actual or potential health risk to the Athlete based on medical or other scientific evidence, pharmacological effect, or experience
- The use of the substance or method violates the spirit of the sport described in the Code by WADA

Furthermore, a substance or method is added to the Prohibited List if it is determined, based on medical or other scientific evidence, pharmacological effects, or experience, that the substance or method has the potential to mask the use of other prohibited substances or prohibited methods. This list is published annually and is separated into the following general parts: substances and methods that are banned permanently, substances that are banned only during competition, and substances that are banned only in particular sports. Furthermore, it is divided into different classes (Figure 1) and contains over 200 compounds.

Prohibited at all times

- S0 Non-approved substances
- S1 Anabolic agents
- S2 Peptide hormones, growth factors, related substances, and mimetics
- S3 Beta-2 agonists
- S4 Hormone and metabolic modulators
- S5 Diuretics and masking agents

Prohibited in competitions

- M1 – M2 – M3 Prohibited Methods
- S6 Stimulants
- S7 Narcotics
- S8 Cannabinoids
- S9 Glucocorticoids

Prohibited in particular sports

- P1 Beta-blockers
- P2 Alcohol

Figure 1: General structuring of the WADA list of prohibited substances in professional sports; blue: substances prohibited at all times; green: substances and methods prohibited during competition; red: substances prohibited in particular sports

Most classes are further divided into subclasses. This thesis is about anabolic androgenic steroids that belong to class S01 anabolic agents. Within this subclass, there are more than 60 different compounds, and most of them belong to the family of AAS. According to ISL, Wada distinguishes between Adverse Analytical Findings (AAF) and Atypical Findings (ATF). The former is a clear positive finding, e.g. a substance or its metabolite has been detected in a sample by a WADA-accredited laboratory, and the latter is a finding that requires further investigation. As shown in Figure 2, anabolic steroids are responsible for at least 40 % of all AAFs, although there has been a slight decline in recent years[13–22]. This is why the detection of AAS remains a major focus of research in the field of anti-doping analysis.

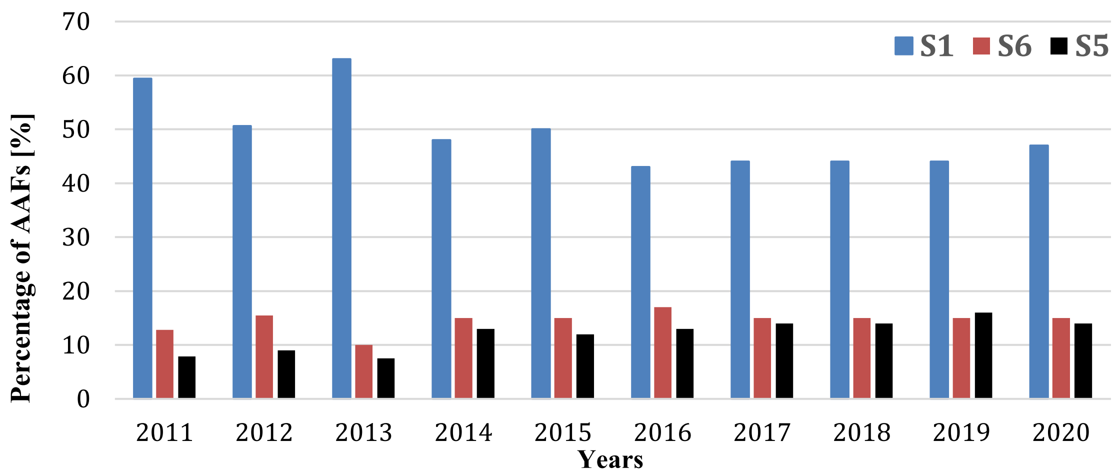

Figure 2: Distribution of Adverse Analytical Findings (AAF) of the three most prominent doping substance classes from 2011-2020; S1: Anabolic agents, S6: Stimulants and S5: Diuretics and masking agents

1.1.2.2 General approach in anti-doping control

Doping control consists primarily of analysing samples, usually body fluids from athletes, to detect the presence of markers for prohibited substances or methods. The main matrix in this field is urine because it is easy and non-invasive to collect. It is also straightforward to obtain a large volume compared to blood, and excretory products such as metabolites accumulate in this matrix[23]. Athletes are tested by the Sample Collection Authority (SCA), which is approved by the Testing Authority (TA). They, in turn, are authorized by the national and international anti-doping organizations[24]. The entire sampling process is subject to a stringent protocol and is documented with the so-called "chain of custody". This form ensures the traceability of each step of the entire sampling and shipping process. Samples can be taken during a competition (=in-competition samples (IC)) and anytime outside a competition (=out-of-competition samples (OOC)). As mentioned above, different rules apply for the respective sample type. Two samples are always taken during a sample collection, the A- and B-sample. The former is used for the actual analysis, and the latter is kept sealed for possible subsequent reanalysis. Blood samples are also taken, albeit to a much lesser extent than urine samples. They are used to determine blood parameters and to check the intake of particular doping substances (e.g. human growth hormone)[25]. After collection, the samples are properly packaged and shipped to a certified anti-doping laboratory, where they are analysed.

1.2 Anabolic androgenic steroids (AAS)

This work is exclusively concerned with the analysis of exogenous anabolic androgenic steroids. As already mentioned, this class of substances is the most frequently detected in professional sports. Furthermore, these agents have arrived in the broad society[26]. Hence, an accurate and efficient analysis is of high importance. In the following chapters, the basic knowledge regarding chemistry, pharmacology, metabolism, and AAS analysis is presented.

1.2.1 Chemistry of AAS

1.2.1.1 Structure and nomenclature

Anabolic steroids can be divided into two forms according to their origin: endogenous when the steroid is biosynthesised in the human body, or exogenous when it is artificially synthesised. All steroids have in common that they are based on the body's steroid testosterone structure. Testosterone is produced mainly in the testicles in men and in smaller quantities in the ovaries and adrenal glands in women[27]. Both endogenous and exogenous steroids share the base backbone structure called sterane (cyclopentanoperhydrophenanthrene), which contains seventeen carbon atoms and is organized in a four-ring system (A-D). The steroid base structure is shown in Figure 3-A. The C-atoms of the basic structure are numbered 1-17. In many AAS, there are two additional methyl groups at angular positions 10 and 13, ascribed 18 and 19 (See Figure 3-B). Steroids that lack the 18- or 19-methyl group are given the prefix "nor" in their designation. Although the nomenclature established by the International Union of Pure and Applied Chemistry (IUPAC) can be consulted in case of doubt, mainly trivial names are used for AAS. The metabolites of the individual steroids are simply described by the name of the parent substance, the letter "M" and a number.

Steroids' physical, chemical and physiological properties are determined by the nature and location of substituent groups and unsaturation within the steroid backbone[28]. The overall conformation of the steroid contributes to its specific interactions with cellular components such as hormone receptors, enzymes, or proteins (See 1.2.2). AAS are chemically distinguished by various modulations on several of the seventeen carbon atoms, such as the addition of various alkyl, keto, hydroxyl, or hydroxymethyl groups

and the introduction of one or more double bonds or, in the case of some exogenous steroids, the addition of heteroatoms, halogens or pyrazole rings (see Figure 4)[29]. Figure 3 shows, as examples, the steroid backbone sterane (A), the endogenous steroid testosterone (B) and the exogenous steroids drostanolone (C).

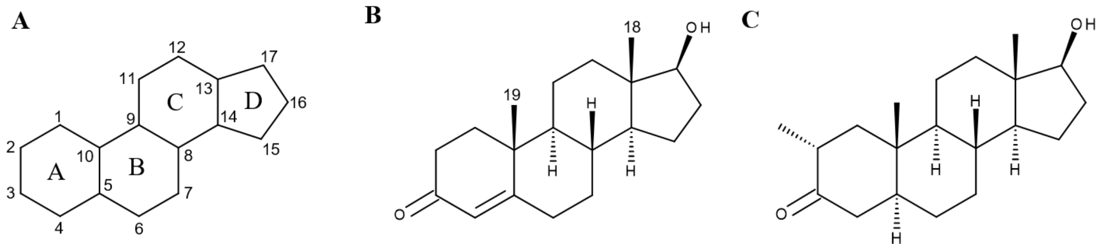

Figure 3: Chemical structure of the steroid backbone sterane (A), the endogenous steroid testosterone (B) and the exogenous steroid drostanolone (C)

1.2.1.2 Designer steroids

Artificial exogenous steroids, also called designer steroids, have been developed to enhance the desired effects of steroids. The emphasis has been on reducing androgenic effects and enhancing anabolic effects such as muscle growth, strength gain, body fat loss, and faster recovery. There are many known structural properties of steroids that can cause such an effect, as shown in Figure 4. Based on this knowledge, a large number of different designer steroids have been synthesized.

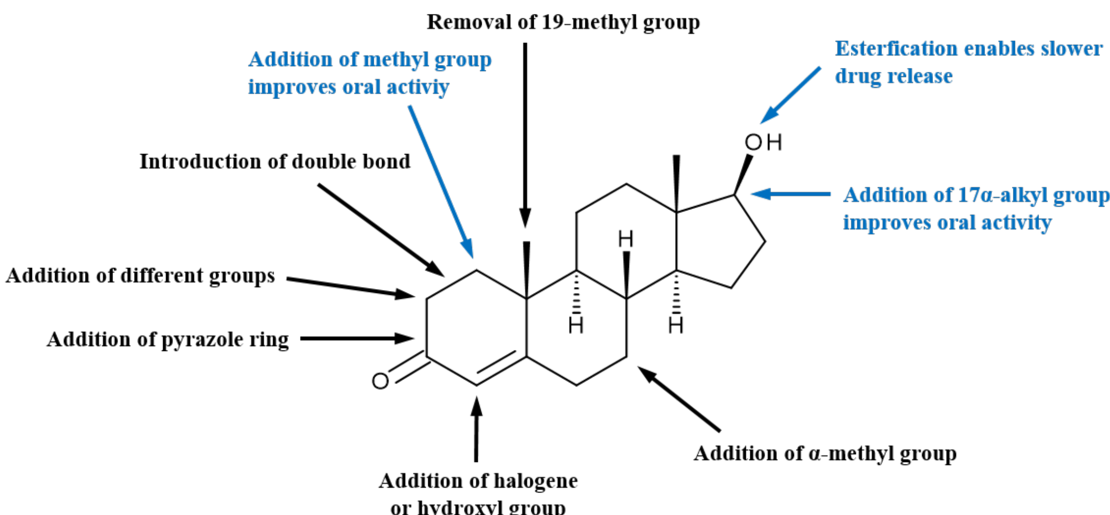

Figure 4: Structural modifications of testosterone that increase anabolic activity (black) and bioavailability (blue)

Furthermore, efforts have been made to simplify the use of the drug (e.g. by improving oral bioavailability) and to reduce negative side effects[30–32]. The most common routes of administration of AAS are oral pills, injectable solutions, or preparations for topical application such as gels or creams[33]. Substitution of 17α-H on the steroid skeleton with a methyl or ethyl group enhances oral activity. The alkyl function impedes the oxidation of the 17β-hydroxyl group, preventing the deactivation of the steroid by first-pass metabolism. Both steroids studied in this thesis, stanozolol and dehydrochlormethyltestosterone, are 17α-alkylated. Another approach to increase oral bioavailability is to add a methyl group to C1, as is the case with the steroids methenolone and mesterolone. However, the effect is much stronger with 17α-alkylated steroids. The escape of first-pass metabolism also gives these steroids their liver-toxic properties when taken over a long period of time. In intramuscular preparations, the 17β-hydroxyl group is esterified with an acid moiety to prevent rapid absorption from the oily carrier, resulting in the slow release of the drug over time. The steroid's activity duration can last up to months, depending on the type of esterification[28].

1.2.2 Pharmacology of AAS

This work is exclusively concerned with the analysis of exogenous anabolic androgenic steroids. AAS are small molecules from the class of hormones that have a variety of functions in the human body. As their name suggests, anabolic and androgenic effects belong to their main tasks. The development of male secondary sexual characteristics, such as the growth of the body and facial hair, and the perpetuation of reproductive function are attributed to androgenic effects. The growth and formation of skeletal muscle and bone belong to the category of anabolic effects[34,35]. These two categories of effects are inextricably linked to each other. The main pharmacological effects of steroids can be divided into two different physiological levels, cellular and molecular. These are explained in more detail in the following sections.

1.2.1.1 Steroid activities at the cellular level

Androgenic anabolic steroids are part of the class of androgen receptor ligands, also called androgens. The effects of these type of substances are based on the interaction with the androgen receptor (AR). This high-molecular protein complex acts as a DNA-binding transcription factor regulating gene expression. Such binding triggers different signalling

cascades that lead to various androgenic and anabolic effects in the body. Androgen receptors occur in different variants and amounts in different tissues. The activities produced by the binding of androgens to the receptor depend strongly on the nature of the ligand. The enzymatic setup of a cell defines an important control mechanism of ligand-receptor binding. The intracellular enzyme composition varies greatly depending on the cell or tissue type. In certain reproduction-associated cell types (e.g. prostate tissue), the enzyme 5α-reductase, which converts testosterone to dihydrotestosterone (DHT), is present in large quantities[36]. DHT binds more strongly than testosterone to the AR, thus dominating its binding and subsequently triggering enhanced androgenic effects in the respective tissue. However, in human skeletal muscle tissue, 5α-reductase activity is negligible. This, in turn, causes testosterone to dominate and trigger anabolic effects. In other tissue types, such as adipose tissue, testosterone (and other AAS) can be converted by the enzyme aromatase into the estrogen estradiol. This hormone, in turn, is associated with the development of secondary sexual characteristics in women. These were some examples of testosterone being converted into more androgenic steroids. However, a reverse mechanism is also possible. Enzymes such as 3α-hydroxysteroid dehydrogenase or 17β-hydroxysteroid hydrogenase are also known to convert DHT to weaker androgens in certain tissues (e.g., skeletal muscle), again leading to enhanced anabolic effects[37]. Therefore, the varying presence of different AAS catalysing enzymes, has a strong influence on the pharmacological effect of steroids in the respective tissue. The well-studied AAS nandrolone is a good example to illustrate this pharmacological aspect. In

5α-reductase active tissue, this steroid is converted into 5α-dihydro-19-nortestosterone[38], as illustrated in Figure 5.

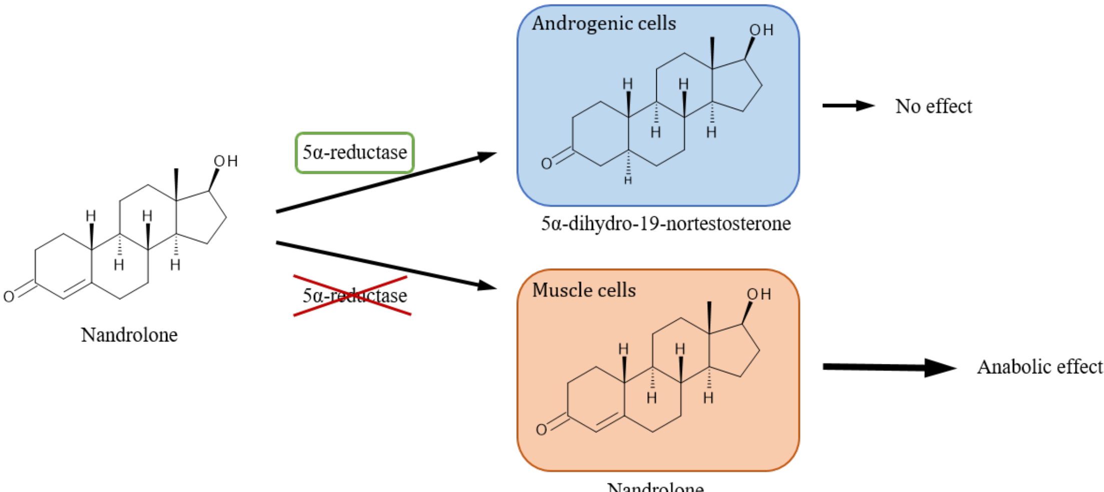

Figure 5: Pharmacological effect of the AAS nandrolone depending on cell type

This metabolite has a very weak affinity for the AR and therefore elicits hardly any effect in these cell types. In contrast, in 5α-reductase inactive cells, such as muscle tissue, nandrolone is not metabolized and binds with high affinity to the AR. This, in turn, leads to strong anabolic effects. Overall, nandrolone has significantly higher anabolic effects than androgenic making this substance very popular for abuse in sports.

1.2.1.2 Steroid activities at the molecular level

Steroids are small molecules that can passively diffuse into cells and bind to the ligand-binding domain of the androgen receptor. See Figure 6. The resulting dissociation of several complexes, such as the heat-shock protein Hsp90 or chaperone protein p23, and subsequent receptor conformational changes cause it to become active. The active receptor is transported into the nucleus, where it binds to deoxyribonucleic acid (DNA) on the androgen receptor binding domain and interacts with other proteins, including transcription factor (TF) and coactivator proteins (Co-A)[39]. This, in turn, leads to gene activation and subsequent transcription and translation into proteins that result in cell function, growth, or differentiation activities. As early as 1984, Saartok *et al.* showed that AAS bind to the androgenic receptor with varying affinities by *in vitro* studies[40]. However, the physical binding of ligand and receptor does not fully explain the activity of an AAS. Many other factors, such as the bioavailability of the steroid, also play an

important role in this matter. It is suggested that the composition of the gene regulatory machinery varies due to structurally different steroids[41]. The variation happens due to different conformational changes of the receptor and the resulting interactivity with transcription factors and co-regulators. This, in turn, influences gene expression and the resulting biological effects of the steroid.

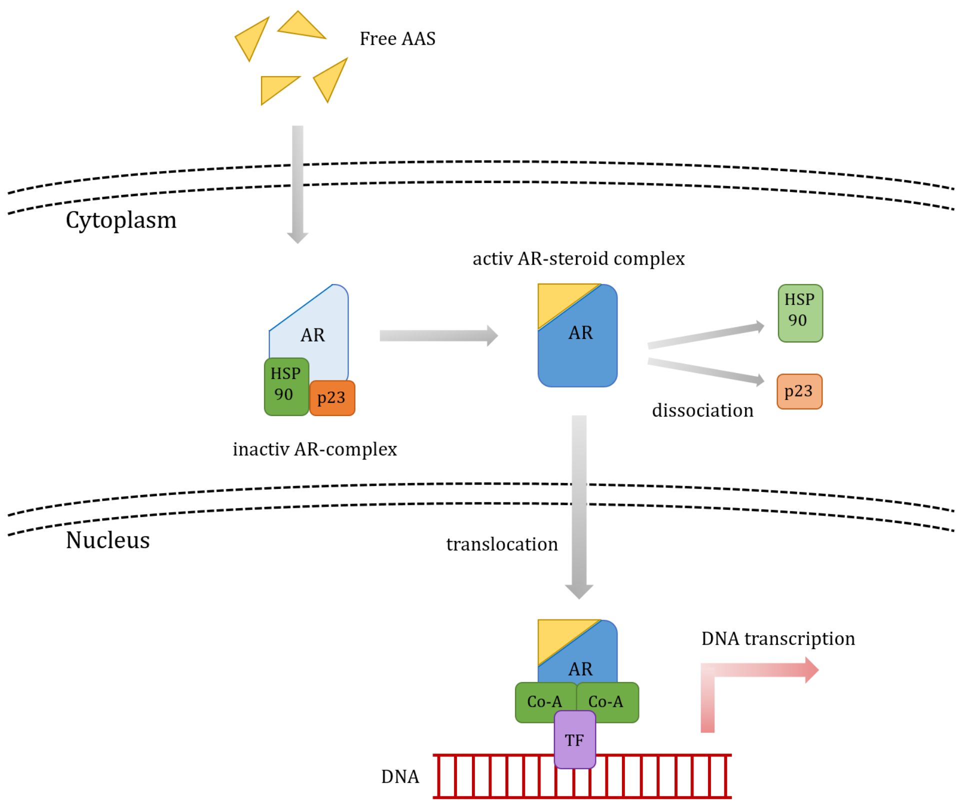

Figure 6: Simplified model of AAS activity at the molecular level; AAS = anabolic androgenic steroids; AR = androgen receptor; HSP = heat-shock protein; p23 = chaperone protein; TF = transcription factor; Co-A = Coactivator protein; DNA = deoxyribonucleic acid

In addition, it is described that AAS may affect glucocorticoid receptor interactions[42]. Glucocorticoids (e.g. cortisol) regulate protein breakdown to ensure the mobilization of energy reserves in the human body[43]. In this process, the skeletal muscle serves as an important amino acid store. It is suggested that AAS can bind to these receptors to a small extent or affect their expression at the genetic level[44,45]. If AAS inhibits regulation, this leads to an anti-catabolic effect, which can also be interpreted as an anabolic effect.

There are a number of other pharmacological-physiological mechanisms attributed to AAS. However, these are still poorly understood, and have little relevance in the context of this work, so they will not be discussed further.

1.2.1.3 Effects and side effects of AAS

Regarding sports, it has been convincingly demonstrated that the use of anabolic androgenic steroids can significantly increase fat-free mass, muscle size, strength, and performance[28]. However, several side effects can also occur with the uncontrolled use of these substances. Disorders of the hypothalamic-pituitary-gonadal axis can lead to symptoms such as testicular atrophy, infertility, and secondary amenorrhea. In addition, mood disorders such as increased aggression and propensity to violence as well as psychosis may occur. Irreversible disorders such as voice changes and clitoral hypertrophy have been described in women. Obviously, athletes are more interested in the anabolic effects than the androgenic ones. But also for medical purposes, great efforts have been made to develop synthetic molecules that enhance the anabolic effect of AAS and attenuate the androgenic effect. AAS are used in many medical conditions to stimulate muscle and bone growth. They are also applied for the treatment of chronic wasting conditions such as acquired immunodeficiency syndrome (AIDS) or cancer. In addition, steroids are utilized for the desired induction of male puberty or other gender-related treatments. However, a complete separation of the anabolic and androgenic effects has not yet been achieved. For these purposes, the substance group of selective androgen receptor modulators (SARM) plays a significant role[46]. This class of non-steroidal androgen receptor ligands promises a significantly better ratio of anabolic effects to androgenic effects than steroids. SARMs are, however, not a further topic in this work.

1.2.3 Metabolism of AAS

AAS are metabolised fast and extensively in the human body so that it is usually not the steroids themselves but their metabolic products that are detected during anti-doping analysis[28]. Therefore, for the detection and monitoring of steroid abuse, knowledge of their metabolism is of high importance. The metabolism of androgenic anabolic steroids in the human body is categorised into phase-I and phase-II. Phase-I reactions, catalysed by different enzymes, aim to inactivate the drug and prepare it for excretion from the body. This is accomplished by adding functional groups to the molecule or altering existing functional groups, resulting in lower activity or toxicity of the drug. In phase-II metabolism, the steroids are conjugated. Highly polar molecules, such as glucuronides or sulfates, are enzymatically attached to the steroids to increase their water solubility and

enable urinary excretion[27]. Both phase-I and phase-II metabolites can be used for the detection of steroid abuse. Accordingly, the analytical strategy must be adapted (see 1.3).

1.2.3.1 Phase-I metabolism

During phase I metabolism, AAS are chemically modified in various ways. Due to many enzymes involved, which catalyse a large number of different reactions, a complex network of different metabolites is formed for each individual AAS. The most commonly observed chemical changes are exemplified in Figure 7.

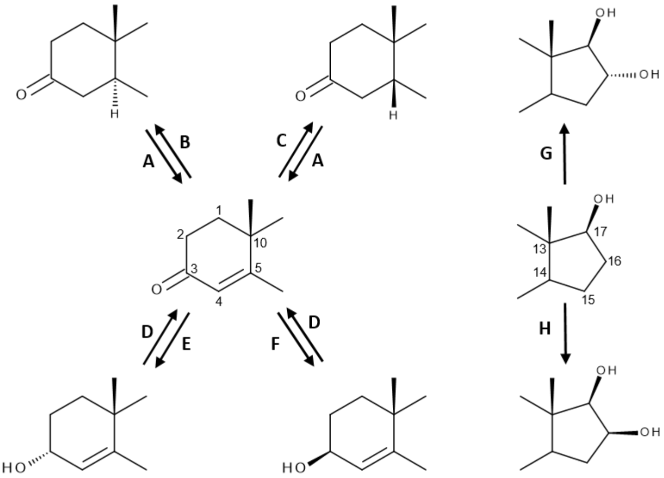

Figure 7: Most common phase-I metabolism reactions of anabolic androgenic steroids; **A:** dehydrogenation; **B,C:** α,β-reduction; **D:** oxidation; **E,F:** α,β-keto-reduction; **G,H:** α,β-hydroxylation

Dehydrogenases perform oxidation reactions in different places of the steroid backbone, leading to double-bond formation by the loss of two hydrogens (A). On the other hand, reductases catalyse the addition of hydrogens which can lead to α- or β- isomers of the steroid (B, C). These kind of formations appear mainly in the A-ring of the AAS. The next important enzyme type in phase-I metabolism are hydroxysteroid-dehydrogenases, which catalyse the conversion of hydroxyl or keto groups (D) into the respective other, mainly at C3 or C17, but also at other positions on the sterane backbone. Hydroxylation again can result in both α- or β-isomers (E, F). Another common reaction is the addition of one or several hydroxyl groups on different C-atoms on the steroid in both α- or β-configuration(G, H)[27,35]. These hydroxyl groups, in turn, can subsequently be further oxidized to keto groups. Other, less common, phase-I reactions include 17-epimerization or the formation of a hydroxymethyl-group at the C17 position of 17-methylated steroids[47–49]. See Figure 12, section 1.2.4.2. All single reaction steps of phase-I

metabolism can be combined in different ways and in different orders, resulting in many different metabolic pathways and numerous metabolic products. The actual composition of a set of individual phase I reactions varies considerably from AAS to AAS, which makes the study of steroid metabolism very complex and laborious. However, in this work, already described phase-I metabolites were selected, and their corresponding phase-II metabolites were studied. The AAS metabolites selected for this work are discussed in section 1.2.4.

1.2.3.2 Phase-II metabolism

Phase-II metabolism results in the formation of more hydrophilic and polar metabolites. Phase-II reactions are characterized by conjugation reactions, where high-polar molecules are attached to the molecule and/or their phase-I metabolites. These highly polar conjugates are mainly glucuronic acid or sulfate, but other molecules such as glutathione, succinic acid, or cysteine are also described in the literature[50–56]. The conjugations contribute to the excretion of steroids from the body via the urine. In this work, the focus was on the conjugation reaction with glucuronic acid as substrate, also called glucuronidation. Glucuronidation is catalysed by the enzyme family of uridine 5'-diphospho glucuronosyltransferases (UGT) that use uridine-5'-diphosphate glucuronic acid (UDP-GC) as a co-substrate. UGTs are membrane-bound enzymes located in the endoplasmic reticulum, mainly in the liver but also in other tissues[28]. At least 16 different variants of these membrane-bound enzymes are known to be expressed in the human body[57]. Comprehensive studies with recombinant human hepatic UGTs have shown that different variants of the enzyme exhibit varying degrees of region-selectivity in the catalytic reactions performed. The selectivity of each UTG is related to the 17-β or 3-α groups and the proton position at C5 on the steroidal backbone. The orientation of the H^+ at this position has a dramatic effect on the shape of the ring structure. This, in turn, leads to a change in the position of the 3-α group. A 5-β position leads to an equatorial position of the 3-α bond and 5-α to an axial one. These different three-dimensional structures influence the binding ability of the enzymes. Further studies have shown inter-ethnic differences in the presence of different UTGs. For example, deletion polymorphisms of the enzyme UGT2B17 resulted in a significant reduction in the glucuronidation of testosterone in an Asian population compared with a Caucasian population[58,59]. These properties of UTGs should be considered when investigating potential phase-II

glucuronide metabolites. The general conjugation reaction is shown in Figure 8 using testosterone as an example.

5'-diphospho glucuronosyltransferases

Testosterone

Testosterone glucuronide

UDP-GC

UDP

Figure 8: Glucuronidation reaction of testosterone; UDP-PC: Uridine-5'-diphosphate glucuronic acid; UDP: Uridine-5'-diphosphate

In AAS, glucuronidation usually takes place at the 3α-hydroxyl-position and 3β-hydroxyl is more likely to be conjugated with sulfates. Both glucuronidation and sulfation can also occur at the 17-α- or 17-β-hydroxyl position. In some cases, glucuronidation can be observed at other positions, e.g., stanozolol is glucuronidated at its two nitrogen positions and its 3'-hydroxy metabolite is glucuronidated at position 3' on the pyrazole ring (see section 1.2.4.1)[60,61]. Although almost all AAS are excreted in the urine as phase-II metabolites, a few, less than 3%, are excreted unconjugated[62]. Furthermore, doubly conjugated glucuronides and sulfates or a combination of both are also described, even though these only represent a small percentage of steroid phase-II metabolism[63–65]. In doping analysis, steroids have traditionally been studied by hydrolysing the samples to separate parent substances and phase-I metabolites from their phase-II conjugates and subsequent analysis with gas chromatography[66]. Modern approaches are able to analyse phase-II conjugates directly (see section 1.3).

1.2.3.3 Long-term metabolites

The concept of long-term metabolites, first mentioned in 1995, has become indispensable in modern anti-doping analysis[67]. As the name suggests, these are metabolic products that show prolonged half-lives and are therefore detectable for a long time. There is no strict definition of "long-term", but it is generally understood to mean several weeks rather than

only a few days. The need to detect such metabolites was born out of a time when there were no out-of-competition controls. Especially steroids are taken during the training phase and were then often no longer detectable during a competition. Although OOC-testing was introduced in the late 1980s, interest in long-term metabolites has not waned. In general, there are two approaches to ensure the long-term detection of a substance. First, analytical methods are improved so that even very low concentrations of a metabolite can be detected in the body. Second, new metabolites of a drug are discovered that naturally remain in the body for a long time. Both approaches are pursued in modern anti-doping research and are also part of the present thesis. The introduction of high-resolution mass spectrometry in anti-doping analysis, for example, brought a significant improvement in the long-term detection of metabolites. In addition, the discovery of new structural features of metabolites has also repeatedly played an important role. An important example is the discovery of the 17β-Hydroxymethyl-17α-methyl-18-nor-androst-13-ene metabolite and its epimere of the AAS metandienone by the team of W. Schänzer in 2006[48]. These metabolites showed large elimination windows up to 18 days and provided the impetus for further discoveries of long-term metabolites of 17-α-methyl steroids. Based on this work, long-term metabolites with the same structural characteristics were identified for the AAS oxandrolone and dehydrochloromethyltestosterone[68,69]. Regardless of which approach is chosen, constantly improving the detection windows of substances is an essential part of anti-doping research.

1.2.4 Selected AAS

This thesis focuses on two of the most important AAS in doping analysis, stanozolol and dehydrochloromethyltestosterone. Both have been around for a very long time, but have lost none of their significance to this day. In 2020, these two even headed the list of the most frequently detected steroids worldwide according to WADA statistics[22]. The following sections briefly describe the history, characteristics, metabolism and analytics of the respective substances.

1.2.4.1 Stanozolol

The exogenous anabolic androgen stanozolol was chosen for this work because, according to WADA statistics[13–21], at least for the last ten years, it has been the most

frequently detected substance within the group of AAS. Therefore, a fast and simple approach to analysing this component is of great interest. Stanozolol, or chemically correctly termed 17α-methyl-5α-androst-2-eno[3,2-c]pyrazole-17β-ol, was first synthesised in 1959 and holds a unique position in the family of AAS with its special structure containing a pyrazole ring fused to the A-ring, as shown in Figure 9[70]. Besides the extra ring, an additional methyl group was added at position C17α of the basic structure of the endogenous steroid dihydrotestosterone.

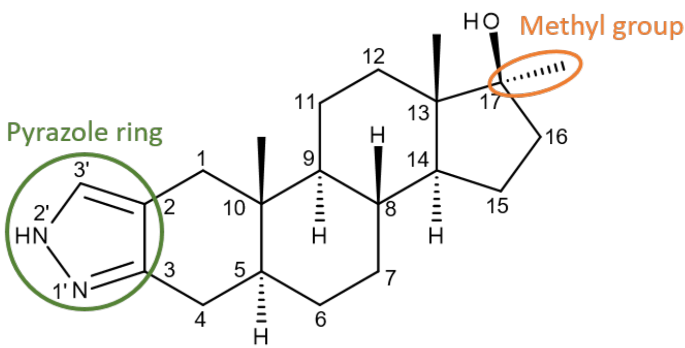

Figure 9: Chemical structure of stanozolol based on the endogenous steroid dihydrotestosterone; chemical modifications are highlighted

Stanozolol was used to treat conditions such as various vascular disorders, hereditary angioedema, corticosteroid-induced myopathy, chronic infections, decubitus ulcers, and severe trauma[71]. The strong anabolic and weak androgenic effects, as well as the high oral bioavailability, are the reasons why stanozolol is so popular among athletes, although it has been banned in professional sports along with many other anabolic steroids since 1974. Popular brand names are Winstrol and Stromba. Reported side effects include menstrual disorders, virilising effects, and depression in women, liver dysfunction, gastrointestinal irritation, but also various cardiovascular, endocrine and reproductive neurological disorders[72,73]. Since 1986, many different techniques and methods have been developed for the analysis of stanozolol and a large number of its metabolites. Most of these approaches are based on mass spectrometric techniques coupled to either gas (GC–MS) or liquid chromatography (LC–MS)[60,61,82–84,74–81]. Both phase-I and phase-II metabolism have been described. The most relevant phase-I metabolites and N-associated glucuronides investigated in this study are illustrated in Figure 10.

Figure 10: Most relevant phase-I metabolites and N-associated glucuronides of stanozolol; 1: stanozolol parent substance (blue); 2: 17-epistanozolol; 1-1: stanozolol-1'N-glucuronide; 1-2: stanozolol-2'N-glucuronide; 2-1: 17-epistanozolol-1'N-glucuronide; 2-2: 17-epistanozolol-2'N-glucuronide; 3: 4β-OH-stanozolol; 4: 3'OH-stanozolol; 5: 16β-OH-stanozolol; 3-1? to 5-2?: theoretical N-associated glucuronides

Compound 1 represents the parent compound stanozolol and 2 its 17-epimer. Metabolites 1-1 to 2-2 are the N-associated glucuronides studied in the first two papers of this thesis. Structures 3 and 4 are the major phase-I metabolites of stanozolol. 3-1? to 5-2? represent theoretically possible N-associated glucuronides of the respective phase-I metabolite that have not been studied to date. In addition, phase-II metabolites associated with OH-groups of stanozolol have also been characterized[61]. However, these metabolites do not show as much potential for long-term detection and are therefore not the subject of this work.

1.2.4.2 Dehydrochloromethyltestosterone (DHCMT)

Dehydrochloromethyltestosterone is, as the name suggests, a direct derivative of the endogenous steroid testosterone. In this molecule, a methyl group was added at the C17α position, a chlorine atom at the C4 position, and a double bond was established between the C1 and C2 positions, as visualized in Figure 11.

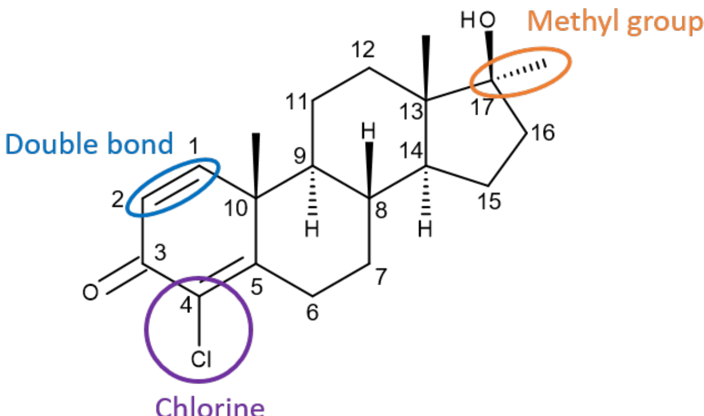

Figure 11: Chemical structure of dehydrochloromethyltestosterone based on the endogenous steroid testosterone; chemical modifications are highlighted

DHCMT, chemically named 4-chloro-17β-hydroxy-17*α*-methylandrosta-1,4-dien-3-on or also known as 4-chlorometandienone or oral-Turinabol, is one of the most detected anabolic steroids in professional sports according to WADA statistics. In 2020, DHCMT was the second most frequently detected steroid after stanozolol[22]. This drug was introduced for clinical use in 1965 and was used in the GDR from 1966 onwards as part of the state-organised doping of male and female high-performance athletes. Thus, it was the first so-called designer steroid used in the context of professional sports[85]. It was produced by the state-owned pharmaceutical company, VEB Jenapharm (Jena, Thuringia, GDR). From the mid-1970s onwards, DHCMT spread rapidly throughout the Eastern Bloc countries and the Western world, including West Germany and the USA[86]. Like stanozolol, DHCMT has a high oral bioavailability and high anabolic effects, but also considerable androgenic effects. The consequences of androgenic side effects could be particularly observed in female athletes from this period. Like all 17-alpha alkylated steroids, DHCMT is hepatoxic and has similar side effects to stanozolol and many other steroids: irregular menstruation, amenorrhoea, acne, hirsutism, impaired libido, impaired potency, fertility, and more. The successful detection of abuse of dehydrochloromethyltestosterone began in 1970 with the introduction of the first analytical method[87]. To date, many approaches have been published for the analysis of the parental substance and a number of metabolites[69,88–90]. All of them are based on GC-MS techniques. In this thesis, the focus is on the long-term metabolite 4α-chloro-18-nor-17β-hydroxymethyl-17α-methyl-5β-androst-13-en-3α-ol, called M3 in literature. The structural feature of this metabolite is the presence of 17α-methyl-17β-hydroxymethyl

groups and a completely reduced A-Ring. First mentioned in 2012, a recent study suggests the following metabolic pathways (Figure 12) for the formation of metabolite M3 and two other potential long-term metabolites (M2 and M4)[91].

Figure 12: Proposed metabolic pathway for the formation of dehydrochloromethyltestosterone metabolite M3 and other potential long-term metabolites; blue: parent substance; green: confirmed metabolites; red: suggested metabolites

Furthermore, this study also confirmed another metabolite (M4) and suggested one more (M2). Both also have a 17α-methyl-17β-hydroxymethyl group but a semi-reduced A-ring. Furthermore, it is assumed that epimers of the metabolites M3 and M4 also exist. Phase-II metabolic findings have not yet been obtained for any of these metabolites, but they might be expected to be similar, based on their very similar structure.

1.3 Analysis of anabolic androgenic steroids

1.3.1 General approach

As mentioned above, urine is the most commonly tested body fluid in routine doping control analysis. For this matrix and small molecule analysis, mass spectrometry combined with chromatography is the most important and widely used technique in anti-doping analysis. The long-standing gold standard for analysis of steroids in urine samples recommended by WADA is the following approach[92]. Athletes' urine samples are enzymatically or chemically treated to perform hydrolysis, where phase-II conjugates are separated from phase-I metabolites and parent compounds. The remaining compounds are then extracted with liquid/liquid extraction (l/l), trimethylsilyl-derivatised, and finally analysed with GC-MSMS[66,93] (see 1.3.2). A more modern way is to analyse phase-I steroids by LC-MSMS analysis without derivatisation. This approach saves time and is particularly important for substances that have, even with derivatisation, insufficient gas chromatographic properties, are thermolabile or non-volatile[94–97]. The general analytical approaches of AAS analysis in urine are shown in Figure 13.

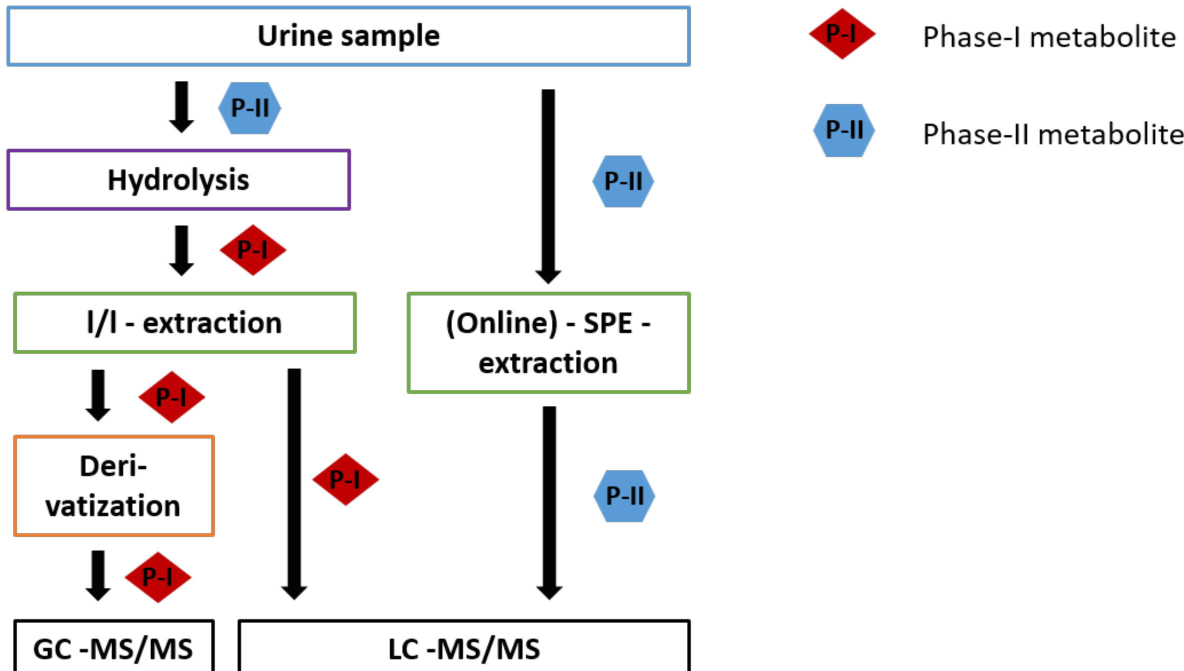

Figure 13: Overview of general analytical approaches fort detection of anabolic androgenic steroids

Even without the derivatisation step both methods are very time and resource-consuming. Both approaches involve many transfer steps, which are potential sources of error and require a high level of chemicals.

Furthermore, the hydrolysis step can be crucial. Hydrolysis is carried out with the enzyme β-glucuronidase from various bacterial sources or chemical methods. It is known that the efficiency of β-glucuronidase depends on the structure of the steroid itself and many other external factors such as matrix influences, sample preparation, or bacterial contamination[98–102]. Chemical hydrolysis approaches show limitations such as additional matrix interferences or degradation of analytes[103,104]. Furthermore, some AAS glucuronides, e.g. stanozolol-N-glucuronides, have shown to be partially or completely resistant to enzymatic hydrolysis[61,98]. Considering all these possible limitations, it is unlikely that a comprehensive analysis of phase-II metabolism is purposeful with this kind of indirect approach. However, with the advent of increasingly powerful LC-MSMS instruments, modern approaches for the analysis of AAS or doping substances, in general, have been developed. Many studies have shown that direct detection of steroid phase-II conjugates with LC-MSMS is a proper approach for steroid detection[60,61,113,114,105–112]. See right side in Figure 13. These methods have the great advantage that the time-consuming steps of enzymatic hydrolysis and derivatisation are entirely omitted. In many cases, depending on the target analytes, solid-phase extraction (SPE) is used instead of l/l-extraction. With highly sensitive instruments, sample preparation can even be reduced completely to diluting the sample with water. These approaches are called 'dilute-and-shoot' or 'direct injection' methods[115–117]. Most AAS are still analysed using the traditional GC-MSMS approach in routine anti-doping analysis, but the increasing importance of steroid analysis with LC methods is observable. One goal of this thesis was to further develop the idea of the dilute-and-shoot approach. Our approach was to combine solid-phase extraction directly with LC-MS measurement. Therefore, the subject of the first paper of this dissertation was the development and validation of a fully automated method for the analysis of phase-II metabolites of anabolic androgenic steroids.

1.3.2 Chromatography techniques

1.3.2.1 Gas chromatography

Gas chromatography falls into the category of adsorption and partition chromatography and uses gas as the mobile phase and a long, thin capillary as the stationary phase. The actual separation process takes inside this capillary wall in an immobilized viscous liquid. Inert and unreactive gases such as helium, argon, nitrogen, or hydrogen are usually used as the mobile phase[118]. In GC, mixtures of substances are vaporized at high temperatures and then separated. Therefore, this method is only applicable for components that are gaseous or can be vaporized without decomposition. The extensive use of gas chromatography for AAS detection in the field of anti-doping analysis began with the pioneering derivatization method introduced by the German chemist and professional cyclist Manfred Donike in 1969[119,120]. This reaction, based on the reagent N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA), is catalysed by trimethyliodosilane (TMIS), resulting in per-TMS derivatives that dramatically improve sensitivity for most steroids in GC-MS analysis due to the decrease of polarity and improvement of evaporability. Polar, actives groups such as hydroxyl or keto groups are exchanged by trimethylsilyl protecting groups, as illustrated in Figure 14 by the example of testosterone.

Figure 14: N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) derivatisation of testosterone for GC-MSMS analysis; TMIS: Trimethyliodosilane; OTMS: Trimethylsiloxy group

This approach, combined with preceding enzymatic hydrolysis with β-glucuronidase from *E. coli* and l/l-extraction, gives the gold standard method for AAS analysis. This The method is recommended by WADA to analyse endogenous and exogenous steroids and is therefore used by anti-doping laboratories around the world[85,92,93,121]. The method offers very low detection limits for many steroids but is also laborious and requires a non-negligible amount of hazardous chemicals. This work used this basic procedure to perform confirmation analysis of potential phase-I metabolites using synthesized

reference standards. One of the main objectives of this work was to emancipate from this method and develop a much more economical method for the detection of AAS phase-II metabolites by LC-MS analysis.

1.3.2.2 Liquid chromatography

As the name implies, this method uses a liquid medium as the mobile phase instead of gas. Packed separation columns of different sizes and adsorbent materials serve as stationary phase. The separation is based on the different degrees of adsorption of the analyte molecules on the surface of the stationary phase. Nowadays, the abbreviation LC is mainly used for high-performance liquid chromatography (HPLC), which involves very high pressures and small column diameters. This more recent chromatographic method made its first major appearance in the field of anti-doping analysis at the 2004 Athens Olympics. It was used to identify several classes of compounds, including anabolic steroids, narcotics, β2-agonists, and corticosteroids in urine samples[23]. However, a first approach for the analysis of steroids with liquid chromatography coupled to mass spectrometry using electron spray ionization (ESI) was published as early as 1992[122]. Unconjugated steroids and their metabolites, such as trenbolone or boldenone, which have large conjugated or cross-conjugated electron systems and therefore sufficient proton affinities, are well suited for analysis with LC-MS analysis[123]. Moreover, AAS that provide heteroatoms, e.g. nitrogen in stanozolol, are also suitable for this type of analysis. However, most AAS do not have any of the above properties and are therefore not directly detectable in their unconjugated form by LC-MS. For these compounds, the direct analysis of phase-II metabolites by LC-MS is of high interest. One of the first approaches of this kind was already carried out in 1997 by K. Bean and D. Henion[124]. So far, many papers have been published on this type of analysis, as already described in section 1.3.1.

Nowadays, ultrahigh-pressure liquid chromatography (UHPLC) is state of the art for the analysis of doping substances via LC-MS[125]. This approach is characterized by small particle and pore size of the stationary phase and the resulting very high pressure during the chromatographic process. This leads to short run times and high separation efficiency. In terms of stationary and mobile phase material, the most commonly used chromatographic method for the analysis of polar substances is the so-called "reversed-phase LC". The method utilizes a non-polar stationary phase and a polar mobile phase.

This means that the more nonpolar a substance is in the sample to be separated, the more likely it is to bind to the hydrophobic stationary phase and be retained on the column. Conversely, the more polar a component is, the faster it will move in the hydrophilic, mobile phase. The basic materials usually used for the stationary phase are C8, C18, biphenyl, or phenyl/hexyl. There are many different variants of these materials, which can contain a variety of chemical modifications. In addition, LC columns can be purchased in various lengths and diameters or with different particle and pore sizes. This offers a large space for compound-specific optimizations. Water, methanol, and acetonitrile are typically used as mobile phases. Mobile phases are usually applied in gradient mode. This means that two solvents are mixed dynamically during a run period to achieve a uniform increase in the organic solvent during the time of the analysis. This leads to an increase in eluotropic strength over time, resulting in improved separation efficiency. Modifiers such as formic acid or acetic acid and ammonium formate or ammonium acetate can be added to the mobile phase to improve chromatographic behaviour and ionization. The right choice of all these parameters contributes to a successfully designed LC method.

1.3.3 Mass spectrometric techniques

After successful chromatographic separation of the analytes, the individual substances must be detected. Besides UV or fluorescence detectors, mass spectrometric analysers are the most commonly used detectors for chromatographic systems. Since the introduction of mass spectrometry in the field of anti-doping analysis at the 1972 Summer Olympics in Munich, this technique has been dramatically developed[23]. Nowadays, triple quadrupole analysers are the most commonly used mass analysers due to their great sensitivity, selectivity, and comparatively low price. High-resolution analysers give even better sensitivity and selectivity but are significantly more expensive[126]. Since the triple quadrupole analyser was used only to a minimal extent in this work, it will not be discussed further.

The connection between the chromatographic and the mass spectrometric system is an essential component. The ionization source assumes this role. It transfers the analytes from an uncharged to a charged state, which can be detected by mass spectrometry. Different ionization techniques are used depending on the chromatographic technique and

the requirements of the analysis and the substances to be detected. Both molecular size and polarity play a significant role in the selection of the ion source. While electron impact ionization (EI) is mainly used for GC-MSMS, electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), or atmospheric pressure photoionization (APPI) sources dominate for LC-MSMS applications[127,128]. The application areas of the most commonly used ion source types are shown in Figure 15.

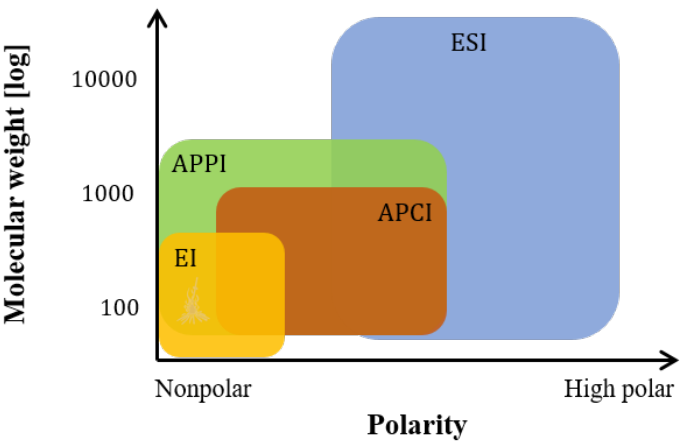

Figure 15: Application areas of the most commonly used ion source types for GC- and LC-MS; EI: electron impact ionization; APCI: atmospheric pressure chemical ionization; APPI: atmospheric pressure photoionization; ESI: electrospray ionization

Since mainly highly polar phase-II metabolites were analysed in this work, ESI was used almost exclusively. EI was only used in paper 3 to confirm a phase-I metabolite with GC-MSMS.

1.3.3.1 Electron Spray Ionization

Electron spray ionization is a weak, atmospheric pressure ionization (API) method, which means that ions remain intact during the ionization process. This can have both advantages and disadvantages. On one hand, this has the advantage that the data obtained is very easy to interpret, but on the other hand, it provides less compound-specific information. However, the biggest advantage is that ESI can ionize non-volatile and thermally labile molecules. The electrospray process can be considered as an electro-chemical reaction[129]. In this process, ions are generated that originate from a solution. The solution flows through a small capillary and is subjected to a high voltage. As a result, charging of the solvent takes place, which causes electrons to flow to or from the metal capillary, depending on the polarity. Furthermore, the voltage causes the formation of an

electric field between the capillary and a counter electrode, in this case, the mass spectrometer, as shown in Figure 16.

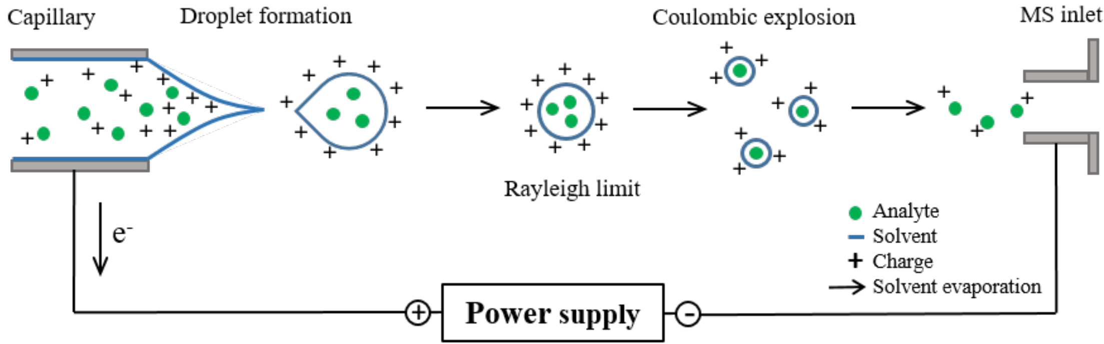

Figure 16: Schematic representation of an electrospray ionization in positive mode

When a sufficiently high voltage is applied between the solution and the MS inlet, the solution begins to break down into fine droplets. Further evaporation of the solvent leads to a steady decrease of the droplets until the so-called Rayleigh stability limit is reached. At this point, the Coulombic explosions occur. This process produces very small, multiply charged droplets containing single or very few molecules. The charges are then transferred from the droplet surface to the analytes during further evaporation. This is a simplification of the exact mechanism of charge formation. There are several models that attempt to explain exactly how charge formation occurs[130]. The electrospray can be used with a positive as well as with a negative voltage, resulting in the formation of cations (positive) or anions (negative). In the positive ion mode, charging generally occurs by protonation, and in the negative mode, charging occurs by deprotonation of the analyte[131]. In addition, the charging can also result from adduct formation with sodium, potassium and ammonium[132]. The efficiency of the charging process depends on the type of the analyte, the solvent, and other matrix components. In the end, depending on the size of the analytes, singly or multiply charged ions are generated, which are then transferred to the mass analyser via the electric field. This is a very gentle ionization method, as very little residual energy is transferred to the analyte at the end of the process. As a result, even very large molecular complexes remain intact and are transferred into the gas phase.

1.3.3.1.1 Ion suppression and enhancement

A well-known phenomenon in ESI is the influence of various factors on ionization efficiency. These factors can lead to both positive and negative effects. On one hand,

there may be an increase in ion yield, called ion enhancement. On the other hand, there can also be a reduction in ion yield, called ion suppression. The following issues are considered to be the most important factors influencing ion efficiency[133]:

- Various matrix influences competing with the analytes for charges
- Ions are neutralized by acid/base reactions in the gas phase
- Additives in the mobile phase
- Instrumental design

All these factors can have different consequences. Ion suppression can reduce detection capability by decreasing the signal intensity of the analyte. Inter-sample variability in matrix effects may affect precision, ion ratios, linearity, and quantitation. In general, the reliability of the analytical method may be reduced. Ion enhancement can increase the detection capacity and thus improve parameters such as the detection limit or the signal-to-noise ratio. However, the negative effects on the reliability of the analytical method are the same as for ion suppression. To counteract the undesirable effects, a number of countermeasures can be taken. The best ways to reduce variations in ionization efficiency are as follows:

- Optimized sample preparation and purification of the extract
- Use of suitable internal standards or standard addition
- Optimization of chromatographic conditions
- Use of suitable additives or modifiers in the mobile phase (e.g. formic acid)

Since it is usually impossible to eliminate all ionization effects, it is recommended that the suppression or enhancement of ions be determined by comprehensive validation. This contributes significantly to better evaluating and assessing the results obtained. This can be done by various types of experiments. A comparable simple method is to compare the signal of the analyte spiked in a post-extraction blank sample with the signal of the analyte spiked in the same concentration in pure mobile phase or solvent. A decrease or increase in signal intensity provides information about ion suppression or enrichment. In papers 1 and 2 of this thesis, the ionization effects of the method used were acquired as part of the validation.

1.3.3.2 Orbitrap high-resolution mass spectrometer

The mass spectrometric instrument mainly used for this thesis was an Orbitrap high-resolution system. At this point, it should be briefly explained what constitutes high-resolution mass analysers. The most important parameter used to describe the performance of a mass spectrometer is the mass resolving power. Resolving power is the ability of an instrument to separate closely spaced mass peaks. The term "resolution" is often used in the literature as an analogy for resolution power. According to the IUPAC, mass resolving power R is defined as the ratio of the mass m of a mass peak and the difference Δm to a second peak m + Δm, which is just separated from the first peak[134] (Figure 17). Signals are considered to be separated if the valley between the two peaks does not exceed a defined value of the peak height with the lower intensity.

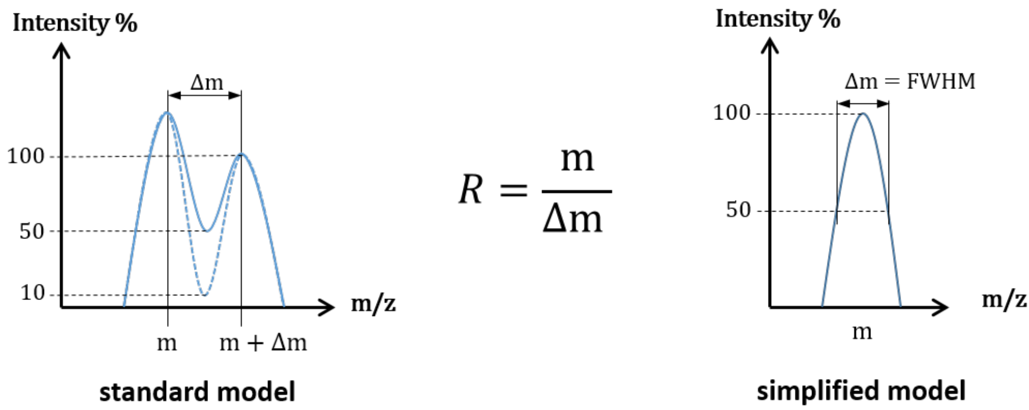

Figure 17: Mass spectrometry resolving power (R) definitions

Typically defined peak heights are 10% or 50%, expressed as Δm10% or Δm50%, the intensity of the lower peak as illustrated on the left side in Figure 17. In addition to this standard model, there is a simplified model that is used more frequently in practice. For the latter, only one peak is considered, and the so-called Full Width at Half Maximum (FWHM) is used instead of Δm as illustrated on the right side in Figure 17. This value corresponds to the peak width at a peak height of 50%. And this value is then used in place of Δm in the formula for R. In practice, high-resolution mass spectrometers are defined to have $R_{50\%} = m/\Delta m50\% > 10\ 000$ or $R_{FWHM} = m/FWHM > 20\ 000$[135,136]. The resolving power of (triple) quadrupole mass spectrometers is significantly lower than this value. Another important parameter is the mass measurement accuracy. This value, expressed in parts per million (ppm), gives the difference between the measured mass and

the exact atomic mass of the observed ion, depending on its molecular composition. Modern high-resolution mass spectrometry techniques include time-of-flight (TOF), Fourier transform ion cyclotron resonance (FT-ICR), and Orbitrap detectors.

The Orbitrap system, commercially introduced in 2005, was the device of choice for this work[137]. The actual instrument used in all three papers in this thesis was a Thermo Fisher Q-Exactive Orbitrap system (Austin, Texas, USA). This instrument is characterized by its combination of an atmospheric-pressure ionization source (e.g. ESI), a quadrupole mass filter, the so-called C-trap, a collision cell, and a high-resolution mass detector[138]. The schematic structure of this instrument is shown in Figure 18[139].

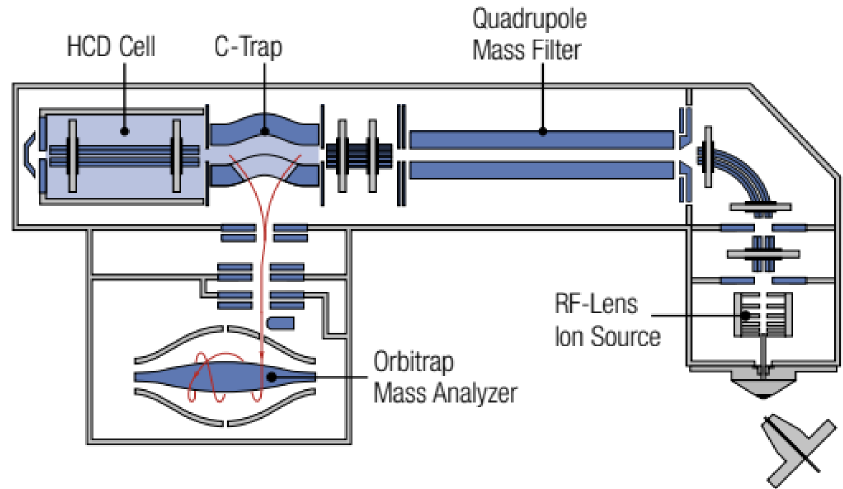

Figure 18: Schematic structure of an Orbitrap mass spectrometer[139]

The quadrupole mass filter allows the preselection of precursor ions. The following C-trap is a gas-filled, curved quadrupole without mass filtering that enables the intermediate storage of ions. From there, the ions can then be transferred by short pulse injection either to the Orbitrap mass analyser or to a high energy collision-induced dissociation (HCD) cell. In the HCD cell, the ions are then fragmented and subsequently transported to the mass analyser via the C-trap. A spindle-shaped electrode is located in the centre of the mass analyser of the Orbitrap. The ions from the C-trap are injected radially to this electrode into the Orbitrap and move on paths around the central electrode due to electrostatic attraction. The decentrally injected ions oscillate around the central electrode and simultaneously in a longitudinal direction to it. The frequency of this oscillation generates signals at the surrounding detector plates, which are converted into the corresponding m/z ratios by Fourier transformation. According to the manufacturer, this

device achieves a resolution R_{FWHM} of 70 000 and a mass accuracy of less than 1ppm. This system can be used to perform Selected Ion Monitoring (SIM), Parallel Reaction Monitoring (PRM), and full-scan operation, among others. Furthermore, an online polarity switching is possible.

1.3.3.2.1 Parallel Reaction Monitoring

Besides the full scan mode, the PRM method was mainly used in this work. This approach results from combining a quadrupole and the Orbitrap[140]. One or more precursor ions can be selected in the quadrupole, which are subsequently fragmented in the HCD cell. All ions resulting from fragmentation of single or multiple precursor ions are then detected simultaneously in one high-resolution MS/MS scan as visualized in Figure 19.

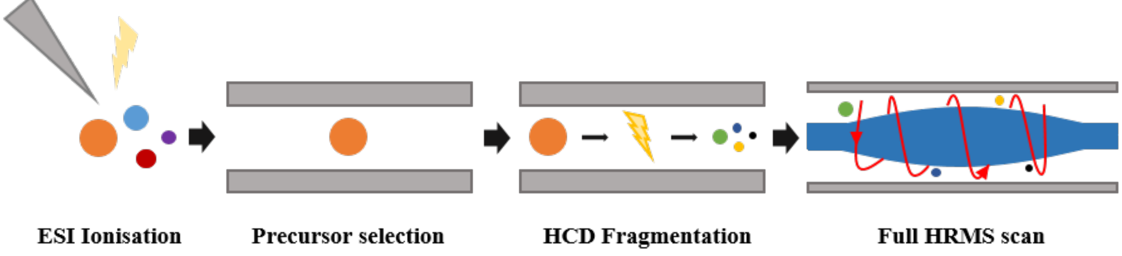

Figure 19: Parallel Reaction Monitoring principle; ESI = Electron Spray Ionization; HCD = high energy collision-induced dissociation; HRMS = high-resolution mass spectrometry

A high-resolution full scan of all generated fragments is generated over the entire measurement period. From these data, extracted ion chromatograms (XIC) can be generated, including any of the generated fragments and the precursor ion. From these chromatograms, in turn, it is very easy to select the most specific or sensitive fragments, which can then be used for qualitative and quantitative purposes. This results in a significantly higher number of mass transitions within one measurement compared to the otherwise very common selected reaction monitoring (SRM) or multiple reaction monitoring modes. Furthermore, the PRM is very well suited for the identification of unknown molecules. Structural information can be obtained by pre-setting the theoretical atomic mass of the suspected molecule in the quadrupole and analysing the resulting high-resolution data of the resulting fragments. In this thesis, PRM was used to identify and characterize unknown molecules and quantify known substances using reference standards.

1.3.4 Online-SPE

Given the stringent requirements of anti-doping analysis, it is essential to reduce negative matrix effects and increase the methods' sensitivity, selectivity, and robustness. On the other hand, a time-saving and economical operation is in the interest of every practicing laboratory. Dilute-and-shoot or direct injection methods meet these requirements but have limitations in selectivity, sensitivity, robustness, or quantification[116]. To solve these problems, the next step in method development has been the introduction of the so-called online-SPE approaches. These techniques combine the analytical advantage of SPE sample separation with the simplicity of direct injection methods. In principle, such methods involve the installation of a fully automated SPE apparatus directly in front of the LC-MS instrument. Reusable small analytical columns are usually used as sample separation devices. These are installed between the injection device and the analytical column of the LC apparatus through a valve system. The analyte extraction and the measurement of the sample are then carried out within one cycle. This comparatively new type of procedure saves a lot of manual work and thus time and money. There is no best-practice application of this type of method in the field of anti-doping analysis yet, but several research groups have already addressed this problem[141–150]. These approaches were focused on a specific type of analyte and had more or less sophisticated instrumentation. Most worked with a two-valve system, utilizing multiple pump systems and triple quadrupole mass analysers. The various methods differ in how the valves are connected to each other, the sample is injected into the system, the analytes are applied to the column, the separation is performed, and the system is subsequently cleaned. However, all have the common goal of reducing sample preparation to a minimum while still achieving maximum analytical performance. To our knowledge, no online-SPE method has previously been presented for the analysis of phase-II metabolites of steroids. Therefore, the development of such a method was one of the main objectives of this work.

1.3.5 Study of AAS metabolism

Since AAS have manifold metabolic behaviour, it is essential to perform comprehensive metabolic studies to find the best potential markers for detecting steroid abuse. Although suitable metabolites have already been found for many steroids, this topic's continuous development and re-evaluation is an essential part of anti-doping research. In principle, a well-suited metabolite is characterized by the following two properties:

- The metabolite signal should be as abundant as possible
- The metabolite should be detectable for as long as possible

However, in reality, often only one of the two properties can be achieved. Nevertheless, with the continuous improvement of analytical tools and methods, the first point is becoming less important, and the long-term detection of metabolites is increasingly coming to the fore (see 1.2.3.3).

Two general approaches can be distinguished in the detection of new metabolites: targeted and untargeted analysis. In the first case, researchers already have a hypothesis about the structure of the metabolite and attempt to find evidence of that structure in positive samples. The analysis strategy is then purposefully developed and applied to proven positive samples for this task. Untargeted analysis, on the other hand, uses a method that generates extensive data, such as full-scan mass spectrometry. From the data one attempts to gain knowledge without having a hypothesis about the exact structure of the metabolites. For this purpose, data from blank or pre-administration and post-administration samples are compared. The approach is complex and laborious and requires a targeted analysis as final proof. However, this also allows several metabolites to be investigated simultaneously. Regardless of which approach is chosen, performing these studies on many different positive samples is desirable. Individual metabolism is highly dependent on genetics, gender, age, diet, and ethnicity of the subject. Furthermore, the amount and the form of ingestion of the drug can influence metabolism. Ideally, the analyses can additionally be performed with excretion study samples. This allows an estimation of the excretion of metabolites over time. So-called detection windows can be determined for each metabolite. Of course, these *in vivo* approaches in humans are not always possible, as there is no access to positive or excretion study samples. Human excretion studies are challenging to perform due to many ethical considerations. Doping agents are mostly unapproved substances for which no valid pharmacology and toxicology data are available. Access to the substances alone is a significant challenge. Alternatively, animal models, such as chimeric mice or zebrafish, or *in vitro* models, such as cell cultures, are also used in the field of anti-doping research[151–153].

The final step to unequivocally prove the existence of a metabolite is to confirm the postulated structure with authentic standard substances. The reference standards must be

synthesized in high quality and their chemical structure ideally confirmed by nuclear magnetic resonance spectroscopy (NMR). Comparison of the standard substance with a suspect substance in a sample is usually performed using mass spectrometry in conjunction with chromatography techniques. For anti-doping purposes, this kind of approach is recommended and described in a technical document published by the WADA[154]. The identification of an analyte is based on a comparison of the retention times and relative abundances of at least two diagnostic ions of the substance detected in a sample with those of a sample containing the reference standard. The criteria to be met depend on the analysis method used, but also on the signal properties, and are precisely regulated in the above-mentioned paper. In this thesis, these criteria were also used to unambiguously identify newly described metabolites.

2. Aims of the study

The general goal of this thesis was to identify and characterize new phase-II metabolites of AAS for doping control analysis. In addition, the focus was on developing a time- and resource-saving method for routine doping analysis based on high-resolution mass spectrometry coupled with liquid chromatography using AAS phase-II metabolites as targets. Furthermore, basic knowledge of the phase-II metabolism of selected exogenous steroids in the human body should be acquired. These new findings could then find practical application in the global fight against doping abuse.

Two specific AAS were chosen for this work, stanozolol and dehydrochloromethyltestosterone. Although stanozolol has been the most frequently detected AAS for many years, its phase-II metabolism has only been minimally elucidated and is therefore of great interest for research. For DHCMT, there is almost no data on phase-II metabolism and analytical techniques are limited to GC-MS, which is no longer the current standard in anti-doping analysis. Thus, the summarized aims of this thesis were:

1. Development and validation of a time- and resource-saving method for the analysis of AAS phase-II metabolites
2. Gain new insights into phase-II metabolism of stanozolol
3. Gain new insights into phase-II metabolism of dehydrochloromethyltestosterone

3. Methods and material

3.1 Sample material

All positive urine samples used in this work were collected by accredited sample collection authorities according to WADA's collection guidelines[24]. Consistent with the ISL, the athletes previously permitted to use the samples for research purposes[9]. The samples were analysed by the WADA-accredited anti-doping laboratory Seibersdorf Labor GmbH and subsequently anonymized and provided for research purposes. Additionally, WADA proficiency test samples were used for the experiments described in paper 1. These samples, mostly excretion samples, are sent to anti-doping laboratories as part of the external quality assurance system (EQAS). These can also subsequently be used for research projects. Furthermore, a quality assurance program sample provided by the World Association of Anti-doping Scientists (WAADS) was used for the experiments described in paper 3. Both EQAS and WAADS samples have the advantage that other laboratories also receive these samples, and thus good comparability of analytical results can be achieved. The excretion study samples used in the experiments described in paper 2 were provided by the accredited anti-doping laboratory Cologne, Institute of Biochemistry, German Sport University Cologne, Germany. A healthy male subject received a single oral dose of 5 mg of stanozolol (Winstrol®), and urine samples were collected up to 28 days after administration[61]. All blank urine samples used for this thesis were provided by healthy female and male volunteers. Until further use, all samples were stored at -20°C.

3.2 Sample concentration

Oasis HLB cartridges (6 ml, 500 mg) and a vacuum ejector-driven glass chamber were utilized to perform sample concentration in paper 3 (Waters Corporation, Milford, MA, USA). 5 ml of methanol (MeOH) was used for conditioning, and 5 ml of Milli-Q purified water (MQ) was used for washing the cartridges. 5 ml of urine were loaded, and subsequently, the sample was washed with 2 x 5 ml MQ, dried for 5 min and eluted with 2 ml MeOH.

3.3 Sample preparation

For the experiments described in all three papers, sample preparation was reduced to dilute a small volume of urine (500-250µl) 1:1 with MQ. Subsequently, an internal standard solution containing 16,16,17α-d3-testosterone-glucuronide with a final concentration of 30 ng/ml was added, and samples were vortexed for 10 seconds.

3.4 Online solid-phase extraction

The online solid-phase extraction method was introduced in paper 1 and then further used for the sample preparation described in papers 2 and 3. Based on a standard HPLC dual-pump system, the online-SPE was installed via an automated, two-position, six-port HPLC valve (MXT715–000, Rheodyne LLC, Bensheim, Germany). The extraction column, an Accucore Phenyl-Hexyl, 10 × 3 mm column with 2.6 µm particle size and 80 Å pore size (Fischer Scientific, Loughborough, UK), was installed between the injector and the valve using a Universal Uniguard Holder, 2.1/3.0 mm ID (Thermo Scientific, Bellefonte, USA). Capillaries from a Viper finger-tight fittings system (Thermo Fisher, Austin, Texas, USA) connected the single components. The mobile phase of the online-SPE corresponds to that of the LC-HRMS method and is described in 3.8. In the first step of the online SPE procedure, the valve is set to the 1-2 position for two minutes, and the mobile phase stream is directed over the extraction column into a waste container. The analytes are trapped on the column during this step, and unwanted matrix compounds are flushed away. In step 2, the valve switches to position 1-6, and the flow is conducted via the analytical column to the mass spectrometer. At the same time, the solvent gradient is started, and the elution of the analytes from the extraction column begins. Now the purified analytes are transported to the analytical column as in a standard HPLC method.

3.5 LC-HRMS

The measurements described in all three papers were carried out on a Vanquish Horizon UHPLC+ System coupled to a Q-Exactive Orbitrap high-resolution mass spectrometer (Thermo Fisher, Austin, Texas, USA). Water with 0.2% v/v formic acid (FA) (solvent A) and methanol with 0.1% v/v FA (solvent B) were used as mobile phases. After conditioning and loading the extraction column with 10% solvent B for 2 minutes, the solvent gradient is continued from 10% solvent B up to 100% over 7 minutes, then 100%

B is held for 2 minutes, and then lowered again to 10% for 2 minutes to purge and re-equilibrate the system. An injection volume of 25 µl, a constant flow of 0.4 ml/min, and a constant temperature at 25°C were applied.

HRMS was carried out with both positive and negative electrospray ionization in full scan and PRM modes. The spray voltage was set to 3.8kV, and the capillary temperature was 320°C. Nitrogen was used both as sheath and as auxiliary gas. The mass resolution was set to 70 000 at m/z 200. Full scans were performed in the range of m/z 300–600, and PRM measurements were carried out in separate runs. Collision energies (CE) were optimized for each metabolite to obtain the highest signal intensities. For XICs, an ion extraction range of 2 ppm in paper 1 and 5 ppm in paper 2 and 3 were used. Isolation windows were set to ±1 m/z in all cases.

3.6 GC-MSMS

In paper 3, GC-MSMS analysis was used for phase-I metabolite confirmation according to the standard protocol as recommended by WADA[92]. Therefore, 1ml sample was diluted with 1 ml 0.8 M phosphate buffer (pH 7), 25 µl β-glucuronidase and 50 µl IS solution were added and subsequently samples were heated at 50°C for 2 hours to perform enzymatic hydrolysis. After adding of 1 ml of 20% potassium carbonate buffer (pH 9.0) and 5 ml of MTBE, an l/l-extraction by shaking samples for 10 minutes was performed. Then, the organic layer was separated and evaporated to dryness. As the last step, 80 µl derivatization working solution (NH_4I in MSTFA and ethanthiol) was added, and samples were heated at 60°C for 20min to carry out trimethylsilyl derivatization. GC-MSMS measurements were performed on a Trace-1300 gas chromatograph coupled to a TSQ-8000 Evo triple quadrupole mass spectrometer, containing a TriPlus-100 autosampler (Thermo Fisher, Austin, TX, USA). For chromatographic separation, a RTX-1MS fused silica capillary column, 15 m × 0.25 mm ID, 0.11 µm film thickness, was used (Restek, CP-Analytica, Mistelbach, Austria). High-purity helium was used as carrier gas with a constant pressure of 90 kPa. Electron ionization (EI) mode with electron energy of 70 eV was used to perform measurements in SRM mode. The temperature program and ion transitions were optimized for this specific application.

3.7 Sample fractionation

Sample fractionation was performed in paper 3 to isolate single metabolite molecules. This was accomplished by installing a T-piece between the analytical column and the mass spectrometer in the online-SPE-LC-HRMS apparatus described previously. By dividing the stream in two, it was possible to measure and collect fractions simultaneously. Three different fractions in three different retention time windows were collected in ten runs with 50 µl of injected sample each. Subsequently, the single samples were pooled, evaporated, and reconstituted in MQ.

3.8 Derivatisation experiment

In paper 3, a simple derivatization experiment was performed to distinguish between two variants of glucuronic acid conjugation sites. Therefore, under argon atmosphere, 5 ml of tritylation solution (trityl chloride in dimethylformamide (DMF)) was added to 0.5 ml of evaporated sample, and the mixture was stirred at room temperature. Aliquots of 0.5 ml were taken in regular intervals up to 6 days reaction time, and the reaction was quenched by adding 0.25 ml of sat. aq. $NaHCO_3$. Afterwards, water and DMF were removed by stirring under a vacuum, yielding a brown-yellowish solid product. For subsequent analysis, the product was dissolved in 0.5 ml MQ, centrifuged and the aqueous supernatant was transferred into a measuring vial.

3.9 Method Validation

Comprehensive method validations were part of the work for paper 1 and 2. These were intended to demonstrate both the efficacy of the method and the usability of the newly identified metabolites. The validation parameters are based on ISL and are designated for qualitative and semi-quantitative purposes. Therefore, specificity, precision, robustness, linearity, accuracy, matrix effects, carryover, and limit of identification were determined.

4. Summary of results

4.1 Paper 1

Development and validation of a simple online-SPE method coupled to high-resolution mass spectrometry for the analysis of stanozolol-N-glucuronides in urine samples

Lorenz Göschl, Günter Gmeiner, Valentin Enev, Nicolas Kratena, Peter Gärtner, Guro Forsdahl

The first aim of this work was to develop and validate a simple, fast, and highly sensitive online solid-phase extraction method coupled with liquid chromatography – high-resolution tandem mass spectrometry for the analysis of steroid-glucuronides. A detailed description of the installation of the method is a major part of this work. This new approach is characterised by only two manual steps in sample preparation: Dilution of the sample with water and addition of the internal standard. The subsequent extraction step is fully automatically integrated into the measuring process. This method was validated with two newly synthesized phase-II metabolites of the androgenic anabolic steroid stanozolol. With this method and access to the reference material, it was possible for the first time to unequivocally confirm the presence of 1'N- and 2'N-stanozolol glucuronide in stanozolol-positive human urine samples. The validation was based on the WADA specifications and contained parameters such as specificity, intra- and inter-day precision, accuracy, linearity, robustness, carryover, matrix effects, and limit of identification. Remarkable results were achieved during the validation, shown in the following table for both metabolites 1'N- and 2'N-stanozolol glucuronide (1N-STANG and 2N-STANG).

Table 1: Validation results of the developed online-SPE method;

Parameter	c [ng/ml]	n	1N-STANG	2N-STANG
Specificity	1	10+10	10 / 10	10 / 10
Intra-day precision (CV)	1	10+10+10	3.1%	4.9%
	10		5.5%	3.7%
	50		4.8%	3.3%
Inter-day precision (CV)	1	10+10+10	3.4%	7.8%
	10		5.6%	6.8%
	50		4.5%	5.0%
Accuracy	1	10	87.0%	90.2%
	10		91.6%	94.2%
	50		99.7%	102.1%
Linearity r^2	1 - 100	6 x 4	0.999	0.998
Robustness	1	3 x 3	9 / 9	9 / 9
Carryover	200	1	0%	0%
Matrix effects	1	10	160%	151%
LOI	-	3	75 pg/ml	75 pg/ml

In addition to excellent confirmation analysis performance, the method shows sufficient potential for the identification and characterization of unknown metabolites, which was further used for the work described in the following publications.

4.2 Paper 2

Stanozolol-N-glucuronide metabolites in human urine samples as suitable targets in terms of routine anti-doping analysis

Lorenz Göschl, Günter Gmeiner, Peter Gärtner, Georg Stadler, Valentin Enev, Mario Thevis, Wilhelm Schänzer, Sven Guddat, Guro Forsdahl

After establishing an efficient method for the analysis of steroid glucuronides, particularly stanozolol glucuronides, the goal of this paper was to elucidate the overall stanozolol-N-associated phase-II metabolism. In this work, we were able to describe for the first time the unambiguous existence of two new N-associated phase-II, 17-epistanozolol-1'*N*-glucuronide and 17-epistanozolol-2'*N*-glucuronide, in stanozolol-positive human urine samples due to the access to high-quality reference standards. We also performed a comprehensive validation for these two new metabolites using the

previously published online-SPE method and obtained similar good validation parameters as in the previous work. Furthermore, we were able to gain knowledge about the metabolism behaviour of stanozolol-N-glucuronides. Figure 20 shows elimination curves obtained from samples of a stanozolol excretion study.

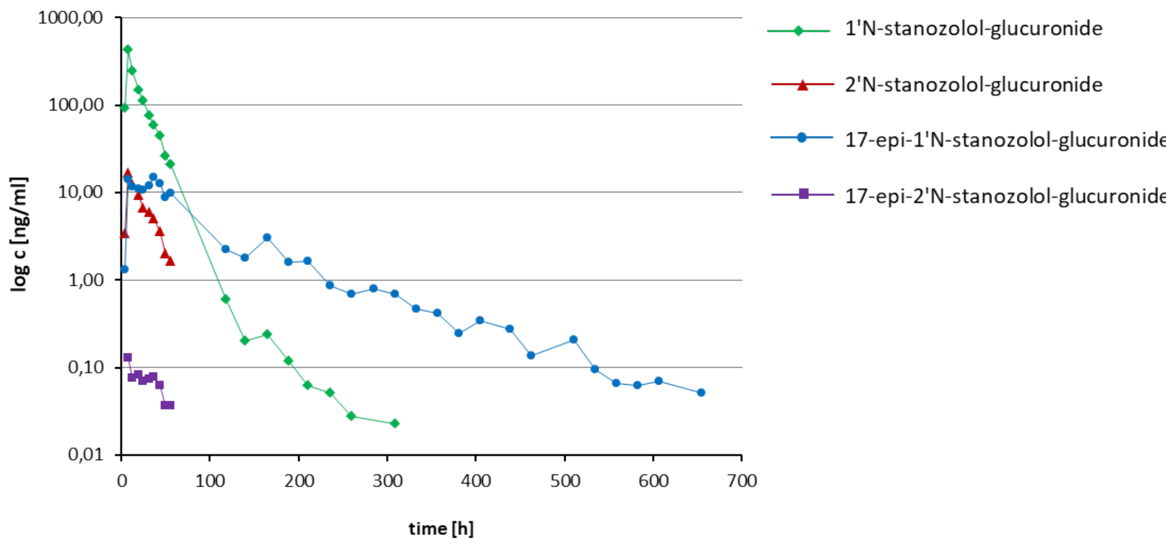

Figure 20: Elimination curve of four stanozolol-N-glucuronides; y axis: Concentration in nanograms per milliliter urine on logarithmic scale, x axis: Time in hours

Large detection windows for stanozolol-1'*N*-glucuronide and 17-epistanozolol-1'*N*-glucuronide up to 12 days and respectively up to almost 28 days can be observed. The other two 2'N- associated metabolites have much shorter detection windows but can still provide information about the time of application of stanozolol if successfully detected. Considering that stanozolol-N-glucuronides are immune to glucuronidase and therefore undetectable in routine doping analysis, the data of this work suggest that doping analysis in the future should focus more on direct detection of phase-II metabolites.

4.3 Paper 3

Detection of DHCMT long-term metabolite glucuronides with LC-MSMS as an alternative approach to conventional GC-MSMS analysis

Lorenz Göschl, Günter Gmeiner, Peter Gärtner, Michael Steinacher, Guro Forsdahl

This paper is about the detection of phase-II metabolites of the AAS dehydrochloromethyltestosterone (DHCMT). The goal was to find long-term metabolites, which are detectable with LC-MS. The conventional method for the analysis of this substance, GC-MSMS, is quite sensitive and selective but also very time- and resource-consuming. In this work, we introduce a new approach for a simple analysis with LC-HRMSMS using the already established online-SPE method. The analysis is based on the direct detection of two newly described and characterized phase-II metabolites of the DHCMT long-term metabolite 4α-chloro-18-nor-17β-hydroxymethyl-17α-methyl-5β-androst-13-en-3α-ol, called M3 in literature. Different methods as LC-HRMSMS, GC-MSMS, fractionation, and derivatization experiments were combined to identify and characterize two different glucuronide-acid conjugates of metabolite M3 in positive human urine samples for the first time. These two metabolites, called DHCMT-M3-3-glucuronide and DHCMT-M3-17-methyl-glucuronide, and the parent substance DHCMT-M3 are shown in Figure 21.

Figure 21: Chemical structures of DHCMT-M3-3-glucuronide, DHCMT-M3 and DHCMT-M3-17-methyl-glucuronide; DHCMT = dehydrochloromethyltestosterone

Furthermore, a third glucuronide metabolite was identified, however without determination of the exact isomeric structure. The detection of these phase-II metabolites is of great interest for confirmatory analyses because this approach requires little sample material and is quite rapid, requiring almost no sample preparation. This work is another stage in the shift of AAS analysis from GC-MS to LCMS, leading to a more recourse and time-saving fight against doping abuse.

5. Discussion

5.1 General aspects

This doctoral thesis's general goal was to improve the routine anti-doping control analysis in terms of increasing efficiency and effectiveness. This is of great importance due to the increasing qualitative and quantitative requirements being imposed on accredited anti-doping laboratories worldwide. From year to year, more and more substances have to be analysed, and a higher analytical performance is required. This is, needless to say, in the interest of fair sporting competition and fair treatment of athletes, but it also confronts the professional laboratories with major challenges. Therefore, a lot of research and development work is being done in this area, which includes this thesis.

To address the mentioned tasks, two general aspects related to improving the analysis of exogenous androgenic anabolic steroids were central in this work. On one hand, the aim was to develop a highly effective analytical method for the detection of steroid phase-II metabolites in human urine. Emphasis was placed on reducing labour and chemical resources to save costs, time and preserve the environment. On the other hand, the focus was on describing and characterizing new AAS phase-II metabolites and determining their utility for routine anti-doping analysis. These goals are closely related to the fact that analysis of anabolic androgenic steroids is generally dominated by the detection of phase-I metabolites using GC-MSMS. However, this type of approach is no longer state of the art in many other areas of analytical chemistry where LC-MS has replaced GC-MS. As already discussed above, LC-MS is characterized by its supposed simplicity, which is justified by the partial elimination of time-consuming sample preparation and the easier interpretation of the analysis data. Since a large number of steroids are too nonpolar for analysis by LC-MS, it is reasonable to select their highly polar phase-II metabolites as targets for detection. Methods for the detection of steroid phase-II metabolites have been developed for many years, but most of them involve laborious sample preparation steps and/or use simple triple quadrupole mass spectrometry systems for detection. Furthermore, online-SPE methods published to date rely on complicated installation systems and have not been optimized to analyse steroid phase-II metabolites. In addition, there exist almost no purchasable reference standards for AAS phase-II metabolites.

In anti-doping analysis, however, not only the effectiveness of a method is important, but also its safety and reliability is a major issue. Both an athlete's and analyst's careers depend on it. A wrongful conviction of an athlete can have consequences that threaten his or her existence and must therefore be prevented by all means. But false-positive results can also have dire consequences for a professional laboratory. This can lead to the closure of the facility, which of course, affects many people. Quality management also plays a very important role in such an area. Therefore, the idea of this work was to increase not only the effectiveness but also the reliability of an analytical result as much as possible. On one hand, this was achieved with the help of extensive validation assessments of the newly developed method. On the other hand, we have endeavoured to prove our hypotheses for new metabolites with high-quality synthesized reference standards. For this purpose, we worked very closely with colleagues from a synthesis laboratory. In reality, generation of reference material was not always feasible and we were forced to use other methods to confirm our hypotheses. The individual focal points of this work will be discussed in the following sections.

5.2 Methodology

5.2.1 Online-SPE method

The field of analytical chemistry is highly dynamic with ongoing improvement processes. The focus is not only on advancing analytical performances but also on reducing costs, chemicals and time. In the first publication of this work, the development and validation of a novel online-SPE method in conjunction with LC-MS is described. It is characterized by its simplicity and optimization for phase-II metabolites of steroids. Although the idea of installing an online-SPE is not new, at the time of writing this thesis, this work was the first to focus on the analysis of AAS phase-II glucuronides. Generally, in the field of anti-doping analysis, this kind of approach is not widespread yet. So far, a few approaches have been published, almost all of which have included a relatively complex installation of the online-SPE system[141,142,155–160,143–150]. However, it must also be mentioned here that a direct comparison of the systems is complicated since all methods have been optimized for particular applications. However, the high complexity of the devices is present in all of them.

Therefore, our goal was, to develop a simple and quick-to-install online-SPE system for confirmation measurements. The idea was that no complicated backwash system is needed if the samples are applied in very small amounts and if the separation column and gradient are well-matched to each other. A slight extension of the total run time should be sufficient to clean and calibrate the system to be ready for the next injection. And the small sample quantity is compensated by the high sensitivity of the HRMS system. This method should be a dilute-and-shoot approach supplemented by a fast, automated sample preparation step. In the first publication, we demonstrated that these ideas work. We clearly showed that installing a fully functioning online-SPE application requires little material and effort. A simple two-position six-way switching valve, some capillaries and fittings, and an additional pre-column are all that is needed to install such a system. Our approach does not require an additional valve, another sample loop, or further LC pump, as is the case with most other published online-SPE methods. This method provides excellent validation parameters in combination with a high-resolution MS/MS measuring mode. However, this method can also be combined with any other mass spectrometric system, e.g. a triple quadrupole mass analyser. The instrumental setup between the injector and the ESI source remains the same. Nevertheless, it is conceivable that less good validation parameters are achieved due to the lower sensitivity and specificity of the detector. A comparison of these two systems was not part of this work but could be an interesting topic for future research. Although HR systems have higher sensitivity and selectivity, triple quadrupole analysers dominate in routine laboratories due to their lower cost. If the sensitivity of a triple quadrupole instrument would not be sufficient for certain applications, online SPE could also be combined with manual sample preparation.

The online-SPE method was used for the work in all three publications only for confirmatory analysis or to identify and characterize new metabolites. An application as a multi-target screening procedure could also be of great interest. It is likely to be expected that not all steroid phase-II metabolites are as sensitive to the detection as stanozolol. Stanozolol is particularly well suited for detection by ESI due to the nitrogen heteroatom, which leads to higher ion yields and thus higher signal intensities. However, Balcells *et al.* demonstrated back in 2017 that many phase-II metabolites of steroids can be well analysed by LC-MS[113]. The implementation of parent substances and phase-I metabolites that can be detected with LC-MS is also an interesting approach for a future

project. The team used a triple quadrupole instrument and performed conventional SPE sample preparation. Therefore, further development of the method using high-resolution mass spectrometry and online-SPE would be an exciting approach.

Besides glucuronide conjugates, sulfate conjugates are the second major group of phase-II metabolites. These were deliberately excluded in this work, but also play an important role in the long-term analysis of steroids[161–164]. For the analysis of sulfates, the online-SPE could also be an auspicious approach. However, the analytical setup would probably have to be changed significantly.

From an economic point of view, online SPE methods make absolute sense and will, therefore, certainly continue to be a component of anti-doping research in the future. Moreover, the use of such a method is entirely in the spirit of green chemistry, especially if it can reduce the use of GC-MSMS and the complex derivatization reactions required for it. For one run with the presented online-SPE-HRMS method including sample preparation, about 2.5 ml MeOH and 3 ml water as well as few µl of FA are needed. In comparison, the standard GC-MSMS method requires at least twice the amount of solvent, not to mention the dangerous derivatization reagents.

5.2.2 Sample fractionation

For the work described in paper 3, we applied novel approaches to identify new metabolites. First of all, fractionation with LC-MSMS is not a common tool for extracting metabolites that are present at very low concentrations. After identifying potential metabolite signals using a conventional LC-MSMS approach, we isolated them directly using the LC-MSMS system. The MS-based sample fractionation apparatus was created with small amounts of additional material, which allowed molecule isolation on the smallest scale. This approach worked very well for AAS phase-II glucuronides but can certainly be used for the extraction of all other LC-suitable substances. The approach allowed us to separate peaks that were very close to each other. One disadvantage, however, is that due to the very small sample quantity, the extraction process must be repeated very often in order to obtain an appropriate quantity of the substances. This results in a time-consuming, manual work step. In addition, a solid-phase extraction step was required to pre-concentrate the analytes. Nevertheless, a total volume of about 25 ml of positive urine was sufficient for all experiments in this work.

5.2.3 Derivatisation experiment

In paper 3, the structural elucidation of the DHCMT metabolites M3-glucuronides was a completely new approach to our knowledge in the field of steroid research. Although trityl chloride is widely used for various applications, e.g., as a protective reagent for amines, alcohols, or thiols, it has not been used for the determination of the conjugation site of glucuronic acid on steroids[165,166]. Evidently, this approach works only in special cases when a primary and secondary alcohol serve as conjugation sites for the glucuronic acid[167]. The reaction is very simple and can be performed with common chemicals and laboratory equipment. Since there were no comparable experiments, we tried various reaction approaches until the desired result was obtained. Fortunately, this was successful in the second application using the reaction conditions described in paper 3. Even if the results indicate a successful reaction, confirmation of the experiment with standard substances on a larger scale would be very helpful. Unfortunately, no comparable steroid glucuronides exist as reference substances to date.

According to the study from Loke *et al.*, at least two other DHCMT metabolites (M2 and M4, see Figure 12) have both a primary and secondary alcohol in their steroidal backbone. If phase-II glucuronides are identified for these metabolites, derivatization with trityl chloride may also be used to determine the conjugation side of the glucuronic acid. In addition, it is also conceivable that other 17α-methyl steroid metabolites also meet these structural requirements, and therefore this derivatization can be applied. Possible candidates would be e.g. metandienone, methyltestosterone, methyl-1-testosterone, and oxandrolone, for which metabolites with a 7β-hydroxymethyl-17α-methyl-18-nor-androst-13-ene structure have already been identified[49].

5.3 Identification of new phase-II metabolites

5.3.1 Stanozolol phase-II metabolites

As the most detected steroid in the last ten years, stanozolol is of special interest in this thesis. This substance is one of the most studied AAS in doping analysis, and many findings on its metabolism, phase-I and phase-II, have been generated so far. The first method to detect a phase-II metabolite was published in 2013 by the team around E. Tudela[60]. The team published the detection of the metabolite 3'-hydroxy-stanozolol

glucuronide using solid-phase extraction and LC-MSMS. In the following years, the teams around W. Schänzer and M. Thevis generated further knowledge about stanozolol phase-II metabolites, especially N-associated glucuronides[61,114]. They proposed many stanozolol phase-II metabolites, including epi-1'N-, 1'N-, and 2'N-stanozolol glucuronide. However, unequivocal proof of the existence of the proposed metabolites could never be provided, as there was always a lack of high-quality, synthetically produced reference substances. In particular, determination of the exact position of the glucuronic acid conjugates on the steroid backbone was not possible without conclusive NMR data.

The subject of the work described in the first paper, in addition to the development of the online SPE method, was the clear and unequivocal detection of 1'N- and 2'N-stanozolol glucuronide in human urine samples positive for stanozolol. In collaboration with a team from the Synthetic Chemistry Department of the Technical University of Vienna, Austria, we were able to synthesize these metabolites on a large scale and in pure quality. Its synthesis and structure confirmation by NMR was published elsewhere[168]. Using the reference standards and the newly developed method, we were able to detect both metabolites in human urine samples without any doubt. In the work described in the second paper, using the same approach, we managed to unambiguously identify the 17-epimerized versions of these metabolites, epi-1'N- and epi-2'N-stanozolol glucuronide. These results are of great importance for the long-term detection of stanozolol abuse since two of these four metabolites have large detection windows, as we found in a study with excretion samples. 1'N-stanozolol glucuronide was detectable up to 12 days and epi-1'N-stanozolol glucuronide up to 28 days. The other two 2'N-associated metabolites have much shorter detection windows to a maximum of a few hours. This fact can be of importance when estimating the time of drug intake. Of course, more extensive excretion studies would have to be undertaken for a more accurate assessment in this matter. Due to the synthesized reference material, we were also able to estimate the concentration of excreted metabolites for the first time. This gives deeper information about the pharmacological behaviour of the steroid in the human body.

Although Schänzer and Thevis proposed the metabolites in part earlier, our work has provided definitive and unequivocal evidence for their existence and unambiguous structural elucidation for the first time. Since a detailed description of the synthesis of these metabolites was also published, other laboratories now have the possibility of using

the reference material for routine doping analysis. Especially in targeted analysis, such knowledge is of utmost importance. If a substance is detected unequivocally using high-quality reference material, the risk of a false-positive result is negligible. The further development of such knowledge thus leads to a fairer fight against doping abuse for both the anti-doping laboratory and the athletes.

In this work, emphasis was placed on the N-associated glucuronide metabolites of the parent molecule of stanozolol, as these promised potential for long-term detection. However, theoretically, a variety of other phase-II metabolites also exist. There are many different phase-I metabolites described, and each of them could theoretically form N-associated or even OH-associated phase-II metabolites (see Figure 10). However, to date, unequivocal evidence, including unequivocal structural elucidation, has been obtained only for the 3'-hydroxystanozolol glucuronide metabolite mentioned above. In addition to the glucuronide metabolites, there is, of course, also a large area of sulfate phase-II metabolites. This area has been deliberately excluded from this work in order not to exceed the scope. Nevertheless, studies have already been carried out here for stanozolol. In their publication from 2016, the team around G. Balcells confirmed eleven different sulfate metabolites, in addition to some stanozolol glucuronides[107]. However, the sulfate metabolites did not show great potential for long-term detection compared to some glucuronide metabolites. Phase-II metabolism and the detection of stanozolol therefore still offer much room for research for the future. And as long as this steroid will continue to be one of the most widely used, it will continue to be an important part of anti-doping research.

5.3.2 Dehydrochloromethyltestosterone phase-II metabolites

In 2020, DHCMT was the second most commonly detected AAS after stanozolol, according to WADA statistics[22]. This is reason enough to give this steroid special attention in anti-doping research. With a few exceptions, the detection of DHCTM abuse is based on GC-MSMS. Therefore, we aimed to develop an analytical approach based on the analysis of phase-II glucuronides by LC-MSMS. To the best of our knowledge, the only work that has already addressed such an approach was presented 2010 at the annual Anti-Doping Workshop in Cologne by the team around M. Fernandez-Alvarez[169]. However, no long-term metabolites were studied. Since detection of the parent compound

or phase-I metabolites by LC is quite difficult due to the non-polar nature of the steroid, direct analysis of the highly polar phase-II metabolites is a more appropriate approach.

Since there is very little knowledge about DHCMT phase-II metabolites so far, this was the aim of the work described in the third publication. So far, many phase-I metabolites have been described, and some of them have even been proposed as long-term metabolites. In particular, in 2012, it was already established that the metabolite M3 has a long-term detection potential[69]. In a recent study, S. Loke's team confirmed the potential of metabolite M3 and suggested several other metabolites for long-term detection[91]. At the beginning of our studies, metabolite M3 was the only one for which high-quality reference material was available[90]. This is one of the reasons for choosing the metabolite for our studies. Compared to the work described in the first two papers, this work took a different approach since we did not have access to phase-II metabolite reference standards. Therefore, we attempted to specifically extract potential glucuronides of DHCMT and confirm them after enzymatic hydrolysis with phase-I reference substances. Using LC-based, mass spectrometry-monitored fractionation, we were able to extract three different potential phase-II glucuronides of the metabolite M3. After successful hydrolysis, we clearly identified two of these three glucuronides as DHCMT M3 conjugates. In the case of the third metabolite, we assume that it is an epimer of M3. The two confirmed phase-II glucuronides of DHCMT M3 are easily analysed by LC-MSMS and can be a good alternative to GC-MSM. In particular, in combination with online-SPE, a fast and resource-efficient analysis is possible.

However, in order to fully meet the stringent quality requirements in the field of anti-doping analysis, an essential goal for the future must be the synthesis of high-quality reference compounds of the newly described phase-II metabolites to confirm our structure proposals. In addition, the study of excretion samples would also provide many important insights into the direct detection of DHCMT phase-II metabolites. Furthermore, detection of DHCMT sulfate metabolites may be of interest. Balcells *et al.* investigated the sulfate metabolism of DHCMT and identified six sulfate conjugates[170]. Five of this metabolites showed potential for long-term detection. Direct analysis of sulfates using the online SPE method, and possibly in combination with the glucuronide fraction, could be a powerful tool for rapidly detecting DHCMT abuse.

6. Future perspectives

Based on the results and discussion in this thesis, the following points can be considered as topics for future projects:

Online SPE:

- Transfer of the online-SPE application to other mass spectrometric systems such as triple quadrupole, time-of-flight (TOF), or other ion-trap systems
- Implementation of parent substances and phase-I metabolites in the online-SPE method
- Implementation of more glucuronides and development of a multi-target screening method, including a comprehensive validation
- Optimization of the online-SPE setup for analysis of phase-II sulfate metabolites
- Implementation of this method for different routine anti-doping applications, e.g. screening and confirmation procedures

Stanozolol phase-II metabolites:

- Identification and characterization of potential N- and OH-associated glucuronides from stanozolol phase-I metabolites
- Identification and characterization of potential sulfate phase-II metabolites of stanozolol and the corresponding phase-I metabolites
- Identification and characterization of other potential phase-II metabolite types, such as glutathione, succinic acid, or cysteine conjugates
- Synthesis of potential stanozolol phase-II metabolites
- Extended excretion studies to evaluate long-term detectability of identified metabolites

Dehydrochlormethyltestosterone phase-II metabolites:

- Synthesis of high-quality reference standards of the suggested DHCMT-M3 glucuronides for unambiguous confirmation of the presented results
- Identification and characterization of phase-II glucuronides of other DHCMT metabolites

- Identification and characterization of phase-II sulfates of DHCMT-M3 and other metabolites
- Extended excretion studies to evaluate the long-term detectability of identified metabolites
- Identification and characterization of other potential phase-II metabolite types such as glutathione, succinic acid or cysteine conjugates
- Optimization of LC-MS conditions for the analysis of newly identified phase-II metabolites of DHCMT

7. Conclusion

This work started with the aim of improving the analysis of anabolic androgenic steroids. The focus was on detecting the highly polar phase-II glucuronides of steroids, which allowed the analysis to be shifted from GC-MS to LC-MS. A novel, simple, and quick-to-install online-SPE method was established in the present work. This newly developed approach was coupled with an LC-HRMSMS system and optimized for the analysis of phase-II glucuronide conjugates of androgenic anabolic steroids. A comprehensive validation revealed excellent analytical parameters for this method.

We selected two specific steroids, stanozolol and dehydrochlormethyltestosterone, as they are among the most commonly detected steroids in professional sports. For stanozolol, we definitively and unequivocally elucidated the formation of all N-associated phase-II glucuronides of the parent compound stanozolol and its major phase-I metabolite 17-epistanozolol. Furthermore, we presented an informative excretion profile that provides a deeper insight into the pharmacology of these metabolites.

In the case of DHCMT, we were able to take a first important step towards long-term analysis with LC-MS. Combining several different techniques, we were able to identify two new glucuronides of the important DHCMT long-term metabolite M3. Although unambiguous confirmation of these metabolites with reference standards is still pending, we have found very strong evidence for their existence. In addition, we presented a completely new approach for distinguishing different positions of the glucuronic acid conjugate.

In conclusion, this work provided valuable contributions both at the methodological level and in the study of phase-II metabolism of anabolic androgenic steroids.

8. References

1. Newerla JG. The history of the discovery and isolation of the male hormone. *N Engl J Med*. 1943;228(2):41-47. doi:10.1056/NEJM194301142280201

2. Yesalis C, Bahrke M. History of doping in sport. *Int Sport Stud*. 2002;24(1):42-76.

3. Klein AM. Pumping Irony: Crisis and Contradiction in Bodybuilding. *Sociol Sport J*. 2016;3(2):112-133. doi:10.1123/ssj.3.2.112

4. Wade N. Anabolic steroids: Doctors denounce them, but athletes aren't listening. *Science*. 1972;176(4042):1399-1403. doi:10.1126/science.176.4042.1399

5. Ljungqvist A. Brief History of Anti-Doping. *Med Sport Sci*. 2017;62:1-10. doi:10.1159/000460680

6. Catlin DH, Fitch KD, Ljungqvist A. Medicine and science in the fight against doping in sport. *J Intern Med*. 2008;264(2):99-114. doi:10.1111/j.1365-2796.2008.01993.x

7. World Anti-Doping Agency (WADA). Lausanne Declaration on Doping in Sport. 1999. Available at: https://www.wada-ama.org/sites/default/files/resources/files/lausanne_declaration_on_doping_in_sport.pdf. Accessed February 1, 2022.

8. World Anti-Doping Agency (WADA). What we do. 1999. Available at: https://www.wada-ama.org/en/what-we-do. Accessed February 1, 2022.

9. World Anti-Doping Agency (WADA). World Anti-Doping Code 2021. 2021. Available at: https://www.wada-ama.org/sites/default/files/resources/files/2021_wada_code.pdf. Accessed February 1, 2022.

10. World Anti-Doping Agency (WADA). Prohibited List 2021. 2021. Available at: https://www.wada-ama.org/sites/default/files/resources/files/2021list_en.pdf. Accessed February 1, 2022.

11. World Anti-Doping Agency (WADA). Accredited Laboratories for Doping Control Analysis. 2021. Available at: https://www.wada-ama.org/sites/default/files/2022-01/WADA_Accredited_Laboratories_EN.pdf. Accessed February 1, 2022.

12. World Anti-Doping Agency (WADA). WADA Guide Operational Independence of National Anti-Doping Organizations under the 2021. World Anti-Doping Code 2020. Available at: https://www.wada-ama.org/sites/default/files/resources/files/guide_on_nado_operational_independence_final_english_0.pdf. Accessed February 1, 2022.

13. World Anti-Doping Agency (WADA). Laboratory Testing Figures 2011. 2011. Available at: https://www.wada-ama.org/sites/default/files/resources/files/WADA-2011-Laboratory-Testing-Figures.pdf. Accessed February 1, 2022.

14. World Anti-Doping Agency (WADA). Anti - Doping Testing Figures Report 2012. 2012. Available at: https://www.wada-ama.org/sites/default/files/resources/files/WADA-2012-Anti-Doping-Testing-Figures-Report-EN.pdf. Accessed February 1, 2022.

15. World Anti-Doping Agency (WADA). Anti - Doping Testing Figures Report 2013. 2013. Available at: https://www.wada-ama.org/sites/default/files/wada_2013_anti-doping_testing_figures_report_en.pdf. Accessed February 1, 2022.

16. World Anti-Doping Agency (WADA). Anti - Doping Testing Figures Report 2014. 2014. Available at: https://www.wada-ama.org/sites/default/files/wada_2014_anti-doping-testing-figures_full-report_en.pdf. Accessed February 1, 2022.

17. World Anti-Doping Agency (WADA). 2015 Anti-Doping Testing Figures. 2015. Available at: https://www.wada-ama.org/sites/default/files/resources/files/2015_wada_anti-doping_testing_figures_report_0.pdf. Accessed February 1, 2022.

18. World Anti-Doping Agency (WADA). 2016 Anti-Doping Testing Figures. 2016. Available at: https://www.wada-ama.org/sites/default/files/resources/files/2016_anti-doping_testing_figures.pdf. Accessed February 1, 2022.

19. World Anti-Doping Agency (WADA). 2017 Anti-Doping Testing Figures. 2017. Available at: https://www.wada-ama.org/sites/default/files/resources/files/2017_anti-doping_testing_figures_en_0.pdf. Accessed February 1, 2022.

20. World Anti-Doping Agency (WADA). 2018 Anti-Doping Testing Figures. 2018. Available at: https://www.wada-ama.org/sites/default/files/resources/files/2018_testing_figures_report.pdf. Accessed February 1, 2022.

21. World Anti-Doping Agency (WADA). 2019 Anti-Doping Testing Figures. 2019. Available at: https://www.wada-ama.org/sites/default/files/resources/files/2019_anti-doping_testing_figures_en.pdf. Accessed February 1, 2022.

22. World Anti-Doping Agency (WADA). 2020 Anti-Doping Testing Figures. 2020. Available at: https://www.wada-ama.org/sites/default/files/2022-01/2020_anti-doping_testing_figures_en.pdf. Accessed February 1, 2022.

23. Thevis M, Schanzer W. Mass Spectrometry in Doping Control Analysis. *Curr Org Chem*. 2005;9(9):825-848. doi:10.2174/1385272054038318

24. World Anti-Doping Agency (WADA). 2021 Code Implementation Support Program Guidelines for Sample Collection. 2021. Available at: https://ita.sport/uploads/2021/02/isti_sample_collection_guidelines_en_final_2_feb_2021.pdf. Accessed February 1, 2022.

25. Thevis M, Thomas A, Schänzer W. Targeting prohibited substances in doping control blood samples by means of chromatographic – mass spectrometric methods. *Anal Bioanal Chem*. 2013;405(30):9655-9667. doi:10.1007/s00216-013-7224-3

26. Ding JB, Ng MZ, Huang SS, Ding M, Hu K. Anabolic-Androgenic Steroid Misuse : Mechanisms , Patterns of Misuse , User Typology , and Adverse Effects. *J Sports Med*. 2021;2021:1-9. doi:https://doi.org/10.1155/2021/7497346

27. Wilhelm Schänzer. Metabolism of anabolic androgenic steroids. *Clin Chem*. 1996;1020(42:7):1001-1020.

28. Makin HLJ, Gower DB. Steroid Analysis – Second Edition. 2010. 753-821. Springer

Dordrecht Heidelberg London New York. doi: 10.1023/b135931. ISBN: 978-1-4020-9774-4

29. Fragkaki AG, Angelis YS, Koupparis M, Tsantili-Kakoulidou A, Kokotos G, Georgakopoulos C. Structural characteristics of anabolic androgenic steroids contributing to binding to the androgen receptor and to their anabolic and androgenic activities. Applied modifications in the steroidal structure. *Steroids*. 2009;74(2):172-197. doi:10.1016/j.steroids.2008.10.016

30. Thieme D, Hemmersbach P, Kazlauskas R. Doping in Sports: Biochemical Principles, Effects and Analysis. 2010. 155-158. Springer - Verlag Berlin Heidelberg. doi: https://doi.org/10.1007/978-3-540-79088-4. ISBN: 978-3-540-79088-4

31. Fourcroy J. Designer steroids : past , present and future. *Curr Opin Endocrinol Diabetes 2006*. 2006;13:306-309. doi:10.1097/01.med.0000224812.46942.c3

32. Thieme D, Hemmersbach P, Thevis M, Schänzer W. Doping in Sports: Biochemical Principles, Effects and Analysis. 2010. 99-126. Springer - Verlag Berlin Heidelberg. doi: https://doi.org/10.1007/978-3-540-79088-4. ISBN: 978-3-540-79088-4

33. Görög S. Recent Advances in the Analysis of Steroid Hormones. *Anal Sci*. 2004;20(05):767-782. doi:10.2116/analsci.20.767

34. Mooradian AD, Morley JE, Korenman SG. Biological Actions of Androgens. *Endocr Rev*. 1987;8(1):1-28. doi:10.1210/edrv-8-1-1

35. Kicman A. Pharmacology of anabolic steroids. *Br J Pharmacol*. 2008;154:502-521. doi:10.1038/bjp.2008.165

36. Thigpen AE, Silver RI, Guileyardo JM, Casey ML, McConnell OD, Russell DW. Tissue distribution and ontogeny of steroid 5α-reductase isozyme expression. *J Clin Invest*. 1993;92(2):903-910. doi:10.1172/JCI116665

37. Massa R, Martini L. Testosterone metabolism: A necessary step for activity? *J Steroid Biochem*. 1974;5(8):941-947. doi:10.1016/0022-4731(74)90089-2

38. Toth M, Zakar T. Relative binding affinities of testosterone, 19-nortestosterone and their 5a-reduced derivatives to the androgen receptor and to other androgen-binding proteins. *J Steroid Biochem*. 1982;17:653-660. doi:10.1016/0022-4731(82)90567-2

39. Weigel NL, Moore NL. Steroid receptor phosphorylation: A key modulator of multiple receptor functions. *Mol Endocrinol*. 2007;21(10):2311-2319. doi:10.1210/me.2007-0101

40. Saartok T, Dahlberg E, Gustafsson J-A. Relative Binding Affinity of Anabolic-Androgenic Steroids : Comparison of the Binding to the Androgen Receptors in Skeletal Muscle and in Prostate , as well as to Sex Hormone-Binding Globulin. *Endocrinology*. 2015;114(6):2100-2106. doi:10.1210/endo-114-6-2100

41. Holterhus PM, Piefke S, Hiort O. Anabolic steroids, testosterone-precursors and virilizing androgens induce distinct activation profiles of androgen responsive promoter constructs. *J Steroid Biochem Mol Biol*. 2002;82(4-5):269-275. doi:10.1016/S0960-0760(02)00220-0

42. Mayer M, Rosen F. Interaction of anabolic steroids with glucocorticoid receptor sites in

rat muscle cytosol. *Am J Physiol*. 1975;229(5):1381-1386. doi:10.1152/ajplegacy.1975.229.5.1381

43. Braun TP, Marks DL. The regulation of muscle mass by endogenous glucocorticoids. *Front Physiol*. 2015;6(02):1-12. doi:10.3389/fphys.2015.00012

44. Hickson A, Czerwinski S, Falduto M. Glucocorticoid antagonism by exercise and androgenic-anabolic steroids. *Med Sci Sports Exerc*. 1990;22(3):331-340.

45. Friedel A, Geyer H, Kamber M, et al. Tetrahydrogestrinone is a potent but unselective binding steroid and affects glucocorticoid signalling in the liver. *Toxicol Lett*. 2006;164(1):16-23. doi:10.1016/j.toxlet.2005.11.006

46. Grata E, Perrenoud L, Saugy M, Baume N. SARM-S4 and metabolites detection in sports drug testing: A case report. *Forensic Sci Int*. 2011;213(1-3):104-108. doi:10.1016/j.forsciint.2011.07.014

47. Schänzer W, Opfermann G, Donike M. 17-Epimerization of 17a-methyl anabolic steroids iu humans: metabolism and synthesis of 17a-hydroxy-17b-methyl steroids. *Steroi*. 1992;57(11):537-550. doi:https://doi.org/10.1016/0039-128X(92)90023-3

48. Schänzer W, Geyer H, Fußhöller G, et al. Mass spectrometric identification and characterization of a new long-term metabolite of metandienone in human urine. *Rapid Commun Mass Spectrom*. 2010;24:1457-1466. doi:10.1002/rcm

49. Parr MK, Fußhöller G, Gütschow M, Hess C, Schänzer W. GC-MS (/ MS) investigations on long-term metabolites of 17-methyl steroids. Available at: https://www.dshs-koeln.de/fileadmin/redaktion/Institute/Biochemie/PDF/Long-term_metabolites_Parr_2010.pdf. Accessed February 1, 2022.

50. Hobkirk R. Steroid sulfotransferases and steroid sulfate sulfatases: characteristics and biological roles. *Can J Biochem Cell Biol*. 2009;63(11):1127-1144. doi:10.1139/o85-141

51. King CD, Rios GR, Green MD, Tephly TR. UDP-Glucuronosyltransferases. *Curr Drug Metab*. 2000;1:143-161. doi:10.2174/1389200003339171

52. Strott CA. Steroid Sulfotransferases. *Endocr Rev*. 1996;17(6):670-697. doi:https://doi.org/10.1210/edrv-17-6-670

53. Mackenzie P, Rodbourne L, Stranks S. Steroid UDP glucuronosyltransferases. *J Steroid Biochem Mol Biol*. 1992;43(8):1099-1105. doi:https://doi.org/10.1016/0960-0760(92)90338-J

54. Jancova P, Anzenbacher P, Anzenbacherova E. Phase II drug metabolizing enzymes. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub*. 2010;154(2):103-116. doi:10.5507/bp.2010.017

55. Pozo ÓJ, Ibáñez M, Sancho J V, et al. Mass Spectrometric Evaluation of Mephedrone In Vivo Human Metabolism : Identification of Phase I and Phase II Metabolites , Including a Novel Succinyl Conjugate s. *Drug Metab Dispos*. 2015;43(2):248-257. doi:10.1124/dmd.114.061416

56. Fabregat A, Kotronoulas A, Marcos J, et al. Detection, synthesis and characterization of metabolites of steroid hormones conjugated with cysteine. *Steroids*. 2013;78(3):327-336.

doi:10.1016/j.steroids.2012.11.017

57. Kuuranne T, Kurkela M, Thevis M, Schänzer W, Finel M, Kostiainen R. Glucuronidation of anabolic androgenic steroids by recombinant human UDP-glucuronosyltransferases. *Drug Metab Dispos*. 2003;31(9):1117-1124. doi:10.1124/dmd.31.9.1117

58. Jakobsson J, Ekstro L, Inotsume N, et al. Large Differences in Testosterone Excretion in Korean and Swedish Men Are Strongly Associated with a UDP-Glucuronosyl Transferase 2B17 Polymorphism. *J Clin Endocrinol Metab*. 2006;91(May):687-693. doi:10.1210/jc.2005-1643

59. Martin-Escudero P, Muoz-Guerra J, Prado N Del, Canales MG, Mart P. Impact of UGT2B17 gene deletion on the steroid profile of an athlete. *Physiol Rep*. 2015;3(12):1-7. doi:10.14814/phy2.12645

60. Tudela E, Deventer K, Van Eenoo P. Sensitive detection of 3'-hydroxy-stanozolol glucuronide by liquid chromatography-tandem mass spectrometry. *J Chromatogr A*. 2013;1292(2010):195-200. doi:10.1016/j.chroma.2013.01.001

61. Schänzer W, Guddat S, Thomas A, Opfermann G, Geyer H, Thevis M. Expanding analytical possibilities concerning the detection of stanozolol misuse by means of high resolution/high accuracy mass spectrometric detection of stanozolol glucuronides in human sports drug testing. *Drug Test Anal*. 2013;5(11-12):810-818. doi:10.1002/dta.1516

62. Gomes RL, Meredith W, Snape CE, Sephton MA. Analysis of conjugated steroid androgens: Deconjugation, derivatisation and associated issues. *J Pharm Biomed Anal*. 2009;49(5):1133-1140. doi:10.1016/j.jpba.2009.01.027

63. Pranata A, Fitzgerald CC, Khymenets O, et al. Synthesis of steroid bisglucuronide and sulfate glucuronide reference materials: Unearthing neglected treasures of steroid metabolism. *Steroids*. 2019:25-40. doi:10.1016/j.steroids.2018.11.017

64. Murai T, Iwabuchi H, Ikeda T. Repeated glucuronidation at one hydroxyl group leads to structurally novel diglucuronides of steroid sex hormones. *Drug Metab Pharmacokinet*. 2005;20(4):282-293. doi:10.2133/dmpk.20.282

65. Jänne O, Vihko R, Sjövall J, Sjövall K. Determination of human steroid mono-and disulfates in human plasma. *Clin Chim Acta*. 1969;23:405-412. doi:https://doi.org/10.1016/0009-8981(69)90340-4

66. Parr MK, Schänzer W. Detection of the misuse of steroids in doping control. *J Steroid Biochem Mol Biol*. 2010;121(3-5):528-537. doi:10.1016/j.jsbmb.2009.12.008

67. Schänzer W, Fußhöller G, Guddat S, Geyer H, Piper T, Thevis M. Long Term Metabolites – Historical Aspects and the Cologne Strategy. 2017. 99-126. Available at: https://fis.dshs-koeln.de/portal/files/3422830/Sch_nzer_Long_term_metabolites_8.28.17.pdf. Accessed February 1, 2022.

68. Guddat S, Fußhöller G, Beuck S, et al. Synthesis, characterization, and detection of new oxandrolone metabolites as long-term markers in sports drug testing. *Anal Bioanal Chem*. 2013;405(25):8285-8294. doi:10.1007/s00216-013-7218-1

69. Sobolevsky T, Rodchenkov G. Detection and mass spectrometric characterization of novel long-term dehydrochloromethyltestosterone metabolites in human urine. *J Steroid Biochem Mol Biol*. 2012;128(3-5):121-127. doi:10.1016/j.jsbmb.2011.11.004

70. Clinton R. O., Manson A. J., Stonner F. W., Beyler A. L. PG 0. and AA. Steroidal [3,2-c] pyrazoles. *J Am Chem Soc*. 1959;81:1513-1514.

71. National Center for Biotechnology Information. 2021. PubChem Annotation Record for Stanozolol, Source: Hazardous Substances Data Bank (HSDB). Available at: https://pubchem.ncbi.nlm.nih.gov/source/hsdb/3185. Accessed February 1, 2022.

72. Sheffer A, Fearon D, Austen K. Clinical and biochemical effects of stanozolol therapy for hereditary angioedema. *J Allergy Clin Immunol*. 1981;68(3):181-187. doi:10.1016/0091-6749(81)90181-0

73. Kara M, Ozcagli E, Kotil T, Alpertunga B. Effects of Stanozolol on Apoptosis Mechanisms and Oxidative Stress in Rat Cardiac Tissue. *Steroids*. 2018;134:96-100. doi:10.1016/j.steroids.2018.02.004

74. Masse R, Ayotte C, Bi H, Dugal R. Studies on anabolic steroids. III. Detection and characterization of stanozolol urinary metabolites in humans by gas chromatography-mass spectrometry. *J Chromatogr*. 1989;497:17-37.

75. W. Schänzer, G. Opfermann MD. Metabolism of stanozolol: Identification and synthesis of urinary metabolites. *J Steroid Biochem*. 1990;36(1-2):153-174. doi:10.1016/0022-4731(90)90126-D

76. Pozo OJ, Van Eenoo P, Deventer K, et al. Detection and structural investigation of metabolites of stanozolol in human urine by liquid chromatography tandem mass spectrometry. *Steroids*. 2009;74(10-11):837-852. doi:10.1016/j.steroids.2009.05.004

77. Schänzer W, Delahaut P, Geyer H, Machnik M, Horning S. Long-term detection and identification of metandienone and stanozolol abuse in athletes by gas chromatography-high-resolution mass spectrometry. *J Chromatogr B Biomed Appl*. 1996;687(1):93-108. doi:10.1016/S0378-4347(96)00187-9

78. Marques MAS, Pereira HMG, De Aquino Neto FR. Improvements in steroid screening in doping control with special emphasis to GC-MS analytical conditions and method validation. *J Braz Chem Soc*. 2006;17(2):382-392. doi:10.1590/S0103-50532006000200024

79. Leinonen A, Kuuranne T, Kotiaho T, Kostiainen R. Screening of free 17-alkyl-substituted anabolic steroids in human urine by liquid chromatography-electrospray ionization tandem mass spectrometry. *Steroids*. 2004;69(2):101-109. doi:10.1016/j.steroids.2003.10.007

80. Guddat S, Thevis M, Kapron J, Thomas A, Schänzer W. Application of FAIMS to anabolic androgenic steroids in sport drug testing. *Drug Test Anal*. 2009;1(11-12):545-553. doi:10.1002/dta.73

81. Thevis M, Fußhöller G, Geyer H, et al. Detection of Stanozolol and Its Major Metabolites in Human Urine by Liquid Chromatography-Tandem Mass Spectrometry. *Chromatographia*. 2006;64(7-8):441-446. doi:10.1365/s10337-006-0043-3

82. Thevis M, Makarov AA, Horning S, Schänzer W. Mass spectrometry of stanozolol and its analogues using electrospray ionization and collision-induced dissociation with quadrupole-linear ion trap and linear ion trap-orbitrap hybrid mass analyzers. *Rapid Commun Mass Spectrom*. 2005;19(22):3369-3378. doi:10.1002/rcm.2204

83. Ahi S, Beotra A, Jain S. Detection of mono-hydroxylated metabolites of stanozolol by HPLC-ESI (+) MS/MS in Indian sports persons. *Drug Test Anal*. 2009;1(11-12):538-544. doi:10.1002/dta.76

84. Thevis M, Dib J, Thomas A, et al. Complementing the characterization of in vivo generated N-glucuronic acid conjugates of stanozolol by collision cross section computation and analysis. *Drug Test Anal*. 2015;7(11-12):1050-1056. doi:10.1002/dta.1907

85. Parr MK, Schänzer W. Detection of the misuse of steroids in doping control. *J Steroid Biochem Mol Biol*. 2010;121(3-5):528-537. doi:10.1016/j.jsbmb.2009.12.008

86. Franke WW, Berendonk B. Hormonal doping and androgenization of athletes: A secret program of the German Democratic Republic government. *Clin Chem*. 1997;43(7):1262-1279. doi:10.1093/clinchem/43.7.1262

87. Schubert K, Wehrberger K. Metabolism of steroid drugs. II. Isolation and identification of metabolites of 4 - chlor - 17 alpha - methyl - 17 beta - hydroxy - 1,4 - androstadien - 3 - one. Endokrinologie. 1970;55:257 - 269.

88. Durbeck HW, Buker I, Scheulen B, Telin B. GC and Capillary Column GC / MS Determination of Synthetic Anabolic Steroids (Oral Turinabol) and Its Metabolites. *J Chromatogr Sci*. 1983;21(9):405-410. doi:10.1093/chromsci/21.9.405

89. Schänzer W, Horning S, Opfermann G, Donike M. Gas Chromatography/Mass Spectrometry Identification of Long-term Excreted Metabolites of the Anabolic Steroid 4-Chloro-1,2- dehydro- 17a-methyltestosterone in Humans. *J Steroid Biochem Mol Biol*. 1996;57(5):363-376. doi:https://doi.org/10.1016/0960-0760(95)00276-6

90. Forsdahl G, Geisendorfer T, Göschl L, et al. Unambiguous identification and characterization of a long-term human metabolite of dehydrochloromethyltestosterone. *Drug Test Anal*. 2018;10(8):1244-1250. doi:10.1002/dta.2385

91. Loke S, de la Torre X, Iannone M, et al. Controlled administration of dehydrochloromethyltestosterone in humans: Urinary excretion and long-term detection of metabolites for anti-doping purpose. *J Steroid Biochem Mol Biol*. 2021;214. doi:10.1016/j.jsbmb.2021.105978

92. World Anti-Doping Agency (WADA). WADA Technical Document - TD2021EAAS. Measurement and Reporting of Endogenous Anabolic Androgenic Steroid (EAAS) Markers of the Urinary Steroid Profile. Available at: https://www.wada-ama.org/sites/default/files/2022-01/td2021eaas_final_eng_v_2.0.pdf. Accessed February 1, 2022.

93. Ayotte C, Goudreault D, Charlebois A. Testing for natural and synthetic anabolic agents in human urine. *J Chromatogr B*. 1996;4347(96):3-25. doi:10.1016/s0378-4347(96)00032-1

94. Leinonen A, Kuuranne T, Kostiainen R. Liquid chromatography / mass spectrometry in

anabolic steroid analysis — optimization and comparison of three ionization techniques : electrospray ionization , atmospheric pressure chemical ionization and atmospheric pressure photoionization. *J Mass Spectrom*. 2002;37(5):693-698. doi:10.1002/jms.328

95. Pozo OJ, Van Eenoo P, Deventer K, Delbeke FT. Ionization of anabolic steroids by adduct formation in liquid chromatography electrospray mass spectrometry. *J Mass Spectrom*. 2007;42(2):497-516. doi:10.1002/jms

96. Politi L, Groppi A, Polettini A. Applications of Liquid Chromatography- Mass Spectrometry in Doping Control. *J Anal Toxicol*. 2005;29:1-14. doi:10.1093/jat/29.1.1

97. Abushareeda W, Fragkaki A, Vonaparti A, et al. Advances in the detection of designer steroids in anti-doping. *Bioanalysis*. 2014;6(6):881-896. doi:10.4155/bio.14.9

98. Fabregat A, Pozo OJ, Marcos J, Segura J, Ventura R. Use of LC-MS/MS for the open detection of steroid metabolites conjugated with glucuronic acid. *Anal Chem*. 2013;85(10):5005-5014. doi:10.1021/ac4001749

99. Hackett LP, Dusci LJ, Ilett KF, Chiswell GM. Optimizing the hydrolysis of codeine and morphine glucuronides in urine. *Ther Drug Monit*. 2002;24(5):652-657. doi:10.1097/00007691-200210000-00012

100. Mareck U, Geyer H, Opfermann G, Thevis M, Schänzer W. Factors influencing the steroid profile in doping control analysis. *J mass Spectrom*. 2008;43:877–891. doi:10.1002/jms

101. Gomes RL, Meredith W, Snape CE, Sephton MA. Conjugated steroids: Analytical approaches and applications. *Anal Bioanal Chem*. 2009;393(2):453-458. doi:10.1007/s00216-008-2451-8

102. Trout GJ, Kazlauskas R. Sports drug testing – an analyst's perspective. *Chem Soc Rev*. 2004;33(1):1-13. doi:10.1039/b201476a

103. Gomes RL, Meredith W, Snape CE, Sephton MA. Analysis of conjugated steroid androgens: Deconjugation, derivatisation and associated issues. *J Pharm Biomed Anal*. 2009;49(5):1133-1140. doi:10.1016/j.jpba.2009.01.027

104. Wynne PM, Batty DC, Vine JH, Simpson NJK. Approaches to the solid-phase extraction of equine urine. *Chromatographia*. 2004;59:51-60. doi:10.1365/s10337-004-0234-8

105. Gómez C, Pozo OJ, Marcos J, Segura J, Ventura R. Alternative long-Term markers for the detection of methyltestosterone misuse. *Steroids*. 2013;78(1):44-52. doi:10.1016/j.steroids.2012.10.008

106. Fragkaki AG, Angelis YS, Kiousi P, Georgakopoulos CG, Lyris E. Comparison of sulfo-conjugated and gluco-conjugated urinary metabolites for detection of methenolone misuse in doping control by LC-HRMS, GC-MS and GC-HRMS. *J Mass Spectrom*. 2015;50(5):740-748. doi:10.1002/jms.3583

107. Balcells G, Matabosch X, Ventura R. Detection of stanozolol O- and N-sulfate metabolites and their evaluation as additional markers in doping control. *Drug Test Anal*. 2017;9(7):1001-1010. doi:10.1002/dta.2107

108. Kotronoulas A, Marcos J, Segura J, Ventura R, Joglar J, Pozo OJ. Ultra high

performance liquid chromatography tandem mass spectrometric detection of glucuronides resistant to enzymatic hydrolysis: Implications to doping control analysis. *Anal Chim Acta*. 2015;895:35-44. doi:10.1016/j.aca.2015.08.043

109. Fabregat A, Pozo OJ, Marcos J, Segura J, Ventura R. Use of LC-MS/MS for the open detection of steroid metabolites conjugated with glucuronic acid. *Anal Chem*. 2013;85(10):5005-5014. doi:10.1021/ac4001749

110. Gomes RL, Meredith W, Snape CE, Sephton MA. Conjugated steroids: Analytical approaches and applications. *Anal Bioanal Chem*. 2009;393(2):453-458. doi:10.1007/s00216-008-2451-8

111. Gomez C, Fabregat A, Pozo ÓJ, Marcos J, Segura J, Ventura R. Analytical strategies based on mass spectrometric techniques for the study of steroid metabolism. *TrAC - Trends Anal Chem*. 2014;53:106-116. doi:10.1016/j.trac.2013.08.010

112. Wang Z, Zhou X, Liu X, Dong Y, Zhang J. A novel HPLC-MRM strategy to discover unknown and long-term metabolites of stanozolol for expanding analytical possibilities in doping-control. *J Chromatogr B Anal Technol Biomed Life Sci*. 2017;1040:250-259. doi:10.1016/j.jchromb.2016.11.006

113. Balcells G, Pozo OJ, Esquivel A, et al. Screening for anabolic steroids in sports: Analytical strategy based on the detection of phase I and phase II intact urinary metabolites by liquid chromatography tandem mass spectrometry. *J Chromatogr A*. 2015;1389:65-75. doi:10.1016/j.chroma.2015.02.022

114. Thevis M, Dib J, Thomas A, et al. Complementing the characterization of in vivo generated N-glucuronic acid conjugates of stanozolol by collision cross section computation and analysis. *Drug Test Anal*. 2015;7(11-12):1050-1056. doi:10.1002/dta.1907

115. Tudela E, Deventer K, Geldof L, Van Eenoo P. Urinary detection of conjugated and unconjugated anabolic steroids by dilute-and-shoot liquid chromatography-high resolution mass spectrometry. *Drug Test Anal*. 2015;7(2):95-108. doi:10.1002/dta.1650

116. Deventer K, Pozo OJ, Verstraete AG, Eenoo P Van. Trends in Analytical Chemistry Dilute-and-shoot-liquid chromatography-mass spectrometry for urine analysis in doping control and analytical toxicology. *Trends Anal Chem*. 2014;55:1-13. doi:10.1016/j.trac.2013.10.012

117. Görgens C, Guddat S, Orlovius A, et al. " Dilute-and-inject " multi-target screening assay for highly polar doping agents using hydrophilic interaction liquid chromatography high resolution / high accuracy mass spectrometry for sports drug testing. *Anal Bioanal Chem*. 2015;407(18):5365-5379. doi:10.1007/s00216-015-8699-x

118. Harvey D. Modern Analytic Chemistry – First Edition. 2000. 563-577. The McGraw-Hill Companies United States of America. ISBN: 0–07–237547–7

119. Donike M. N-methyl-n-trimethylsilyl-trifluoroacetamide a new silylating agent from series of silylated amides. *J Chromatogr*. 1969;42:103-104.

120. Donike M, Zimmermann J. Preparation of trimethylsilyl, triethylsilyl and tert-butyldimethylsilyl enol ethers from ketosteroids for investigations by gas-chromatography and mass-spectrometry. *J Chromatogr*. 1980;202(3):483-486.

doi:10.1016/S0021-9673(00)91836-3

121. Saugy M, Cardis C, Robinson N, Schweizer C. Test methods: Anabolics. *Bailliere's Best Pract Res Clin Endocrinol Metab*. 2000;14(1):111-133. doi:10.1053/beem.2000.0058

122. Poon GK, Chui YC, Jarman M, Rowlands MG, Kokkonen PS, Niessen WM, Greef J. Investigation of conjugated metabolites of 4-hydroxyandrost-4-ene-3,17-dione in patient urine by liquid chromatography-atmospheric pressure ionization mass spectrometry. Drug Metab Dispos.1992;20(6):941-7.

123. Thevis M, Schänzer W. Current role of LC-MS(/MS) in doping control. *Anal Bioanal Chem*. 2007;388(7):1351-1358. doi:10.1007/s00216-007-1131-4

124. Bean KA, Henion JD. Direct determination of anabolic steroid conjugates in human urine by combined high-performance liquid chromatography and tandem mass spectrometry. *J Chromatogr B*. 1997;690:65-75. doi:https://doi.org/10.1016/S0378-4347(96)00403-3

125. Thevis M, Thomas A, Pop V, Schänzer W. Ultrahigh pressure liquid chromatography-(tandem) mass spectrometry in human sports drug testing: Possibilities and limitations. *J Chromatogr A*. 2013;1292:38-50. doi:10.1016/j.chroma.2012.12.048

126. Balogh MP. Ms in Practice. *LC GC Eur*. 2004;17(3):152-159.

127. Li DX, Gan L, Bronja A, Schmitz OJ. Gas chromatography coupled to atmospheric pressure ionization mass spectrometry (GC-API-MS): Review. *Anal Chim Acta*. 2015;891:43-61. doi:10.1016/j.aca.2015.08.002

128. Parr MK, Wüst B, Teubel J, Joseph JF. Splitless hyphenation of SFC with MS by APCI, APPI, and ESI exemplified by steroids as model compounds. *J Chromatogr B Anal Technol Biomed Life Sci*. 2018;1091:67-78. doi:10.1016/j.jchromb.2018.05.017

129. Rohner TC, Lion N, Girault HH. Electrochemical and theoretical aspects of electrospray ionisation. *Phys Chem Chem Phys*. 2004;6(12):3056-3068. doi:10.1039/b316836k

130. Cech NB, Enke CG. Practical implications of some recent studies in electrospray ionization fundamentals. *Mass Spectrom Rev*. 2001;20(6):362-387. doi:10.1002/mas.10008

131. Banerjee S, Mazumdar S. Electrospray Ionization Mass Spectrometry: A Technique to Access the Information beyond the Molecular Weight of the Analyte. *Int J Anal Chem*. 2012;2012:1-40. doi:10.1155/2012/282574

132. Kruve A, Kaupmees K. Adduct Formation in ESI/MS by Mobile Phase Additives. *J Am Soc Mass Spectrom*. 2017;28(5):887-894. doi:10.1007/s13361-017-1626-y

133. Furey A, Moriarty M, Bane V, Kinsella B, Lehane M. Ion suppression; A critical review on causes, evaluation, prevention and applications. *Talanta*. 2013;115:104-122. doi:10.1016/j.talanta.2013.03.048

134. IUPAC Compendium of Chemical Terminology Gold Book – Version 2.3.3. 2014. 886. Available at: https://goldbook.iupac.org/files/pdf/goldbook.pdf. Accessed February 1, 2022. doi: 10.1351/goldbook. ISBN: 0-9678550-9-8

135. Xian F, Hendrickson C, Marshall A. High-resolution mass spectrometry. *Anal Chem*. 2012;84:708-719. doi:10.1126/science.151.3711.641

136. Ojanperä I, Kolmonen M, Pelander A. Current use of high-resolution mass spectrometry in drug screening relevant to clinical and forensic toxicology and doping control. *Anal Bioanal Chem*. 2012;403(5):1203-1220. doi:10.1007/s00216-012-5726-z

137. Makarov A, Denisov E, Kholomeev A, et al. Performance evaluation of a hybrid linear ion trap/orbitrap mass spectrometer. *Anal Chem*. 2006;78(7):2113-2120. doi:10.1021/ac0518811

138. Makarov A, Scigelova M. Coupling liquid chromatography to Orbitrap mass spectrometry. *J Chromatogr A*. 2010;1217(25):3938-3945. doi:10.1016/j.chroma.2010.02.022

139. Eliuk S, Makarov A. Evolution of Orbitrap Mass Spectrometry Instrumentation. *Annu Rev Anal Chem*. 2015;8:61-80. doi:10.1146/annurev-anchem-071114-040325

140. Bourmaud A, Gallien S, Domon B. Parallel reaction monitoring using quadrupole-Orbitrap mass spectrometer: Principle and applications. *Proteomics*. 2016;16(15-16):2146-2159. doi:10.1002/pmic.201500543

141. Saito K, Yagi K, Ishizaki A, Kataoka H. Determination of anabolic steroids in human urine by automated in-tube solid-phase microextraction coupled with liquid chromatography-mass spectrometry. *J Pharm Biomed Anal*. 2010;52(5):727-733. doi:10.1016/j.jpba.2010.02.027

142. Guo F, Shao J, Liu Q, Shi JB, Jiang G Bin. Automated and sensitive determination of four anabolic androgenic steroids in urine by online turbulent flow solid-phase extraction coupled with liquid chromatography-tandem mass spectrometry: A novel approach for clinical monitoring and doping control. *Talanta*. 2014;125:432-438. doi:10.1016/j.talanta.2014.03.010

143. Santos MG, Tavares IMC, Boralli VB, Figueiredo EC. Direct doping analysis of beta-blocker drugs from urinary samples by on-line molecularly imprinted solid-phase extraction coupled to liquid chromatography/mass spectrometry. *Analyst*. 2015;140(8):2696-2703. doi:10.1039/c4an02066a

144. Verplaetse R, Henion J. Quantitative determination of opioids in whole blood using fully automated dried blood spot desorption coupled to on-line SPE-LC-MS/MS. *Drug Test Anal*. 2016;8(1):30-38. doi:10.1002/dta.1927

145. Tretzel L, Thomas A, Piper T, et al. Fully automated determination of nicotine and its major metabolites in whole blood by means of a DBS online-SPE LC-HR-MS/MS approach for sports drug testing. *J Pharm Biomed Anal*. 2016;123:132-140. doi:10.1016/j.jpba.2016.02.009

146. De Wilde L, Roels K, Polet M, Van Eenoo P, Deventer K. Identification and confirmation of diuretics and masking agents in urine by turbulent flow online solid-phase extraction coupled with liquid chromatography-triple quadrupole mass spectrometry for doping control. *J Chromatogr A*. 2018;1579:31-40. doi:10.1016/j.chroma.2018.10.032

147. Liang Y, Liu J, Zhong Q, Huang T, Zhou T. An automatic online solid-phase dehydrate

extraction-ultra-high performance supercritical fluid chromatography-tandem mass spectrometry system using a dilution strategy for the screening of doping agents in human urine. *Anal Chim Acta*. 2020;1101:184-192. doi:10.1016/j.aca.2019.12.011

148. Coppieters G, Judák P, Van Haecke N, Van Renterghem P, Van Eenoo P, Deventer K. A high-throughput assay for the quantification of intact Insulin-like Growth Factor I in human serum using online SPE-LC-HRMS. *Clin Chim Acta*. 2020;510:391-399. doi:10.1016/j.cca.2020.07.054

149. De Wilde L, Roels K, Deventer K, Van Eenoo P. Automated sample preparation for the detection and confirmation of hypoxia-inducible factor stabilizers in urine. *Biomed Chromatogr*. 2021;35(2):1-10. doi:10.1002/bmc.4970

150. De Wilde L, Roels K, Van Eenoo P, Deventer K. Online Turbulent Flow Extraction and Column Switching for the Confirmatory Analysis of Stimulants in Urine by Liquid Chromatography-Mass Spectrometry. *J Anal Toxicol*. 2021;45(7):666-678. doi:10.1093/jat/bkaa136

151. Matos RR, Anselmo C, Sardela VF, Pereira HMG. Phase II stanozolol metabolism study using the zebrafish water tank (ZWT) model. *J Pharm Biomed Anal*. 2021;195. doi:10.1016/j.jpba.2020.113886

152. Lootens L, Van Eenoo P, Meuleman P, et al. Steroid metabolism in chimeric mice with humanized liver. *Drug Test Anal*. 2009;1(11-12):531-537. doi:10.1002/dta.67

153. Savill R, Baues H, Voigt E, Zierau O, Thieme D, Keiler AM. Cell culture as a toolbox to generate phase I metabolites for antidoping screening. *Drug Test Anal*. 2021;13(6):1169-1177. doi:10.1002/dta.3009

154. World Anti-Doping Agency (WADA). WADA Technical Document - TD2021IDCR. Minimum Criteria for Chromatographic-Mass Spectrometric Confirmation of the Identity of Analytes for Doping Control Purposes. Available at: https://www.wada-ama.org/sites/default/files/resources/files/td2021idcr_final_eng_0.pdf. Accessed February 1, 2022.

155. Koch HM, Gonzalez-Reche LM, Angerer J. On-line clean-up by multidimensional liquid chromatography-electrospray ionization tandem mass spectrometry for high throughput quantification of primary and secondary phthalate metabolites in human urine. *J Chromatogr B Anal Technol Biomed Life Sci*. 2003;784(1):169-182. doi:10.1016/S1570-0232(02)00785-7

156. Wang T, Li H, Han Y, et al. A rapid and high-throughput approach to quantify non-esterified oxylipins for epidemiological studies using online SPE-LC-MS/MS. *Anal Bioanal Chem*. 2020;412(28):7989-8001. doi:10.1007/s00216-020-02931-y

157. Schettgen T, Musiol A, Alt A, Ochsmann E, Kraus T. A method for the quantification of biomarkers of exposure to acrylonitrile and 1,3-butadiene in human urine by column-switching liquid chromatography- tandem mass spectrometry. *Anal Bioanal Chem*. 2009;393(3):969-981. doi:10.1007/s00216-008-2510-1

158. Ebert KE, Belov VN, Weiss T, et al. Determination of urinary metabolites of the UV filter homosalate by online-SPE-LC-MS/MS. *Anal Chim Acta*. 2021;1176:338754. doi:10.1016/j.aca.2021.338754

159. Taherzadeh Ghahfarrokhi M, Zeinali S, Bagheri H. Preparation of amine–modified lignin and its applicability toward online micro–solid phase extraction of valsartan and losartan in urine samples. *J Chromatogr A*. 2021;1643:462081. doi:10.1016/j.chroma.2021.462081

160. Saito K, Yagi K, Ishizaki A, Kataoka H. Determination of anabolic steroids in human urine by automated in-tube solid-phase microextraction coupled with liquid chromatography-mass spectrometry. *J Pharm Biomed Anal*. 2010;52(5):727-733. doi:10.1016/j.jpba.2010.02.027

161. Gómez C, Pozo OJ, Garrostas L, Segura J, Ventura R. A new sulphate metabolite as a long-term marker of metandienone misuse. *Steroids*. 2013;78(12-13):1245-1253. doi:10.1016/j.steroids.2013.09.005

162. Rzeppa S, Heinrich G, Hemmersbach P. Analysis of anabolic androgenic steroids as sulfate conjugates using high performance liquid chromatography coupled to tandem mass spectrometry. *Drug Test Anal*. 2015;7(11-12):1030-1039. doi:10.1002/dta.1895

163. Albertsdóttir AD, Van Gansbeke W, Coppieters G, Balgimbekova K, Van Eenoo P, Polet M. Searching for new long-term urinary metabolites of metenolone and drostanolone using gas chromatography–mass spectrometry with a focus on non-hydrolysed sulfates. *Drug Test Anal*. 2020;12(8):1041-1053. doi:10.1002/dta.2818

164. Balcells G, Pozo OJ, Garrostas L, et al. Detection and characterization of clostebol sulfate metabolites in Caucasian population. *J Chromatogr B Anal Technol Biomed Life Sci*. 2016;1022:54-63. doi:10.1016/j.jchromb.2016.03.028

165. Bradshaw JS, Krakowiak KE. Selective Protection of the Primary Amine Functions of Linear Tetraamines Using the Trityl Group. *Synth Commun*. 1998;28(18):3451-3459. doi:https://doi.org/10.1080/00397919808004453

166. Maltese M. Reductive demercuration in deprotection of trityl thioethers, trityl amines, and trityl ethers. *J Org Chem*. 2001;66(23):7615-7625. doi:10.1021/jo0156971

167. Hendrickson J. The Determination of Primary Alcohol Groups in Polyglycols Using Triphenylchlormethane. *Anal Chem*. 1964;36:126-128. doi:10.1021/AC60207A039

168. Kratena N, Enev V, Gmeiner G, Gärtner P. Synthesis and characterization of stanozolol N-glucuronide metabolites. *Monatshefte fur Chemie*. 2019;150(5):843-848. doi:10.1007/s00706-019-02424-4

169. Fernández-Álvarez, M.; Lu, J.; Cuervo, D.; Xu, Y.; Munoz-Guerra, J.; Aguilera R. Detection of new Oral-Turinabol metabolites by LC-QToF. *Recent Adv Doping Anal*. 2014:182-187.

170. Balcells G, Gómez C, Garrostas L, Pozo ÓJ, Ventura R. Sulfate metabolites as alternative markers for the detection of 4-chlorometandienone misuse in doping control. *Drug Test Anal*. 2017;9(7):983-993. doi:10.1002/dta.2101

Appendix

Paper I

Development and validation of a simple online-SPE method coupled to high-resolution mass spectrometry for the analysis of stanozolol-N-glucuronides in urine samples

Lorenz Göschl[1,2], Günter Gmeiner[1], Valentin Enev[3], Nicolas Kratena[3], Peter Gärtner[3], Guro Forsdahl[1,2]

1 Doping Control Laboratory, Seibersdorf Labor GmbH, Seibersdorf, Austria

2 Department of Pharmacy, University of Tromsø – The Arctic University of Norway, Tromsø, Norway

3 Institute of Applied Synthetic Chemistry, Technical University of Vienna, Austria

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Development and validation of a simple online-SPE method coupled to high-resolution mass spectrometry for the analysis of stanozolol-N-glucuronides in urine samples

Lorenz Göschl[1,2] | Günter Gmeiner[1] | Valentin Enev[3] | Nicolas Kratena[3] | Peter Gärtner[3] | Guro Forsdahl[1,2]

[1]Doping Control Laboratory, Seibersdorf Labor GmbH, Seibersdorf, Austria

[2]Department of Pharmacy, University of Tromsø – The Arctic University of Norway, Tromsø, Norway

[3]Institute of Applied Synthetic Chemistry, Technical University of Vienna, Austria

Correspondence
Lorenz Göschl, Department of Pharmacy, University of Tromsø – The Arctic University of Norway, Tromsø, Norway.
Email: lorenz.goeschl@seibersdorf-laboratories.at

Abstract

Stanozolol is still the most commonly used illicit anabolic-androgenic steroid (AAS) in professional sports. Therefore, accurate and fast analysis and long detection windows are of great interest in the field of antidoping analysis. In this work, a very simple, fast, and highly sensitive online solid-phase extraction method coupled with liquid chromatography–high-resolution tandem mass spectrometry (HPLC-HRMSMS) for the analysis of stanozolol-N-glucuronides was developed. This fully validated procedure is characterized by only a few manual steps (dilution and addition of internal standard) in the sample preparation. A limit of identification (LOI) of 75 pg/mL, high accuracy (87.1%–102.1%), precision (3.1%–7.8%), and sensitivity was achieved. Furthermore, good linearity (> 0.99) and robustness, as well as no carry-over effects, could be observed. In addition to excellent confirmation analysis performance, this method shows sufficient potential for the identification and characterization of unknown metabolites. Using this method, it was possible to unambiguously confirm the presence of 1′N- and 2′N-stanozolol-glucuronide in human urine for the first time due to the access to reference material.

KEYWORDS

anabolic androgenic steroids, mass spectrometry, online solid-phase extraction, phase-II metabolite, stanozolol

1 | INTRODUCTION

Since the beginning of doping analysis, knowledge about the window of opportunity for the detection of illicit substances has been of great interest. The discovery of so-called long-term metabolites (LTM) of substances listed on the Prohibited List of the World Anti-Doping Agency (WADA),[1] is still one of the most important topics in current antidoping research.[2] In particular, the analysis of anabolic-androgenic steroids (AAS), which represent the most frequently detected class of illicitly used substances in professional sport,[3] has always been a major priority in the field of doping analysis.[2,4] Conventional steroid analysis is based on the enzymatic cleavage of steroid phase-II metabolite conjugates and the following analysis of remaining parent molecules and phase-I metabolites as their trimethylsilyl-derivatives with gas chromatography-tandem mass spectrometry (GC–MS/MS).[5,6] Although this very sensitive and highly efficient method is still the gold standard for routine steroid analysis in doping control, many previous studies have shown that the direct analysis of steroid phase-II

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conjugates using liquid chromatography–tandem mass spectrometry (LC–MS/MS), is a highly promising approach for the detection of unknown steroid metabolites, respectively LTMs.[7–15] In most cases, phase-II metabolites are excreted in urine as glucuronide- and/or sulfate-conjugates. Both conjugates lead to an increase in water solubility, allowing urinary excretion.[16–19]

According to WADA statistics, stanozolol (17α-methyl-5α-androst-2-eno[3,2-*c*]pyrazol-17β-ol) is the most detected substance within the class of AAS.[3] It was first synthesized in 1959 and due to its special structure with a pyrazole ring fused to the androstane framework, it has a unique place in the family of AAS.[20] To date, many stanozolol metabolites have been described. Donike and Schänzer developed the detection of the metabolite 3′-OH-stanozolol as early as 1986 and successfully implemented the method for the Olympic Games in Seoul in 1988.[21] In the following years, many papers concerning stanozolol metabolites have been published and a high number of stanozolol metabolites have been described.[22–31] Both GC–MS/MS and LC–MS/MS methods were developed and improved and, as a result, the limits of identification (LOI) became lower and the detection windows for stanozolol larger. All these above-mentioned methods use an indirect analytical approach by detecting the remaining hydrolyzed parent molecules or phase-I metabolites. In 2012, Van Enoo et al. developed the first highly sensitive method for the direct detection of 3′OH-stanozolol-glucuronide by LC–MS/MS.[32] Further published methods either use a solid-phase-extraction (SPE) as sample preparation followed by LC–MS/MS analysis or a direct, so-called dilution-and-shoot approach, without any sample preparation, for the analysis of phase-II conjugates.[9,14,15,33,34] The objectives of SPE are the trapping and concentration of analytes and the removal of interfering substances from biological matrices in order to improve the detection in the following instrumental analysis. Therefore, SPE has become one of the most important preparation techniques for the analysis of small molecules in biological samples. However, sample preparation with SPE can be very time and resource consuming. The present work aimed to combine the advantages of SPE with a fast and simple dilute-and-shoot method. The result is the development of a simple, fully automatic online-SPE-LC-HRMS/MS method for the analysis of steroid-glucuronides, in particular for stanozolol-glucuronides. The method validation shows a highly sensitive and specific procedure with minimal sample preparation effort.

Initially developed for the confirmation analysis of stanozolol-glucuronides in routine doping control, our method shows very good selectivity and mass accuracy, allowing us to use it for the identification and characterization of new, unknown metabolites. In 2013, Schänzer et al. demonstrated the utility of direct detection of stanozolol glucuronides by high-resolution mass spectrometry (HRMS) coupled to LC in routine doping control and they additionally found and described two new metabolites, stanozolol-N-glucuronide and 17-epistanozolol-N-glucuronide.[33] These metabolites are resistant to enzymatic hydrolysis with beta-glucuronidase and have a high potential for long-term detection. Stanozolol and corresponding metabolites have two feasible N-atoms (1′N/2′N) for the conjugation of glucuronic acid. However, the exact position of the glucuronic acid on the pyrazole ring was not clarified. In 2015, Thevis et al. suggested the existence of both 1′N- and 2′N-stanozolol-glucuronides, based on experiments with collision cross-section computation, but they could not unequivocally confirm the position of the N-glucuronides.[34] In this work, with the presented method and synthesized reference standards[35] we aimed to confirm unambiguously the presence of 1′N- and 2′N-stanozolol-glucuronide in human urine samples after administration of stanozolol.

2 | EXPERIMENTAL

2.1 | Chemicals, reagents, and solutions

Methanol (MeOH) and water used for HPLC analysis (HPLC grade) were purchased from Biosolve Chimie (Dieuze, France). Formic acid (FA) for HPLC was bought from Merck (Darmstadt, Germany). Water (MQ) for sample dilution was obtained by a Milli-Q water purification system (Millipore, Reference A+, Burlington, Massachusetts, USA). Methanol for standard solutions was supplied by Chem-Lab (Zedelgem, Belgium). The internal standard (IS) 16,16,17α-d3-testosterone-glucuronide was purchased from the National Measurement Institute Australia (Sydney, Australia). Both 1′N- and 2′N-stanozolol-glucuronide standards were synthesized and characterized by nuclear magnetic resonance spectroscopy in a previously published study.[35] The chemical structures of all substances involved are illustrated in Figure 1. A concentration of 1 ng stanozolol-glucuronide corresponds to approximately 0.65 ng free stanozolol.

Both the IS solution and standard stock solutions were prepared by dissolving 1 μg of standard substance in 1 mL MeOH (1 μg/mL). A standard working solution was made by diluting stock solutions with MeOH. All solutions were stored at −20°C. For reference samples, the methanolic working solution was directly added to blank urine.

2.2 | Urine samples

The positive urine samples shown in this work were collected by accredited sample collection authorities in compliance with WADA's collection guidelines.[36] The anonymized samples were received, analyzed, and subsequently provided by the WADA accredited anti-doping laboratory Seibersdorf Labor GmbH. Other already characterized stanozolol metabolites had previously been confirmed in these samples. Before the analysis, the athletes gave permission to use the urine samples for research purposes. This is in accordance with the International Standard for Laboratories (ISL).[37] Additionally, WADA proficiency test samples were used, which are excretion samples and sent to antidoping laboratories as part of the educational external quality assessment scheme (EQAS). Blank urine samples were collected from healthy female and male volunteers working at Seibersdorf Labor GmbH. All urine samples were stored frozen at −20°C until analysis.

FIGURE 1 Chemical structures of (A) 1′N-stanozolol-glucuronide (1 N-STANG), (B) 2′N-stanozolol-glucuronide (2 N-STANG), and (C) internal standard: 16,16,17α-d3-testosterone-glucuronide (D3-TESG)

2.3 | Sample preparation

For the analysis, 0.5 mL of urine was diluted with 0.5 mL MQ and 30 μL IS (final concentration: 30 ng/mL) solution was added. Afterwards, the samples were vortexed for 10 seconds.

2.4 | Online solid-phase extraction (online SPE)

The online-SPE is based on a standard UHPLC dual-pump system, in which an additional extraction column is attached before the analytical column via a valve system. As an extraction column, an Accucore Phenyl-Hexyl, 10 × 3 mm column with 2.6 μm particle size and 80 Å pore size was used (Fischer Scientific, Loughborough, UK). As illustrated in Figure 2, the extraction column was installed upstream to a two-position, six-port UHPLC valve (MXT715–000, Rheodyne LLC, Bensheim, Germany). The column was connected to the valve by installing a Universal Uniguard Holder 2.1/3.0 mm ID (Thermo Scientific, Bellefonte, USA). For step 1 (see also Figure 2), the sample loading and washing, the valve is set to position 1–2, which directs the liquid stream through the extraction column and into a waste container. This procedure traps analytes on the

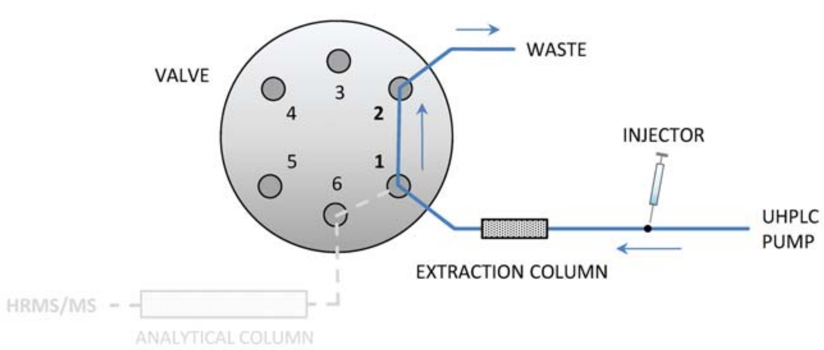

FIGURE 2 Online solid-phase extraction procedure: (A) Schematic illustration of the valve switch system; above: Step 1, Loading of analytes on extraction column and washing; below: Step 2, Eluting analytes from extraction column, separating on analytical column and HRMS measurement (B) Temporal progression of the experimental procedure [Colour figure can be viewed at wileyonlinelibrary.com]

pre-column and flushes matrix compounds, such as proteins or salts, into the waste. After 2 minutes the valve switches from position 1–2 to 1–6, thereby leading the stream via the analytical column to the mass spectrometer. Simultaneously with valve switching (step 2), the solvent gradient is started and the elution of analytes from the extraction column begins. After finishing the separation, the system is flushed and re-equilibrated. Connections between single components were established with Viper Capillary finger-tight fittings (Thermo Fisher, Austin, Texas, USA).

2.5 | Liquid chromatography – high-resolution mass spectrometry (LC-HRMS)

Measurements were performed on a Vanquish Horizon UHPLC$^+$ System coupled to a Q-Exactive Orbitrap high-resolution mass spectrometer (Thermo Fisher, Austin, Texas, USA). As an analytical column, a Kinetex EVO C-18, 100 × 2.1 mm column with 2.6 μm particle- and 100 Å pore size was used (Phenomenex, Aschaffenburg, Germany). Chromatography was carried out with mobile phases containing water with 0.2% v/v FA (solvent A) and methanol with 0.1% v/v FA (solvent B). The separation was performed with a constant flow of 0.4 mL/min and constant temperature at 25°C. After loading and washing the pre-column with 10% solvent B for 2 minutes, the solvent gradient continues as follows: start with 10% solvent B up to 100% over 7 minutes, hold 100% B for 2 minutes and again 10% B for 2 minutes to flush and re-equilibrate the system. The sample injection volume was 25 μL.

High-resolution mass spectrometry (HRMS) was carried out in positive electrospray ionization (ESI+) in the modes full scan and parallel reaction monitoring (PRM) with the following common settings: The spray voltage was 3.8 kV and the capillary temperature was set to 320°C. Nitrogen was used as the sheath gas (pressure 25 units) as well as auxiliary gas (pressure 8 units) and the auxiliary gas heater temperature was set to 310°C. The s-lens RF level was set to 55 and the sweep gas flow rate was 0. The mass resolution was set to 70 000 at m/z 200 and automatic gain control (AGC) to 2×10^5 ions. The maximum IT was set to 100 ms. Internal calibration with the lock-mass m/z 391.2843 (di-isooctyl phthalate) was used. Full scanning was performed in the range of m/z 300–600. PRM measurements were carried out in separate runs. Isolation windows were set to 1 m/z. Collision energies (CE) were optimized to obtain the most abundant signal intensities. Spectrometric parameters were optimized by injection of the methanolic compound solutions. The chosen diagnostic ions and corresponding CEs are summarized in Table 1. Data were processed and monoisotopic masses were calculated with Thermo Xcalibur Qual Browser 4.1.45. All systems were controlled with Xcalibur 4.0 (Thermo Fischer).

2.6 | Method validation

The method introduced in this work was validated for qualitative and semi-quantitative purposes according to the ISL using the parameters presented below. Samples described in the following sections were measured with the above described PRM-method. To create extraction ion chromatograms (XIC), product ion 1 and 2 shown in Table 1 with an ion extraction range of 2 ppm were used. For all quantitative parameters the peak area of product ion 1 was taken. Values were corrected with the internal standard and calculated with a calibration curve, which was established for each measurement sequence. Data processing was carried out with Thermo Xcalibur Quan Browser 4.1.45 and calculations were performed with Microsoft Excel 2010.

A concentration of 1 ng/mL, used for most of the parameters, represents 50% of the minimum required performance level for free stanozolol, defined by WADA.[38] For specificity, robustness, and limit of identification (LOI), the comparison of retention times and ratios of relative abundances of two ion transitions must fulfil WADA identification criteria.[39]

2.7 | Specificity

Five different male and five different female urine samples from healthy volunteers were spiked with 1 ng/mL standard. Additionally, five male and five female blank urine samples were analyzed (n = 10). The absence of interferences for both diagnostic ions was verified. Retention times and relative abundances of two ion transitions (peak area) were compared.

2.7.1 | Precision

Ten replicates of urine samples were spiked with standard working solution at three different concentrations, low 1 ng/mL, medium 10 ng/mL, and high 50 ng/mL ($n = 3 \times 10$). Samples were measured on 3 consecutive days and the coefficient of variation (CV) for intra- and inter-day precision was calculated.

TABLE 1 Mass transitions applied for parallel reaction monitoring

Substance	Formula	Precursor ion	Species	Product ion 1	Product ion 2
		[m/z]		[m/z] / [eV]	[m/z] / [eV]
1 N-STANG	$C_{27}H_{40}N_2O_7$	505.2908	[M + H]+	329.2587/60	81.0447/70
2 N-STANG	$C_{27}H_{40}N_2O_7$	505.2908	[M + H]+	329.2587/60	81.0447/70
D3-TESG	$C_{25}H_{32}D_3O_8$	468.2671	[M + H]+	109.0645/35	97.0651/35

2.7.2 | Robustness

Urine samples spiked with 1 ng/mL standard working solution at various pH values (3, 6,9) and specific gravities (0.005, 0.010, 0.015, 0.025, 0.032) were measured and different injection volumes (15 μL, 25 μL, 35 μL) were tested. Retention times and relative abundances of two ion transitions were compared.

2.7.3 | Linearity

Four replicates of urine samples, spiked with standard working solution at six different concentrations, (1, 10, 25, 50, 75, and 100 ng/mL, n = 4 × 6), were measured and a calibration curve was generated. Linearity (r^2) was calculated with the software Thermo Quan Browser.

2.7.4 | Accuracy

Ten replicates of urine samples, spiked with standard working solution at three different concentrations, low 1 ng/mL, medium 10 ng/mL, and high 50 ng/mL (n = 3 × 10), were measured. Accuracy was calculated (determined concentration/nominal concentration*100%).

2.7.5 | Matrix effects

Six urine samples obtained from six different volunteers and one matrix-free sample (MQ), spiked with 1 ng/mL standard working solution, were measured and average ion suppression or enhancement effects were calculated by comparison of the signal area (normalized with IS) of urine samples with the matrix-free samples.

2.7.6 | Carryover

A urine sample spiked with 200 ng/mL standard working solution was measured directly prior to a blank urine specimen. The presence of signals in the blank sample was calculated (%).

2.7.7 | Limit of identification (LOI)

Three urine samples collected from three different volunteers, spiked with standard working solution at three concentrations (0.025, 0.05, 0.075 ng/mL, n = 3 × 3), close to an estimated LOI, were measured. The LOI was defined as the lowest concentration level at which a compound could be clearly identified. Therefore, retention times and relative abundances of two ion transitions were compared. The term LOI, used by WADA, is to be equated to the limit of detection (LOD).

TABLE 2 Summary of validation results for 1 N-STANG and 2 N-STANG

	Specificity	Intra-day pr. CV			Inter-day pr. CV			Accuracy			Linearity r^2	Robustness	Carryover	ME (RSD)	LOI
n	10	10	10	10	10	10	10	10	10	10	6 x 4	3 + 5 + 3	1	6	n = 3
c [ng/mL]	1	1	10	50	1	10	50	1	10	50	1–100	1	200	1	-
1 N-STANG	10/10	3.1%	5.5%	4.8%	3.4%	5.6%	4.5%	87.0%	91.6%	99.7%	0.999	11/11	0%	160% (12%)	75 pg/mL
2 N-STANG	10/10	4.9%	3.7%	3.3%	7.8%	6.8%	5.0%	90.2%	94.2%	102.1%	0.998	11/11	0%	151% (15%)	75 pg/mL

c, concentration; CV, coefficient of variation; ME, matrix effects; n, number of samples; pr., precision; RSD, relative standard deviation.

2.7.8 | Identification of stanozolol-N-glucuronides

In antidoping analysis, an unequivocal identification of newly discovered metabolites is extremely important. A proper way to achieve this is a combination of chromatography, high-resolution mass spectrometry, and nuclear magnetic resonance spectroscopy. In this work, reference standards of 1′N- and 2′N-stanozolol-glucuronide were compared with human urine samples that were confirmed to be positive for stanozolol.

3 | RESULTS AND DISCUSSION

3.1 | Method validation

The obtained method validation parameters are summarized in Table 2. Good specificity and robustness were demonstrated in all 10 of 10 and 11 of 11 samples for both 1′N- and 2′N-stanozolol-glucuronide, respectively. In all samples, no interfering signals were observed and WADA identification criteria for ion ratios and retention time were fulfilled. The method shows excellent precision and accuracy values. The CV for intra-day precision ranged from 3.1% to 5.5% for both metabolites and the CV for inter-day precision was between 3.4% and 7.8%. Accuracy varied between 87.0% and 102.1%. An improvement in the accuracy with increasing concentration of the substance could be observed. A linear signal response of both metabolites with increasing concentration in the sample matrix was shown. No carryover could be observed after injection of high substance concentrations. The high matrix effects of above 150% may be explained by the lack of elaborate sample preparation, but it does not seem to have any influence on the precision and accuracy of the method. Rather a signal enhancement was observed in all samples. Therefore for quantitative issues, a matrix-matched calibration is needed. We could detect both metabolites with a concentration of 25 pg/mL and S/N > 3 in all samples. However, at this concentration level, the ion ratios did not fulfill WADA's identification criteria. Even though in analytical chemistry S/N > 3 is often consulted for the LOI (LOD) definition, we decided to consider the stricter LOI criteria defined by WADA. This applies to a concentration of 75 pg/mL glucuronide, which corresponds to approximately 50 pg/mL of free stanozolol. Most likely, an additional conventional SPE sample separation prior to analytical measurement could significantly improve the LOI if it is required.

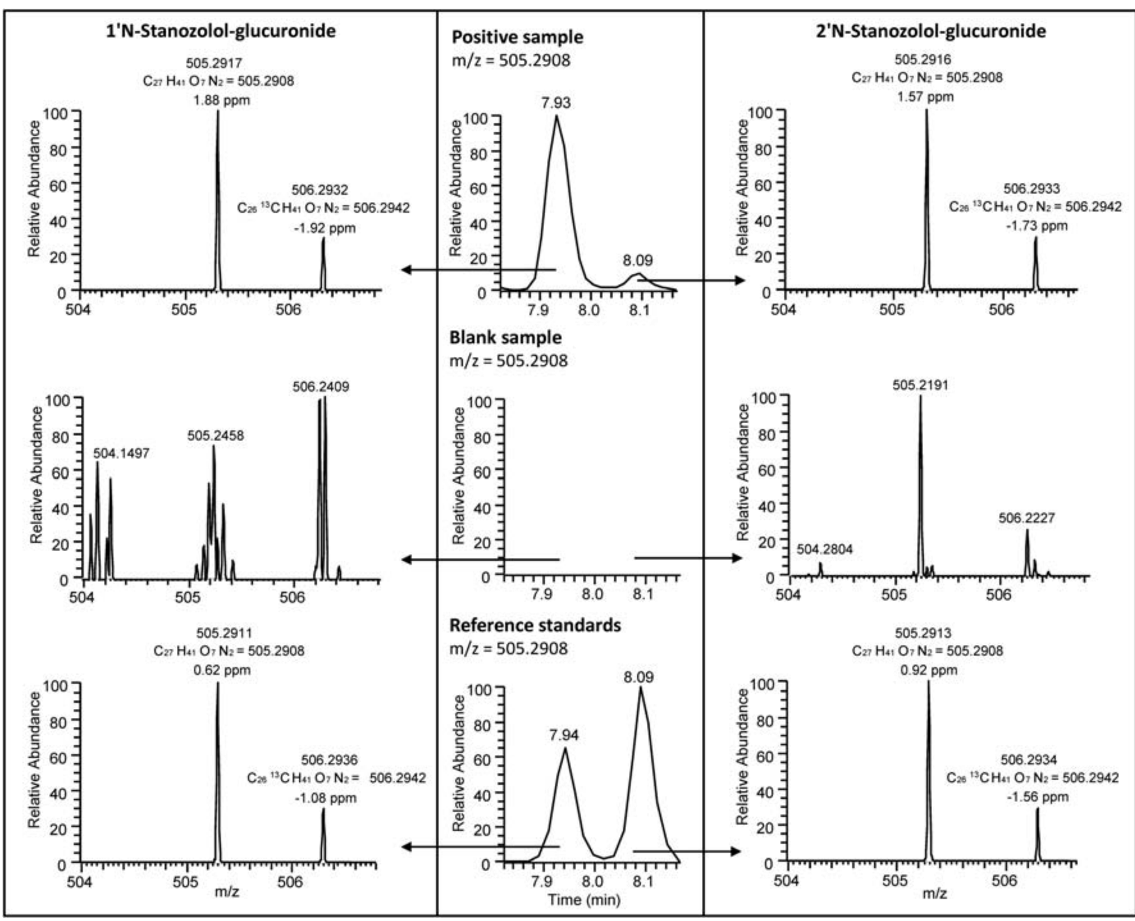

FIGURE 3 Results of full-MS scan; XIC (middle, m/z 505.2908, ESI+, 2 ppm mass tolerance) and corresponding HRMS spectra (left and right) of 1 N-STANG and 2 N-STANG in positive urine, blank urine, and reference standards

3.2 | Identification of synthesized products

The metabolites 1′N- and 2′N-stanozolol-glucuronide were identified by using mass spectrometric techniques. For definitive proof of the existence of these two metabolites, positive doping samples for stanozolol were compared with reference standards and blank urine samples. The above-described method was used for the acquisition of both full-MS scans and MS/MS measurements.

3.3 | HPLC-full-HRMS

As a first step for the identification of synthesized products, HPLC-full-HRMS scans of positive samples for stanozolol, reference standards, and blank samples were measured. When the MS-range was set to m/z 505.2908 (mass tolerance 2 ppm) which corresponds to the theoretical mass of [stanozolol-mono-glucuronide + H] $^{+}$, the results showed a perfect match for both retention times and full-HRMS spectra for both metabolites. The deviations of the retention times were close to zero and the differences between theoretical mass and experimental mass were below 2 ppm in all cases. No signals at all could be observed in blank urine samples. The chromatographic and mass-spectrometric results are shown in Figure 3.

3.4 | HPLC-HRMS/MS

As a second step for the identification of unknown metabolites, PRM was performed on a positive stanozolol sample, reference standards, and blank urine samples. The precursor ion was filtered at m/z 505.2908. Fragmentation with 60 eV collision energy and a following scan were carried out. The chromatograms, again with an ion extraction range of 2 ppm, and resulting fragment spectra are shown in Figure 4. Again, the deviations of the retention times were close to zero and the differences between theoretical mass and experimental mass were below 4 ppm for the 329 fragments and below 6 ppm for the 81 fragments. More interferences in the smaller mass range, as visible in Figure 4, led to a higher deviation of the mass accuracy. All four signals show a highly similar fragmentation pattern, with the most abundant peak at m/z 329 and the second most abundant peak at m/z 81. The 329 ion represents the parent stanozolol molecule, generated after the loss of the glucuronic acid. The 81 ion is a characteristic fragment for stanozolol, which has previously been accurately described in the literature.[30] It represents a stable six-membered heterocyclic ring structure formed by the pyrazole ring and an additional carbon from the steroidal framework. Again, no signals could be observed in blank urine samples.

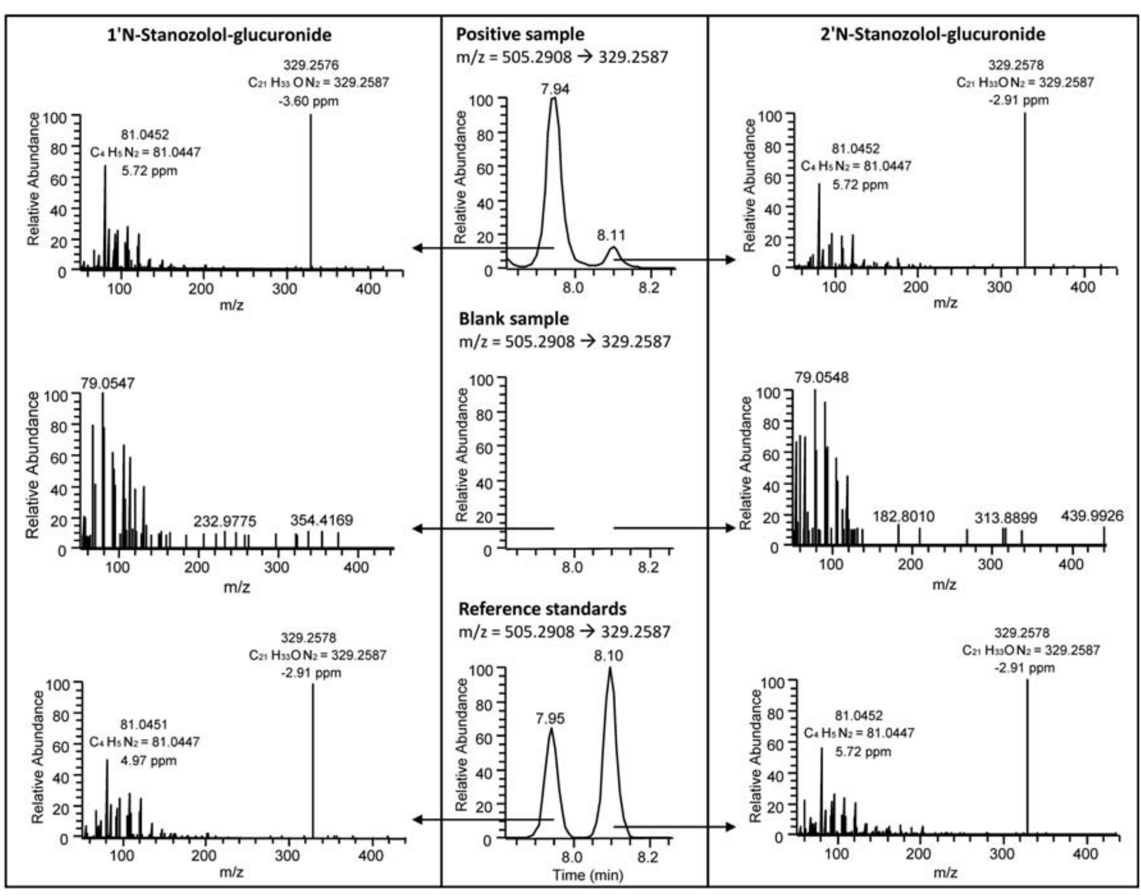

FIGURE 4 Results of PRM measurements; XIC (middle; m/z 505.2908 - > 329.2587 (60 eV), ESI+, 2 ppm mass tolerance) and corresponding PRM spectra (left and right) of 1 N-STANG and 2 N-STANG in positive urine, blank urine, and reference standards

TABLE 3 (A) Retention time comparison and (B) Relative abundances of two MS/MS transitions for a positive stanozolol sample and reference standards; IS, internal standard

A

Substance	Retention time IS [min]		Retention time samples [min]		Rel. Difference Max. Tolerated: 1%
	Sample	Reference	Sample	Reference	
1 N-STANG	7.77	7.77	7.94	7.95	0.1%
2 N-STANG	7.77	7.77	8.11	8.10	0.1%

B

Substance	Transition [m/z] 505.2908	Relative abundance		Difference	Maximum tolerance Window
		Sample	Reference		
1 N-STANG	→ 329.2587 (60 eV)	100%	100%	0.67%	8.5% - 18.5% (±5)
	→ 81.0447 (70 eV)	13.5%	12.9%		
2 N-STANG	→ 329.2587 (60 eV)	100%	100%	0.39%	8.3% - 18.3% (±5)
	→ 81.0447 (70 eV)	13.3%	12.9%		

According to the WADA identification criteria, the ratios of at least two MS/MS-transitions of the targeted analyte in a positive sample and a reference sample have to be compared. Additionally, the retention times have to match in both sample types. In Table 3 the compared retention times, the chosen transitions with corresponding relative abundances, and maximum tolerated values are shown. The abundance of the transitions was determined from the peak area. The relative abundance was calculated by dividing the area of the less intense signal by the area of the more intense signal (100%).

For both metabolites, the retention times in the positive sample match with the retention times in the reference sample. The relative difference in both cases is far below the tolerated level of 1%. Similar good matches were achieved for the relative abundances. In both cases, the relative difference is below 1%, which is much lower than the accepted ±5%. These data clearly confirm the presence of 1′N- and 2′N-stanozolol-glucuronide in human urine.

4 | CONCLUSION

In the present study, we introduce a novel, highly functional analytical method for the analysis of stanozolol-glucuronides for doping control analysis. Sample preparation is reduced to diluting the sample with water and adding an internal standard solution. We established a very simple approach for installing an automatic online solid-phase extraction coupled with UHLPC-HRMS/MS. The method is characterized by satisfactory validation parameters. The LOI of 75 pg/mL, excellent specificity, precision, and accuracy as well as good linearity and robustness make our method interesting for very fast and sensitive confirmation procedures, but also for the identification and characterization of unknown metabolites. We could unambiguously identify the presence of 1′N- and 2′N-stanozolol-glucuronide in human urine with the present method. We suggest that these two metabolites can be used as additional information for initial testing and confirmation procedures for the analysis of stanozolol in human urine. In this paper, we focus on the analysis of N-stanozolol-glucuronides, but our method also showed highly promising results for 3-OH-stanozolol-glucuronides (data not shown).

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ORCID

Lorenz Göschl https://orcid.org/0000-0002-6018-1961
Günter Gmeiner https://orcid.org/0000-0003-3968-2048
Guro Forsdahl https://orcid.org/0000-0001-9049-9463

REFERENCES

1. World Anti Doping Agency (WADA). Prohibited List WADA 2019. Available at: https://www.wada-ama.org/sites/default/files/wada_2019_english_prohibited_list.pdf.
2. Thevis M, Kuuranne T, Geyer H. Annual banned-substance review: analytical approaches in human sports drug testing. *Drug Test Anal*. 2018;10(1):9-27. https://doi.org/10.1002/dta.2549
3. World Anti Doping Agency (WADA). *Anti-Doping Testing Figures*. 2017;2017:9. Available at: https://www.wada-ama.org/sites/default/files/resources/files/2017_anti-doping_testing_figures_en_0.pdf
4. Pozo OJ, De Brabanter N, Fabregat A, et al. Current status and bioanalytical challenges in the detection of unknown anabolic androgenic steroids in doping control analysis. *Bioanalysis*. 2013;5:2661-2677. https://doi.org/10.4155/bio.13.242
5. Parr MK, Schänzer W. Detection of the misuse of steroids in doping control. *J Steroid Biochem Mol Biol*. 2010;121(3-5):528-537. https://doi.org/10.1016/j.jsbmb.2009.12.008
6. Abushareeda W, Fragkaki A, Vonaparti A, et al. Advances in the detection of designer steroids in anti-doping. *Bioanalysis*. 2014;6:881-896. https://doi.org/10.4155/bio.14.9

7. Gómez C, Pozo OJ, Marcos J, Segura J, Ventura R. Alternative long-term markers for the detection of methyltestosterone misuse. *Steroids*. 2013;78(1):44-52. https://doi.org/10.1016/j.steroids.2012.10.008
8. Fragkaki AG, Angelis YS, Kiousi P, Georgakopoulos CG, Lyris E. Comparison of sulfo-conjugated and gluco-conjugated urinary metabolites for detection of methenolone misuse in doping control by LC-HRMS. *GC-MS and GC-HRMS. J Mass Spectrom*. 2015;50(5):740-748. https://doi.org/10.1002/jms.3583
9. Balcells G, Matabosch X, Ventura R. Detection of stanozolol O- and N-sulfate metabolites and their evaluation as additional markers in doping control. *Drug Test Anal*. 2017;9(7):1001-1010. https://doi.org/10.1002/dta.2107
10. Kotronoulas A, Marcos J, Segura J, Ventura R, Joglar J, Pozo OJ. Ultra high performance liquid chromatography tandem mass spectrometric detection of glucuronides resistant to enzymatic hydrolysis: implications to doping control analysis. *Anal Chim Acta*. 2015;895:35-44. https://doi.org/10.1016/j.aca.2015.08.043
11. Fabregat A, Pozo OJ, Marcos J, Segura J, Ventura R. Use of LC-MS/-MS for the open detection of steroid metabolites conjugated with glucuronic acid. *Anal Chem*. 2013;85(10):5005-5014. https://doi.org/10.1021/ac4001749
12. Gomes RL, Meredith W, Snape CE, Sephton MA. Conjugated steroids: analytical approaches and applications. *Anal Bioanal Chem*. 2009;393(2):453-458. https://doi.org/10.1007/s00216-008-2451-8
13. Gomez C, Fabregat A, Pozo ÓJ, Marcos J, Segura J, Ventura R. Analytical strategies based on mass spectrometric techniques for the study of steroid metabolism. *TrAC: Trends Anal Chem*. 2014;53:106-116. https://doi.org/10.1016/j.trac.2013.08.010
14. Wang Z, Zhou X, Liu X, Dong Y, Zhang J. A novel HPLC-MRM strategy to discover unknown and long-term metabolites of stanozolol for expanding analytical possibilities in doping-control. *J Chromatogr B Anal Technol Biomed Life Sci*. 2017;1040:250-259. https://doi.org/10.1016/j.jchromb.2016.11.006
15. Balcells G, Pozo OJ, Esquivel A, et al. Screening for anabolic steroids in sports: analytical strategy based on the detection of phase I and phase II intact urinary metabolites by liquid chromatography tandem mass spectrometry. *J Chromatogr A*. 2015;1389:65-75. https://doi.org/10.1016/j.chroma.2015.02.022
16. Hobkirk R. Steroid sulfotransferases and steroid sulfate sulfatases: characteristics and biological roles. *Can J Biochem Cell Biol*. 2009;63:1127-1144. https://doi.org/10.1139/o85-141
17. King CD, Rios GR, Green MD, Tephly TR. UDP-glucuronosyltransferases. *Curr Drug Metab*. 2000;1(2):143-161. https://doi.org/10.2174/1389200003339171
18. Paxton, J.. Topics on drug metabolism. Chapter 2: 35-49. Croatia, Intech 2012. Available at: http://library.umac.mo/ebooks/b28113676.pdf.
19. Kuuranne T. Phase-II metabolism of androgens and its relevance for doping control analysis. *Handb Exp Pharmacol*. 2009;195:65-75. https://doi.org/10.1007/978-3-540-79088-4_3
20. Potts GO, Arnold A, Clinton RO, Manson AJ, Stonner FW, Beyler AL. Steroidal [3,2-c] pyrazoles. *J Am Chem Soc*. 1959;81:1513-1514.
21. Thevis M. Characterization of prohibited substances and doping control analytical assays. In: *Mass Spectrometry in Sports Drug Testing*. Wiley; 2010. https://doi.org/10.1002/9780470626634
22. Masse RDR, Ayotte C, Bi HG. Studies on anabolic steroids. III. Detection and characterization of stanozolol urinary metabolites in humans by gas chromatography-mass spectrometry. *J Chromatogr B Biomed Appl Ther*. 1989;497:17-37. https://doi.org/10.1016/0378-4347(89)80002-7
23. Schänzer MDW, Opfermann G. Metabolism of stanozolol: identification and synthesis of urinary metabolites. *J Steroid Biochem*. 1990;36(1-2):153-174. https://doi.org/10.1016/0022-4731(90)90126-d
24. Pozo OJ, Van Eenoo P, Deventer K, et al. Detection and structural investigation of metabolites of stanozolol in human urine by liquid chromatography tandem mass spectrometry. *Steroids*. 2009;74(10-11):837-852. https://doi.org/10.1016/j.steroids.2009.05.004
25. Schänzer W, Delahaut P, Geyer H, Machnik M, Horning S. Long-term detection and identification of metandienone and stanozolol abuse in athletes by gas chromatography-high-resolution mass spectrometry. *J Chromatogr B Biomed Appl*. 1996;687(1):93-108. https://doi.org/10.1016/s0378-4347(96)00187-9
26. Marques MAS, Pereira HMG, De Aquino Neto FR. Improvements in steroid screening in doping control with special emphasis to GC-MS analytical conditions and method validation. *J Braz Chem Soc*. 2006;17:382-392. https://doi.org/10.1590/S0103-50532006000200024
27. Leinonen A, Kuuranne T, Kotiaho T, Kostiainen R. Screening of free 17-alkyl-substituted anabolic steroids in human urine by liquid chromatography-electrospray ionization tandem mass spectrometry. *Steroids*. 2004;69(2):101-109. https://doi.org/10.1016/j.steroids.2003.10.007
28. Guddat S, Thevis M, Kapron J, Thomas A, Schänzera W. Application of FAIMS to anabolic androgenic steroids in sport drug testing. *Drug Test Anal*. 2009;1(11-12):545-553. https://doi.org/10.1002/dta.73
29. Thevis M, Fußhöller G, Geyer H, et al. Detection of stanozolol and its major metabolites in human urine by liquid chromatography-tandem mass spectrometry. *Chromatographia*. 2006;64(7-8):441-446. https://doi.org/10.1365/s10337-006-0043-3
30. Thevis M, Makarov AA, Horning S, Schänzer W. Mass spectrometry of stanozolol and its analogues using electrospray ionization and collision-induced dissociation with quadrupole-linear ion trap and linear ion trap-orbitrap hybrid mass analyzers. *Rapid Commun Mass Spectrom*. 2005;19(22):3369-3378. https://doi.org/10.1002/rcm.2204
31. Ahi S, Beotra A, Jain S. Detection of mono-hydroxylated metabolites of stanozolol by HPLC-ESI (+) MS/MS in Indian sports persons. *Drug Test Anal*. 2009;1:538-544. https://doi.org/10.1002/dta.76
32. Tudela E, Deventer K, Van Eenoo P. Sensitive detection of 3′-hydroxy-stanozolol glucuronide by liquid chromatography-tandem mass spectrometry. *J Chromatogr A*. 2013;1292:195-200. https://doi.org/10.1016/j.chroma.2013.01.001
33. Schänzer W, Guddat S, Thomas A, Opfermann G, Geyer H, Thevis M. Expanding analytical possibilities concerning the detection of stanozolol misuse by means of high resolution/high accuracy mass spectrometric detection of stanozolol glucuronides in human sports drug testing. *Drug Test Anal*. 2013;5(11-12):810-818. https://doi.org/10.1002/dta.1516
34. Thevis M, Dib J, Thomas A, et al. Complementing the characterization of in vivo generated N-glucuronic acid conjugates of stanozolol by collision cross section computation and analysis. *Drug Test Anal*. 2015;7(11-12):1050-1056. https://doi.org/10.1002/dta.1907
35. Kratena N, Enev V, Gmeiner G, Gärtner P. Synthesis and characterization of stanozolol N-glucuronide metabolites. *Monatshefte für Chemie*. 2019;150(5):843-848. https://doi.org/10.1007/s00706-019-02424-4
36. World Anti Doping Agency (WADA). Urine sample collection guidelines. Int. Stand. Test. Investig. 2014. Available at: https://www.wada-ama.org/en/resources/world-anti-doping-program/guidelines-urine-sample-collection.
37. World Anti Doping Agency (WADA). World Anti-Doping Code - International Standard. 2019. Available at: https://www.wada-ama.org/en/resources/laboratories/international-standard-for-laboratories-isl-2019-newly-approved.
38. World Anti Doping Agency (WADA). Technical document - TD2019MRPL. 2019. Available at: https://www.wada-ama.org/en/resources/science-medicine/td2019mrpl.

39. World Anti Doping Agency (WADA). Technical Document – TD2015IDCR spectrometric confirmation of the identity of analytes WADA – TD2015IDCR. 2015:1–5. Available at: https://www.wada-ama.org/en/resources/science-medicine/td2015idcr.

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Paper II

Stanozolol-*N*-glucuronide metabolites in human urine samples as suitable targets in terms of routine anti-doping analysis

Lorenz Göschl[1,2], Günter Gmeiner[1], Peter Gärtner[3], Georg Stadler[3], Valentin Enev[3], Mario

Thevis[4], Wilhelm Schänzer[4], Sven Guddat[4], Guro Forsdahl[1,2]

1 Doping Control Laboratory, Seibersdorf Labor GmbH, Seibersdorf, Austria

2 Department of Pharmacy, University of Tromsø – The Arctic University of Norway,
Tromsø, Norway

3 Institute of Applied Synthetic Chemistry, Technical University of Vienna, Austria

4 Institute of Biochemistry/Center for Preventive Doping Research, German Sport University Cologne, Cologne, Germany

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RESEARCH ARTICLE

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Stanozolol-N-glucuronide metabolites in human urine samples as suitable targets in terms of routine anti-doping analysis

Lorenz Göschl[1,2] | Günter Gmeiner[1] | Peter Gärtner[3] | Georg Stadler[3] | Valentin Enev[3] | Mario Thevis[4] | Wilhelm Schänzer[4] | Sven Guddat[4] | Guro Forsdahl[1,2]

[1]Doping Control Laboratory, Seibersdorf Labor GmbH, Seibersdorf, Austria

[2]Department of Pharmacy, University of Tromsø—The Arctic University of Norway, Tromsø, Norway

[3]Institute of Applied Synthetic Chemistry, Technical University of Vienna, Austria

[4]Institute of Biochemistry/Center for Preventive Doping Research, German Sport University Cologne, Cologne, Germany

Correspondence
Lorenz Göschl, Doping Control Laboratory, Seibersdorf Labor GmbH, Seibersdorf, Austria.
Email: lorenz.goeschl@seibersdorf-laboratories.at

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Abstract

The exogenous anabolic-androgenic steroid (AAS) stanozolol stays one of the most detected substances in professional sports. Its detection is a fundamental part of doping analysis, and the analysis of this steroid has been intensively investigated for a long time. This contribution to the detection of stanozolol doping describes for the first time the unambiguous proof for the existence of 17-epistanozolol-1′N-glucuronide and 17-epistanozolol-2′N-glucuronide in stanozolol-positive human urine samples due to the access to high-quality reference standards. Examination of excretion study samples shows large detection windows for the phase-II metabolites stanozolol-1′N-glucuronide and 17-epistanozolol-1′N-glucuronide up to 12 days and respectively up to almost 28 days. In addition, we present appropriate validation parameters for the analysis of these metabolites using a fully automatic method online solid-phase extraction (SPE) method already published before. Limits of identification (LOIs) as low as 100 pg/ml and other validation parameters like accuracy, precision, sensitivity, robustness, and linearity are given.

KEYWORDS
anabolic androgenic steroids, glucuronide, high-resolution mass spectrometry, phase-II metabolite, stanozolol

1 | INTRODUCTION

The family of anabolic-androgenic steroids (AAS) belongs to one of the most common illicitly used substance class in the world of professional sports. Within this large group of different drugs, the synthetic steroid stanozolol (17α-methyl-5α-androst-2-eno[3,2-*c*]pyrazol-17β-ol) attributes to the highest number of positive cases according to World Anti-Doping Agencies (WADA) statistics.[1,2] This exogenous steroid is well known analytically and various strategies for its detection are described in the literature. Because this steroid was synthesized in the late 1950s, there was plenty of time to develop many different approaches to analyze stanozolol and its metabolites.[3] In 1986, the team around Donike and Schänzer developed the first method for the analysis of the metabolite 3′-OH-stanozolol applying gas chromatography–mass spectrometry (GC–MS).[4] In the following 35 years, many other techniques, primarily based on mass spectrometric techniques coupled to on either gas (GC–MS) or liquid chromatography (LC–MS), for analyzing a large number of different stanozolol metabolites, were published.[5–20]

In general, the traditional approach for the simultaneous analysis of several different steroids is to perform enzymatic hydrolysis to cleave highly polar phase-II conjugates, like glucuronic acids and

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sulfates, followed by liquid–liquid extraction and the analysis of remaining phase-I metabolites and parent molecules with GC– or LC–MS.[21,22] For the measurement with GC–MS, the analytes are additionally derivatized with N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) to reduce their polarity. This kind of approach is the gold standard nowadays and is commonly performed by anti-doping laboratories worldwide for the routine initial testing procedure (ITP), often including the detection of stanozolol parent or phase-I metabolites.

However, with the emergence of more powerful LC–MS devices, a new, modern way of steroid analysis was developed. With this approach, time- and resource-consuming steps of enzymatic hydrolysis, extraction and derivatization are omitted. Phase-II conjugates of steroids are analyzed directly without further extraction or concentration steps.[15–20,23–29] In 2015, the team around G. Balcells already proposed the analysis of a high number of relevant phase-II metabolites for anti-doping screening purposes.[16] Nowadays, high-resolution (HR) LC–MS devices are frequently used in order to increase sensitivity and selectivity of the measurement. In 2013, Van Eenoo et al. showed the promising potential of this approach for the detection of stanozolol abuse for the first time.[17] The team developed an approach for the direct analysis of 3′-OH-stanozolol glucuronide in human urine.

This idea was adopted by developing a simple but powerful method for the detection of phase-II metabolites of steroids, as previously published. This approach was optimized by placing a fully automated online solid-phase extraction (SPE) procedure upstream of the analytical measurement with LC-HRMS.[18] Next to the aspect of saving time and resources by direct analysis of phase-II conjugates, no enzymatic hydrolysis step using, for example, β-glucuronidase from *Escherichia coli* is required. Consequently, issues like incomplete or inhibited hydrolysis to yield phase-I metabolites, as necessary for GC–MS methods, are no longer relevant. Literature and own experience demonstrates that, for example, stanozolol-N-glucuronides are hardly hydrolyzed with enzymes commonly used in anti-doping laboratories.[19] As a consequence, these metabolites are usually not detected in routine ITP at all.

We have observed that the excretion profile for stanozolol-N-glucuronides is consistent in most positive samples, depending on the drug's application time. Figure 1 shows a typical extracted ion chromatogram (XIC, $m/z = 505.3 \rightarrow 329.3$) for stanozolol-mono-glucuronides of a positive urine sample and the known corresponding metabolite structures, which are based on the metabolically unchanged molecule of stanozolol.

The structures behind Peaks A–C were already suggested by Schänzer et al. in 2013 and Thevis et al. in 2015.[19,20] Peak A represents stanozolol-17′O-glucuronide, and Peaks B and C represent two N-glucuronides of stanozolol. These two metabolites were identified and characterized in our previous work.[18] These two phase-II metabolites were identified as stanozolol-1′N- (B) and stanozolol-2′N-glucuronide (C). Aim of the present study was to use this method for characterization of the two remaining metabolites D and E. Schänzer and Thevis already suggested the appearance of a 17-epistanozolol-glucuronide in above-mentioned studies. However, in both cases, an unambiguous identification was not successful due to the lack of

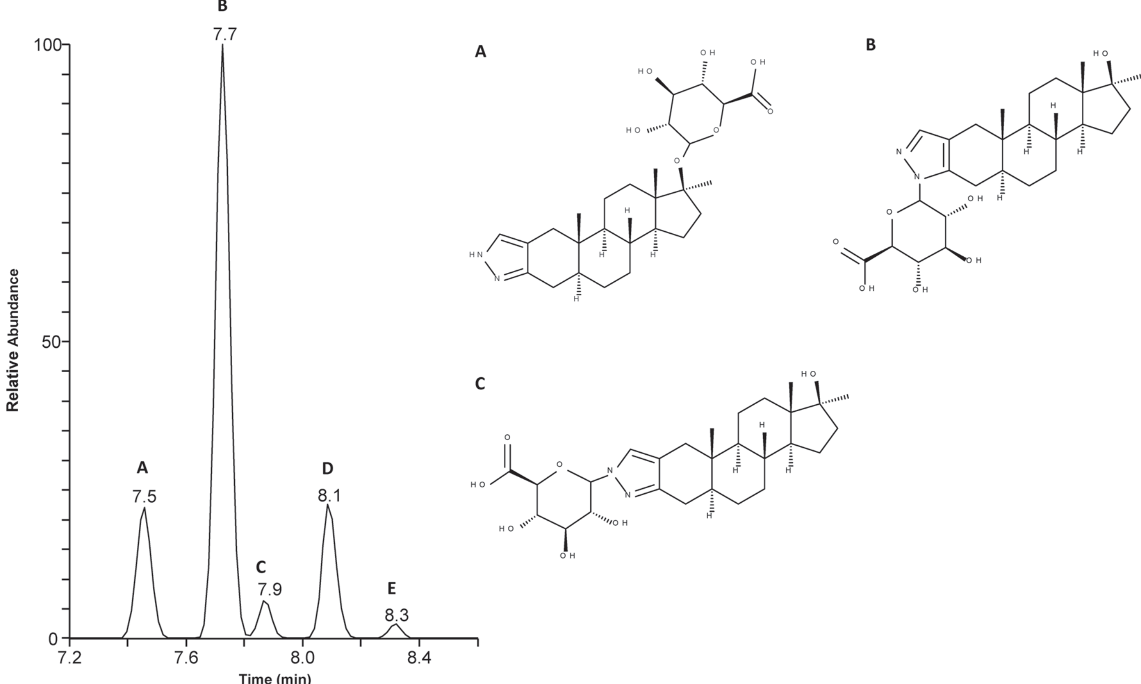


FIGURE 1 Extracted ion chromatogram (XIC) ($m/z = 505.3 \rightarrow 329.3$) of stanozolol-mono-glucuronides and corresponding chemical structures: (a) stanozolol-17′O-glucuronide, (b) stanozolol-1′N-glucuronide, (c) stanozolol-2′N-glucuronide, (d) 17-epistanozolol-1′N-glucuronide, and (e) 17-epistanozolol-2′N-glucuronide (see text below)

high-quality reference material. Furthermore, differentiation of 17-epistanozolol-1′N- and 17-epistanozolol-2′N-glucuronide metabolites was not performed. The structures of these two new metabolites and the parent molecule are shown in Figure 2.

At the Institute of Applied Synthetic Chemistry, Technical University of Vienna, Austria, these two metabolites were synthesized in an amount suitable to confirm their structures with nuclear magnetic resonance (NMR) spectrometry. The detailed description of the synthesis procedure and NMR analysis is still in progress and will soon be published elsewhere. Unambiguous identification and characterization of 17-epistanozolol-1′N- (II) and 17-epistanozolol-2′N-glucuronide (III) is given by using mass spectrometric techniques to compare these reference standards with stanozolol positive human urine samples from excretion experiments or actual athletes. Additionally to the characterization of these two new metabolites, the potential of all four N-associated metabolites for a routine anti-doping analysis of stanozolol is demonstrated. A comprehensive validation and the application of the validated method to an excretion study for stanozolol demonstrates the fitness for purpose of this analytical method as well as the window of detection for stanozolol abuse.

2 | EXPERIMENT

2.1 | Chemicals, reagents, and solutions

Water (high-performance liquid chromatography, HPLC grade) and Methanol (MeOH, HPLC grade) used for HPLC analysis were bought from Biosolve Chimie (Dieuze, France). Formic acid (FA) used for HPLC was purchased from Merck (Darmstadt, Germany). Water (MQ) used for sample dilution was provided by a Milli-Q water purification system (Millipore, Reference A+, Burlington, Massachusetts, USA). Methanol used to prepare standard solutions was supplied by Chem-Lab (Zedelgem, Belgium). The 16,16,17α-d3-testosterone-glucuronide used as the internal standard (IS) was bought from the National Measurement Institute Australia (Sydney, Australia). All stanozolol metabolite standards were synthesized by the team of Peter Gärtner at the Technical University of Vienna and characterized by NMR spectroscopy. Chemical structures are shown in Figure 2.

Stock solutions with a concentration of 1 μg/ml for IS and standard substances were prepared by dissolving 1 μg of standard substance in 1-ml MeOH. Standard working solutions were prepared by diluting stock solutions with MeOH. Until use, solutions were stored at −20°C. Reference samples were prepared by adding working solutions directly to blank urine.

2.2 | Urine samples

According to WADA's collection guidelines, all positive urine samples used in this project were collected by accredited sample collection authorities.[30] The samples have previously been analyzed by the accredited anti-doping laboratory Seibersdorf Labor GmbH. All samples are unanimously confirmed positive for stanozolol. The samples were subsequently anonymized and approved for research. Previously, the athletes gave permission to use the urine samples for research purposes, according to the International Standard for Laboratories (ISL).[31] Samples used for the excretion study were provided by the accredited anti-doping laboratory Cologne, Institute of Biochemistry—German Sport University Cologne, Germany. For these samples, a male healthy volunteer received a single oral dose of 5 mg of stanozolol (Winstrol®). Urine samples were then collected up to 28 days after administration of the substance. A written agreement was received from the participant and the project was accepted by the local ethical committee.[19] The anonymized blank urine samples were provided from healthy female and male volunteers. Until analysis, all urine samples were stored at −20°C.

2.3 | Sample preparation

For sample preparation, 250 μl of urine was diluted with 250 μl of MQ, 15 μl of IS (30 ng/ml) solution was added, followed by vortexing samples for 10 s.

I II III

FIGURE 2 Chemical structures of **I**: Stanozolol, **II**: 17-epistanozolol-1′N-glucuronide, and **III**: 17-epistanozolol-2′N-glucuronide

2.4 | Online SPE coupled to liquid chromatography HR mass spectrometry (online-SPE-LC-HRMS)

An online-SPE-LC-HRMS approach was chosen as analytical method. The method is described in detail in a previous publication.[18] Analytes extraction is carried out fully automatically upstream the injection into the Vanquish Horizon UHPLC+ system (Thermo Fisher, Austin, Texas, USA). An Accucore Phenyl-Hexyl, 10 × 3-mm column with 2.6-μm particle and 80-Å pore size (Fischer Scientific, Loughborough, UK) was used as extraction column. As analytical column, a Kinetex EVO C-18, 100 × 2.1-mm column with 2.6-μm particle- and 100-Å pore size (Phenomenex, Aschaffenburg, Germany) was applied. For chromatography, mobile phases containing water with 0.2% v/v FA (Solvent A) and methanol with 0.1% v/v FA (Solvent B), constant flow of 0.4 ml/min, constant temperature at 25°C, and an injection volume of 25 μl were used. Following gradient was carried out: 10% Solvent B for 2 min to load and wash the pre-column, 10% Solvent B up to 100% over 7 minutes, hold 100% B for 2 min and again 10% B for 2 min to flush and re-equilibrate the system.

HR mass spectrometric measurements were carried out on a Q-Exactive Orbitrap system (Thermo Fisher, Austin, Texas, USA) in positive electrospray ionization mode (ESI+) using the following settings: spray voltage was set to 3.8 kV, and capillary temperature was 320°C. Nitrogen was used as sheath gas (pressure 25 units) and as auxiliary gas (pressure 8 units, temperature 310°C). Sweep gas flow rate was set to 0 and s-lens radio frequency (RF) level was 55. A mass resolution of 70,000 at *m/z* 200 and automatic gain control (AGC) to 2×10^5 ions were carried out.

Parallel reaction monitoring (PRM) was chosen as measuring method. To extract ion chromatograms (XIC), transitions shown in Table 1 with an ion extraction range of 5 ppm were used. Isolation windows were set to ±1 *m/z*. Collision energies (CEs) were optimized by injection of methanolic working solutions of reference substances. Diagnostic ions and corresponding CEs are also shown in Table 1. The software Thermo Xcalibur Qual Browser 4.1.45 was used for data procession and calculation of monoisotopic masses. All systems were supervised with Xcalibur 4.0 (Thermo Fischer).

2.5 | Method validation

Method validation parameters for qualitative and semi-quantitative purposes were used according to the ISL. The following parameters were acquired: specificity, precision, robustness, linearity, accuracy, matrix effects, carryover and limit of identification (LOI). Detailed descriptions of all parameters are given below. Method validation was carried out by using the above described PRM method. Peak areas gained from product ion 1 were used for all semi-quantitative parameters. Concentrations were corrected with the IS and calculated with an internal calibration curve measured in each sequence. Data processing used the software Thermo Xcalibur Quan Browser 4.1.45 and parameters were calculated with Microsoft Excel 2010. The minimum required performance level (MRPL) for free stanozolol is 2 ng/ml, as defined in the WADA Technical Document TD2019MRPL.[32] Therefore, 50% of MRPL, 1 ng/ml, were used for most validation parameters. According to the WADA identification criteria, comparison of retention times and ratios of relative abundances of two ion transitions were used to evaluate the specificity, robustness and LOI.[33] For comparisons, matrix-free (MQ) samples were spiked with reference substances at the respective concentrations.

2.6 | Specificity

Five different female and five different male blank urine samples from healthy volunteers were analyzed ($n = 10$). Furthermore, a second set of these 10 samples were spiked with 1-ng/ml standard working solution. Relative abundances (peak area) of two ion transitions and retention times were compared in order to verify the absence of interferences for both diagnostic ions.

2.6.1 | Precision

Three sets of 10 replicates of blank urine samples were spiked with standard working solution at three different concentrations, low 1 ng/ml, medium 10 ng/ml, and high 50 ng/ml ($n = 3 \times 10$) and were analyzed. Coefficient of variation (CV) of areas (normalized with IS) for intra- and inter-day precision for three concentration levels was calculated by measuring samples on three consecutive days.

2.6.2 | Robustness

Blank urine samples with various specific gravities (0.005, 0.010, 0.020, 0.025, and 0.030) and different pH values (3, 4.5, 6, 7.5, and 9) were spiked with 1-ng/ml standard working solution and were analyzed. Additionally, increasing injection volumes (15, 20, 25, 30, and 35 μl) were tested ($n = 15$). Comparison of retention times and relative abundances of two ion transitions was carried out.

TABLE 1 Mass transitions used for parallel reaction monitoring (PRM) for 17-epistanozolol-1′N- and 17-epistanozolol-2′N-glucuronide (e1N-SG and e2N-SG) and IS d3-testosterone-glucuronide (D3-TG)

Substance	Formula	Precursor ion (*m/z*)	Species	Product ion 1 (*m/z*)/(eV)	Product ion 2 (*m/z*)/(eV)
e1N-SG	C27H40N2O7	505.2908	[M + H]+	329.2587/60	81.0447/70
e2N-SG	C27H40N2O7	505.2908	[M + H]+	329.2587/60	81.0447/70
D3-TG	C25H32D3O8	468.2671	[M + H]+	109.0645/35	97.0651/35

2.6.3 | Linearity

Calibration curves were generated by measuring four replicates of urine samples spiked with standard working solution at six different concentrations (1, 10, 25, 50, 75, and 100 ng/ml, $n = 4 \times 6$). The Software Thermo Quan Browser was used to calculate linearity (R^2).

2.6.4 | Accuracy

Three sets of 10 replicates of blank urine samples were spiked with standard working solution at three different concentrations, low 1 ng/ml, medium 10 ng/ml, and high 50 ng/ml ($n = 3 \times 10$) and were measured. Accuracy (determined concentration/nominal concentration*100%) was calculated.

2.6.5 | Matrix effects

Six different blank urine samples and one matrix-free sample (MQ) were spiked with 1-ng/ml standard working solution and measured. Average matrix effects (ion suppression or enhancement) were calculated by comparing signal area (normalized with IS) of urine samples to the matrix-free sample.

2.6.6 | Carryover

Blank urine sample was spiked with 400-ng/ml standard working solution and measured directly prior to a blank urine sample. The intensity of signal area (normalized with IS) in the blank sample was calculated (%).

2.6.7 | Limit of identification

Three sets of three different blank urine samples were spiked with standard working solution at three concentrations (0.05, 0.075, and 0.1 ng/ml, $n = 3 \times 3$), close to an estimated LOI and were analyzed. According to WADA specifications, LOI was defined as the lowest concentration level at which the analytical signal meets the regulations for relative abundance and retention times. The acronym LOI, used by WADA, is coequal with the more known term limit of detection (LOD).

3 | RESULTS AND DISCUSSION

3.1 | Method validation

The method validation parameters of the 17-epistanozolol-N-glucuronides are quite similar to the values observed for stanozolol-N-glucuronides in our previous work.[18] In Table 2, the

TABLE 2 Summary of validation results for 17-epistanozolol-1′N- and 17-epistanozolol-2′N-glucuronide (e1N-SG and e2N-SG)

	Specificity	Intra-day pr. CV			Inter-day pr. CV			Accuracy			Linearity r^2	Robustness	Carryover	ME (RSD)	LOI
n	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**6 × 4**	**5 + 5 + 5**	**1**	**6**	**3**
c (ng/ml)	**1**	**1**	**10**	**50**	**1**	**10**	**50**	**1**	**10**	**50**	**1–100**	**1**	**400**	**1**	**-**
e1N-SG	10/10	4.8%	3.5%	2.3%	6.7%	7.4%	6.3%	99.6%	91.3%	99.6%	0.999	14/15	0%	177% (11%)	100 pg/ml
e2N-SG	10/10	3.9%	3.1%	1.9%	4.3%	3.2%	2.7%	90.6%	94.2%	102.1%	0.997	15/15	0%	184% (11%)	100 pg/ml

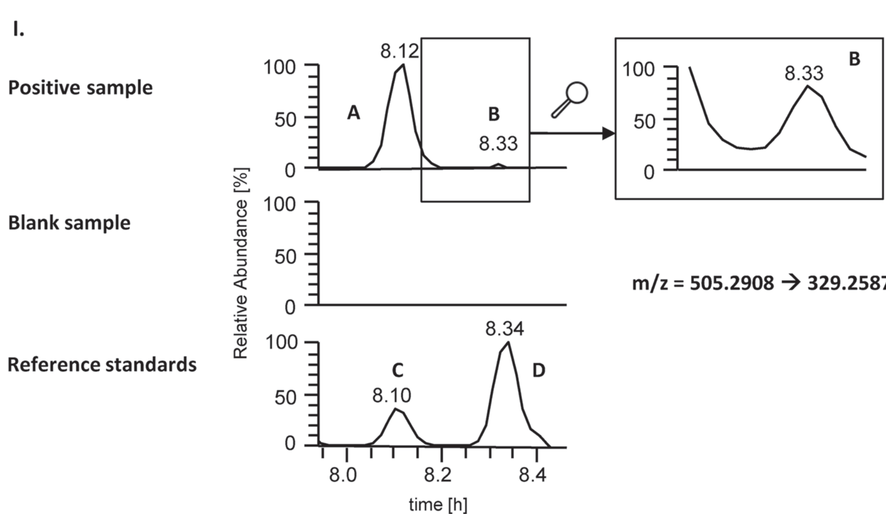
Abbreviations: c, concentration; CV, coefficient of variation; LOI, limit of identification; ME, matrix effects; *n*, number of samples; pr., precision; RSD, relative standard deviation.

determined validation parameter values for 17-epistanozolol-1′N- and 17-epistanozolol-2′N-glucuronide are summarized.

Passing the WADA identification criteria in 10 of 10 samples for both metabolites reflects this method's high specificity. No interfering signals could be observed. Furthermore, suitable intra- (CV 1.9%–4.8%) and inter-day (CV 2.7%–7.4%) precision values and satisfying accuracy parameters (90.6%–102.1%) were achieved. R^2 values (0.999 and 0.997) confirm a linear signal response development with increasing substance concentration for both metabolites. Suitable robustness in 15 of 15 samples was accomplished for the 17-epistanozolol-2′N metabolite. However, for the 17-epistanozolol-1′N metabolite, only 14 of 15 samples passed the identification criteria. The sample with an injection volume of 35 μl could not pass the criteria. In this sample, product ion 2 (*m/z* 81) showed a disproportionately increased abundance compared to product ion 1 (*m/z* 329), leading to a bigger area ratio than a reference sample without matrix and with smaller injection volume. No carryover effect at all was observed after injection of a high concentration sample. Probably due to the lack of comprehensive sample preparation, high matrix effects (177% and 184%) were observed, which, however, do not seem to have a negative influence on precision and accuracy of the method. Nevertheless, for pure quantitative measurements a matching deuterated IS is recommended. Fulfilling WADAs identification criteria, we could detect both 17-epistanozolol-1′N-glucuronide and 17-epistanozolol-2′N-glucuronide at the lowest concentration of 100 pg/ml. By applying alternative criteria for the calculation of the LOI, for example, a signal/noise ratio of >3, the LOIs would be even lower (50 pg/ml). These suitable validation parameters promise a reliable use of this method for the confirmation of stanozolol doping in routine anti-doping analysis.

3.2 | Identification of 17-epistanozolol-N-glucuronides

In order to identify the two metabolites in question, 17-epistanozolol-1′N- and 17-epistanozolol-2′N-glucuronide, HRMS/MS measurements were performed with the above-described PRM method on stanozolol positive urine samples, blank urine samples, and urine samples spiked with reference standards. Extracted ion chromatograms (XIC) with the transition m/z = 505.2908 → 329.2578 are shown in Figure 3-I.



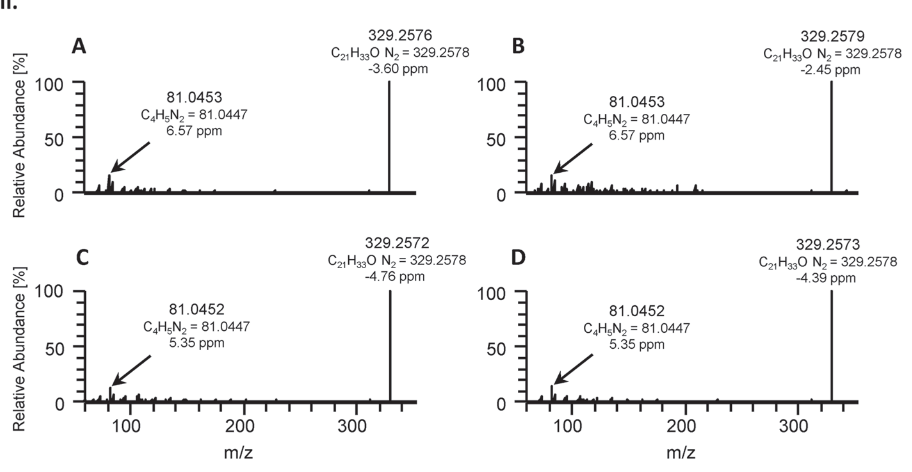


FIGURE 3 Results of parallel reaction monitoring (PRM) measurements; **I**: XIC of positive urine, blank urine and reference standards; *m/z* 505.2908 → 329.2587 (60 eV), ESI+, 5-ppm mass tolerance **II:** Corresponding PRM spectra of 17-epistanozolol-1′N- (a, c) and 17-epistanozolol-2′N-glucuronide (b, d)

The analysis of a number of positive urine samples showed that 17-epistanozolol-2′N-glucuronide (B) is excreted only in significantly lower concentrations, mostly below the detection limit of this method, compared to 17-epistanozolol-1′N-glucuronide (A). In the positive sample shown as an example in this paper, it was possible to provide evidence for the appearance of 17-epistanozolol-2′N-glucuronide (B). In order to visualize the corresponding peak, the chromatogram was expanded at the relevant position. In the blank sample, no signals have been observed. The urine sample spiked with reference standards (2.5 ng/ml) shows excellent signals for both 17-epistanozolol-1′N-glucuronide (C) and 17-epistanozolol-2′N-glucuronide (D). In Figure 3-II, the corresponding PRM mass spectra are shown. All four signals exhibit a highly similar mass spectrometric pattern. Both metabolites form the two stanozolol-glucuronide specific product ions at *m/z* 329 and 81. The product ion at *m/z* 329 is formed by the cleavage of the glucuronic acid and represents the resulting stanozolol aglycone molecule. The product ion at *m/z* 81 is suggested to consist of a robust heterocyclic pyridazine hexagonal ring structure. It is formed by fusing the pyrazole ring with an additional C atom from the sterane backbone during the fragmentation process.[13] In all four cases, the deviation of the theoretical mass from the experimental mass was less than 5 ppm for the ion at *m/z* 329. For the ion at *m/z* 81, the mass deviation is below 7 ppm, explainable by the higher amount of interfering signals in the area of smaller masses.

Comparing retention times and at least two MS/MS transitions of the targeted analyte in a positive sample and a reference sample is required to fulfill WADA identification criteria. The relative abundance of diagnostic ions can be determined from peak areas or heights. In this work, peak areas were used. Table 3 shows the comparative calculations of retention times and abundances, as well as the criteria to be met.

With 0.2% difference for 17-epistanozolol-1′N-glucuronide and 0.1% for 17-epistanozolol-2′N-glucuronide, for both metabolites, the relative differences of retention times were significantly below the maximum tolerance of 1%. Furthermore, the relative area abundances' differences were 0.6% and 0.8%, which is also far below the tolerated 5% aberrance. These data provide the unequivocal proof of the existence of 17-epistanozolol-1′N-glucuronide and 17-epistanozolol-2′N-glucuronide in human urine after ingestion of the exogenous steroid stanozolol.

3.3 | Excretion study

W. Schänzer et al. demonstrated the utility of stanozolol-glucuronides to improve the detection of stanozolol abuse by analyzing excretion study samples in their work in 2013 for the first time.[19] In the following years, further research teams confirmed the usefulness of these metabolites for long-term detection of stanozolol administration in their studies with a higher number of volunteers using oral and intramuscular administration

TABLE 3 Comparison of retention times and relative abundances of two MS/MS transitions for a positive stanozolol sample and reference standards for 17-epistanozolol-1′N- and 17-epistanozolol-2′N-glucuronide (e1N-SG and e2N-SG)

Substance	Retention time IS (min)		Retention time samples (min)		Rel. Difference	Substance	Transition (*m/z*)	Relative abundance		Difference	Maximum tolerance window
	sample	reference	sample	reference	max. tolerated: 1%		→329.2587 (60 eV)	sample	reference		
e1N-SG	7.45	7.45	8.12	8.10	0.2%	e1N-SG	→ 81.0447 (70 eV)	100%	100%	0.6%	10.9%–20.9% (±5)
e2N-SG	7.45	7.45	8.33	8.34	0.1%		→ 329.2587 (60 eV)	15.9%	15.3%		
						e2N-SG	→81.0447 (70 eV)	à 329.2587 (60 eV)	100%	0.8%	9.8%–19.8% (±5)
								14.8%	15.7%		

Abbreviation: IS = internal standard.

of stanozolol.[15,16] However, due to the lack of proper reference substances, in all cases, metabolite elimination data were presented based on relative signal intensities rather than metabolite concentrations. The re-analysis of the same excretion samples used in the work of W. Schänzer provided similar if not equal results including substance concentrations as shown in Figure 4. However, this study focuses only on the analysis of stanozolol-N-glucuronides. The chart shows the concentrations of the four different N-glucuronide metabolites in human urine over time in hours. In order to ensure better comparability of concentrations, values were adjusted for the urine specific gravity according to WADA recommendations and are presented on a logarithmic scale.[34]

These data clearly confirm the large excretion window of 17-epistanozolol-1′N-glucuronide, which is up to almost 4 weeks. Compared to all other known stanozolol metabolites, this metabolite has the largest timeframe for detection.[29] Stanozolol-1′N-glucuronide was detectable up to 12 days. The two 2′N-glucuronides show shorter detection windows up to only 2 days. A major difference in the concentrations of the metabolites can also be observed. At the maximum, stanozolol-1′N-glucuronide is excreted in about 25 times higher concentration compared to 17-epistanozolol-1′N-glucuronide. As already mentioned above, 17-epistanozolol-2′N-glucuronide is only excreted in comparably low concentrations, which is clearly demonstrated in these samples. Almost all data points for this metabolite are below the LOI of the method. Consequently, the concentrations of 17-epistanozolol-2′N-glucuronide below the LOI of 0.1 ng/ml, presented in Figure 4, do not meet the WADA criteria and shall be interpreted as indicative. This metabolite is regarded as of minor importance for the long-term detection of stanozolol doping, but may nevertheless provide information about the time of application of stanozolol, if successfully detected.

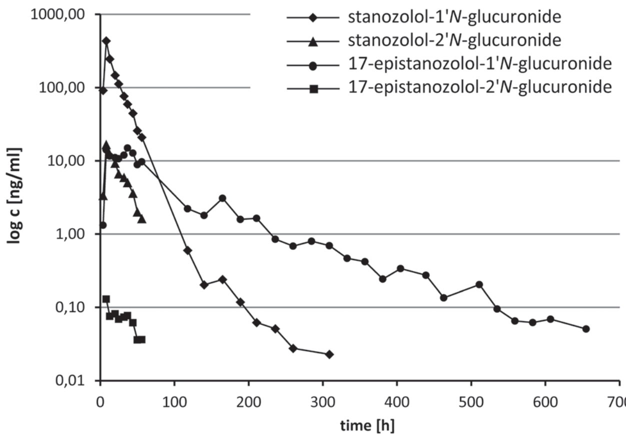


FIGURE 4 Elimination curve of four stanozolol-N-glucuronides monitored in the excretion study samples after oral application of 5 mg of stanozolol; *y* axis: Concentration in nanograms per milliliter on logarithmic scale, *x* axis: Time in hours

4 | CONCLUSION

With the previously developed fully automated SPE-LC-HRMS method, a simple and fast procedure yielding excellent validation parameters for the analysis of 17-epistanozolol-N-glucuronides has been established. Using this method, the presence of 17-epistanozolol-1′N-glucuronide and 17-epistanozolol-2′N-glucuronide in human urine after intake of stanozolol was unequivocally confirmed. Furthermore, due to access to high-quality reference samples, an elimination curve based on the absolute metabolite concentrations of all four stanozolol-N-glucuronides in human urine excretion samples was shown for the first time. The long detection window of up to almost 28 days, the ease of analysis, and the access to synthesized reference standards qualify these metabolites as suitable targets for routine stanozolol analysis.

The fact that these N-glucuronides, some of which exhibit very large detection windows, are resistant to β-glucuronidase means that the long detection time frames of stanozolol are not fully utilized today, because normal ITP relies on the use of β-glucuronidase.

Furthermore, the direct analysis of glucuronide metabolites delivers promising results for many other substances, too. Therefore, consideration should be given to complementing the usual ITP with an approach involving the direct analysis of glucuronide metabolites of doping substances without the use of glucuronidase.

Direct analysis of steroid phase-II metabolites is deemed to bring many advantages to the field of anti-doping analysis. Therefore, the characterization of new unknown metabolites and the subsequent production of reference substances should stay in focus of current research.

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ORCID

Lorenz Göschl https://orcid.org/0000-0002-6018-1961
Günter Gmeiner https://orcid.org/0000-0003-3968-2048
Guro Forsdahl https://orcid.org/0000-0001-9049-9463

REFERENCES

1. World Anti Doping Agency (WADA). Anti-doping testing figures. 2019. Available at: https://www.wada-ama.org/en/resources/laboratories/anti-doping-testing-figures-report. Accessed February 25, 2021.
2. World Anti Doping Agency (WADA). Prohibited list WADA 2021. Available at: https://www.wada-ama.org/en/resources/science-medicine/prohibited-list-documents. Accessed February 25, 2021.
3. Potts GO, Arnold A, Clinton RO, Manson AJ, Stonner FW, Beyler AL. Steroidal [3,2-c] pyrazoles. *J Am Chem Soc*. 1959;81:1513-1514.

4. Thevis M. *Mass Spectrometry in Sports Drug Testing: Characterization of Prohibited Substances and Doping Control Analytical Assays*. Wiley 2010. https://doi.org/10.1002/9780470626634
5. Masse R, Ayotte C, Bi HG. Studies on anabolic steroids. III. Detection and characterization of stanozolol urinary metabolites in humans by gas chromatography-mass spectrometry. *J Chromatogr B Biomed Appl Ther*. 1989;497:17-37. https://doi.org/10.1016/0378-4347(89)80002-7
6. Donike M, Schänzer W, Opfermann G. Metabolism of stanozolol: Identification and synthesis of urinary metabolites. *J Steroid Biochem*. 1990;36(1-2):153-174. https://doi.org/10.1016/0022-4731(90)90126-d
7. Pozo OJ, van Eenoo P, Deventer K, et al. Detection and structural investigation of metabolites of stanozolol in human urine by liquid chromatography tandem mass spectrometry. *Steroids*. 2009;74(10-11):837-852. https://doi.org/10.1016/j.steroids.2009.05.004
8. Schänzer W, Delahaut P, Geyer H, Machnik M, Horning S. Long-term detection and identification of metandienone and stanozolol abuse in athletes by gas chromatography-high-resolution mass spectrometry. *J Chromatogr B Biomed Appl*. 1996;687(1):93-108. https://doi.org/10.1016/s0378-4347(96)00187-9
9. Marques MAS, Pereira HMG, de Aquino Neto FR. Improvements in steroid screening in doping control with special emphasis to GC-MS analytical conditions and method validation. *J Braz Chem Soc*. 2006;17(2):382-392. https://doi.org/10.1590/S0103-50532006000200024
10. Leinonen A, Kuuranne T, Kotiaho T, Kostiainen R. Screening of free 17-alkyl-substituted anabolic steroids in human urine by liquid chromatography-electrospray ionization tandem mass spectrometry. *Steroids*. 2004;69(2):101-109. https://doi.org/10.1016/j.steroids.2003.10.007
11. Guddat S, Thevis M, Kapron J, Thomas A, Schänzera W. Application of FAIMS to anabolic androgenic steroids in sport drug testing. *Drug Test Anal*. 2009;1(11-12):545-553. https://doi.org/10.1002/dta.73
12. Thevis M, Fußhöller G, Geyer H, et al. Detection of stanozolol and its major metabolites in human urine by liquid chromatography-tandem mass spectrometry. *Chromatographia*. 2006;64(7-8):441-446. https://doi.org/10.1365/s10337-006-0043-3
13. Thevis M, Makarov AA, Horning S, Schänzer W. Mass spectrometry of stanozolol and its analogues using electrospray ionization and collision-induced dissociation with quadrupole-linear ion trap and linear ion trap-orbitrap hybrid mass analyzers. *Rapid Commun Mass Spectrom*. 2005;19(22):3369-3378. https://doi.org/10.1002/rcm.2204
14. Ahi S, Beotra A, Jain S. Detection of mono-hydroxylated metabolites of stanozolol by HPLC-ESI (+) MS/MS in Indian sports persons. *Drug Test Anal*. 2009;1(11-12):538-544. https://doi.org/10.1002/dta.76
15. Balcells G, Matabosch X, Ventura R. Detection of stanozolol O- and N-sulfate metabolites and their evaluation as additional markers in doping control. *Drug Test Anal*. 2017;9(7):1001-1010. https://doi.org/10.1002/dta.2107
16. Balcells G, Pozo OJ, Esquivel A, et al. Screening for anabolic steroids in sports: Analytical strategy based on the detection of phase I and phase II intact urinary metabolites by liquid chromatography tandem mass spectrometry. *J Chromatogr a*. 2015;1389:65-75. https://doi.org/10.1016/j.chroma.2015.02.022
17. Tudela E, Deventer K, Van P. Sensitive detection of 3′-hydroxy-stanozolol glucuronide by liquid chromatography-tandem mass spectrometry. *J Chromatogr a*. 2013;1292:195-200. https://doi.org/10.1016/j.chroma.2013.01.001
18. Göschl L, Gmeiner G, Enev V, Kratena N, Gärtner P, Forsdahl G. Development and validation of a simple online-SPE method coupled to high-resolution mass spectrometry for the analysis of stanozolol-N-glucuronides in urine samples. *Drug Test Anal*. 2020;12(8):1031-1040. https://doi.org/10.1002/dta.2805
19. Schänzer W, Guddat S, Thomas A, Opfermann G, Geyer H, Thevis M. Expanding analytical possibilities concerning the detection of stanozolol misuse by means of high resolution/high accuracy mass spectrometric detection of stanozolol glucuronides in human sports drug testing. *Drug Test Anal*. 2013;5(11-12):810-818. https://doi.org/10.1002/dta.1516
20. Thevis M, Dib J, Thomas A, et al. Complementing the characterization of in vivo generated N-glucuronic acid conjugates of stanozolol by collision cross section computation and analysis. *Drug Test Anal*. 2015;7(11-12):1050-1056. https://doi.org/10.1002/dta.1907
21. Parr MK, Schänzer W. Detection of the misuse of steroids in doping control. *J Steroid Biochem Mol Biol*. 2010;121(3-5):528-537. https://doi.org/10.1016/j.jsbmb.2009.12.008
22. Abushareeda W, Fragkaki A, Vonaparti A, et al. Advances in the detection of designer steroids in anti-doping. *Bioanalysis*. 2014;6(6):881-896. https://doi.org/10.4155/bio.14.9
23. Gómez C, Pozo OJ, Marcos J, Segura J, Ventura R. Alternative long-term markers for the detection of methyltestosterone misuse. *Steroids*. 2013;78(1):44-52. https://doi.org/10.1016/j.steroids.2012.10.008
24. Fragkaki AG, Angelis YS, Kiousi P, Georgakopoulos CG, Lyris E. Comparison of sulfo-conjugated and gluco-conjugated urinary metabolites for detection of methenolone misuse in doping control by LC-HRMS, GC-MS and GC-HRMS. *J Mass Spectrom*. 2015;50(5):740-748. https://doi.org/10.1002/jms.3583
25. Kotronoulas A, Marcos J, Segura J, Ventura R, Joglar J, Pozo OJ. Ultra high performance liquid chromatography tandem mass spectrometric detection of glucuronides resistant to enzymatic hydrolysis: Implications to doping control analysis. *Anal Chim Acta*. 2015;895:35-44. https://doi.org/10.1016/j.aca.2015.08.043
26. Fabregat A, Pozo OJ, Marcos J, Segura J, Ventura R. Use of LC-MS/MS for the open detection of steroid metabolites conjugated with glucuronic acid. *Anal Chem*. 2013;85(10):5005-5014. https://doi.org/10.1021/ac4001749
27. Gomes RL, Meredith W, Snape CE, Sephton MA. Conjugated steroids: Analytical approaches and applications. *Anal Bioanal Chem*. 2009;393(2):453-458. https://doi.org/10.1007/s00216-008-2451-8
28. Gomez C, Fabregat A, Pozo ÓJ, Marcos J, Segura J, Ventura R. Analytical strategies based on mass spectrometric techniques for the study of steroid metabolism. *TrAC - Trends Anal Chem*. 2014;53:106-116. https://doi.org/10.1016/j.trac.2013.08.010
29. Wang Z, Zhou X, Liu X, Dong Y, Zhang J. A novel HPLC-MRM strategy to discover unknown and long-term metabolites of stanozolol for expanding analytical possibilities in doping-control. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2017;1040:250-259. https://doi.org/10.1016/j.jchromb.2016.11.006
30. World Anti Doping Agency (WADA). Urine sample collection guidelines. Internations Standards for Testing Investigations. 2014. Available at: https://www.wada-ama.org/sites/default/files/resources/files/wada_guidelines_urine_sample_collection_2014_v1.0_en.pdf. Accessed February 25, 2021.
31. World Anti Doping Agency (WADA). World Anti-Doping Code - International Standard. 2021. Available at: https://www.wada-ama.org/en/what-we-do/the-code. Accessed February 25, 2021.
32. World Anti Doping Agency (WADA). Technical document—TD2019MRPL. 2019. Available at: https://www.wada-ama.org/en/resources/science-medicine/td2019mrpl. Accessed February 25, 2021.
33. World Anti-Doping Agency (WADA). Identification criteria for qualitative assays incorporating column chromatography and mass spectrometry. Technical document - TD2010IDCR. Available at: https://www.wada-ama.org/sites/default/files/resources/files/WADA_TD2010IDCRv1.0_Identification%20Criteria%20for%20Qualitative%20

Assays_May%2008%202010_EN.doc.pdf. Accessed February 25, 2021.

34. World Anti-Doping Agency. Decision limits for the confirmatory quantification of threshold substances. Technical document - TD2019DL. Available at:https://www.wada-ama.org/sites/default/files/resources/files/td2019dl_v2_finalb.pdf. Accessed February 25, 2021.

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Paper III

Detection of DHCMT long-term metabolite glucuronides with LC-MSMS as an alternative approach to conventional GC-MSMS analysis

Lorenz Göschl[1,2], Günter Gmeiner[1], Peter Gärtner[3], Michael Steinacher[3], Guro Forsdahl[1,2]

1 Doping Control Laboratory, Seibersdorf Labor GmbH, Seibersdorf, Austria

2 Department of Pharmacy, University of Tromsø – The Arctic University of Norway, Tromsø, Norway

3 Institute of Applied Synthetic Chemistry, Technical University of Vienna, Vienna, Austria

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Detection of DHCMT long-term metabolite glucuronides with LC-MSMS as an alternative approach to conventional GC-MSMS analysis

Lorenz Göschl[a,b], Günter Gmeiner[a], Peter Gärtner[c], Michael Steinacher[c], Guro Forsdahl[a,b],

a Doping Control Laboratory, Seibersdorf Labor GmbH, Seibersdorf, Austria

b Department of Pharmacy, University of Tromsø – The Arctic University of Norway, Tromsø, Norway

c Institute of Applied Synthetic Chemistry, Technical University of Vienna, Vienna, Austria

Highlights

Analysis of long-term metabolites of oral-turinabol via LC-HRMSMS

Identification of three new phase-II metabolites of dehydrochloromethyltestosterone

Structure determination by fractionation and derivatization experiments

Abstract

Dehydrochloromethyltestosterone (DHCMT) is one of the most detected illicit used anabolic-androgenic steroids in professional sports. Therefore, a fast and accurate analysis of this substance is of great importance for a constructive fight against doping abuse. The conventional method for the analysis of this drug, GC-MSMS, is very sensitive and selective but also very time- and resource-consuming. With the presented work, a new approach for simple detection with LC-HRMSMS without any sample preparation is introduced. The method is based on the direct analysis of two newly described phase-II metabolites of the DHCMT long-term metabolite 4-chloro-18-nor-17β-hydroxymethyl-17α-methyl-5β-androst-13-en-3α-ol (M3). LC-HRMSMS, GC-MSMS, fractionation and derivatization experiments are combined to identify and characterize for the first time two different glucuronide-acid conjugates of this metabolite in positive human urine samples. In addition, a third glucuronide metabolite was identified, however without isomeric structure determination. The detection of these metabolites is particularly interesting for confirmation analyses, as the method is rapid and requires little sample material.

Keywords

Anabolic androgenic steroids, dehydrochloromethyltestosterone, phase-II metabolite, glucuronide, high-resolution mass spectrometry, anti-doping

Introduction

An integral part of professional sports today is regular testing of athletes for doping abuse. Anti-doping measures, as we know them today, began in the early 1960s and have been subject to a constant process of research and improvement ever since[1]. In the field of anti-doping analysis, the long-term detection of prohibited substances, which are defined by the World Anti-Doping Agency (WADA)[2], is one of the most critical issues. The primary sample type in this field is urine, as the sample collection is non-invasive and quick to collect. In this matrix, it is often not the doping substance itself that is detected but its metabolites. Metabolites that are excreted from the human body over a more extended period are called long-term metabolites (LTMs), even though no unequivocal definition for this term exists. The discovery and characterization of new LTMs have always been of high interest in anti-doping research, as previous studies show[3]. The class of anabolic-androgenic steroids (AAS) is particularly focused as it represents the most widely detected family of illicitly used drugs in professional sport[3–5]. The gold standard procedure for routine AAS analysis in doping control is based on enzymatic cleavage to separate parent substances and phase-I metabolites from their phase-II conjugates, followed by liquid-liquid extraction, trimethylsilyl-derivatization and analysis by gas chromatography coupled to tandem mass spectrometry (GC-MSMS)[6]. Only a few steroids can also be measured with liquid chromatography-tandem mass spectrometry (LC-MSMS) directly after extraction due to their higher polarity or capability of ionization at electrospray conditions [7]. However, with the deployment of more powerful LC-MSMS instruments, new approaches for steroid analysis have been developed. Several studies have shown that the direct detection of steroid phase-II conjugates with LC-MSMS is a suitable approach for the detection of steroids[8,9,18–21,10–17]. All of these studies are based on the analysis of the highly polar phase-II metabolites, glucuronide- and sulfate-conjugates[22–25]. The significant advantage of these techniques is that time- and resource-consuming steps of enzymatic hydrolysis and derivatization can be omitted. In many cases, the liquid/liquid extraction is replaced by solid-phase extraction (SPE) or, in some approaches, even the complete sample preparation is skipped. These so-called dilute-and-shoot methods are extremely resource-efficient, but have also disadvantages in terms of sensitivity, specificity and robustness. Another advanced approach combines SPE with direct measurement, so-called online-SPE, where the separation step is fully automated before the LC separation process. This type of method was also used in the presented work.

Dehydrochloromethyltestosterone – DHCMT (4-chloro-17β-hydroxy-17*α*-methylandrosta-1,4-dien-3-on), also known as 4-chlorometandienone or oral-turinabol, is one of the most commonly used illicit anabolic steroids in professional sports according to WADA statistics[4]. Its chemical structure is based on the endogenous steroid testosterone and results from dehydrogenation of positions 1 and 2 and halogenation on position 4. This substance was already used as a doping substance in the early 1970s in East Germany and is still available on the black market today. The detection of DHCMT abuse has an equally long history. The first investigations on DHCMT analysis were published in 1970 by Schubert et al[26,27]. The analysis of the parent compound and three different hydroxyl-metabolites (6β-OH, 16β-OH, 6β,16-di-OH) in human urine samples after application of DHCMT was reported. Subsequently, in 1983, the presence of these metabolites was confirmed, another di-hydroxylated metabolite (6β,12-di-OH) and the epimer of the parent substance (17-epi-DHCMT) were described[28]. Several years later, in 1996, the team around W. Schänzer identified a new metabolite, 4-chloro-3α,6β,17β-trihydroxy-17α-methyl-5β-androst-1-en-16-one, which is detectable up to 14 days after ingestion of DHCMT[29]. As another important step, more and increasingly complex metabolites with detection

windows of up to 22 days were discovered in 2010 [30]. The two newly discovered metabolites 4-chloro-3α,6β,17β-trihydroxy-17α-methyl-5β-androst-1-en-16-one and 4-chloro-18-nor-17β-hydroxymethyl,17α-methylandrosta-1,4,13-trien-3-one, similar to the 18-nor-17-hydroxymethyl metabolite of the well-described steroid metandienone, analyzed with GC-MS/MS, exhibited the largest detection windows. In 2012, based on this knowledge and further investigations, Sobolevsky et al. suggested three structures for new 18-nor-17-hydroxymethyl LTMs, containing a partly or fully reduced A-ring in the steroidal backbone. For the most abundant metabolite, 4-chloro-18-nor-17β-hydroxymethyl-17α-methyl-5β-androst-13-en-3α-ol (M3) and its 17α-epimer, they estimated a detection window of 40-50 days after ingestion of a single dose of 40 mg of DHCMT[31]. However, the correct conformational isomerism of this metabolite was still unknown. In the presented work, we adopted the acronym "M3" for this long-term metabolite. In 2018, Forsdahl et al. analyzed eight different, synthesized isomeric variants of metabolite M3 and compared them with DHCMT positive urine samples[32]. One of these metabolites matched, so the study concluded that the correct structure of the DHCMT long-term metabolite M3 is 4α-chloro-18-nor-17β-hydroxymethyl-17α-methyl-5α-androst-13-en-3α-ol (Figure 1). With the knowledge of the correct structure and access to high-quality synthesized reference standards, the analysis of DHCMT metabolite M3 has become the most crucial tool for the detection of DHCMT abuse. The fact that M3 is detectable for quite a long time and its analysis by GC-MSMS offers high sensitivity and great selectivity makes this approach currently the most widely used technique to expose DHCMT abuse. However, as mentioned above, the analysis by GC-MSMS with enzymatic hydrolysis, liquid-liquid extraction and derivatization is very time and resource consuming.

The goal of our study was to shift the detection of the important DHCMT metabolite M3 from GC-MSMS to LC-MSMS analysis. In 2014, Fernandez-Alvarez M. et al. had already undertaken studies in this direction, but no long-term metabolites were investigated[33]. In the presented work, we aimed to identify usable phase-II metabolites of the DHCMT metabolite M3 with a focus on glucuronide conjugates. Theoretically, there are two possible sites for glucuronide conjugation, the 17-hydroxymethyl- and the 3-hydroxy-group, as shown in Figure 1.

DHCMT-M3-3-glucuronide
Formula: $C_{26}H_{39}O_8Cl$
Exact mass: 514.2334

DHCMT-M3
Formula: $C_{20}H_{31}O_2Cl$
Exact mass: 338.2013

DHCMT-M3-17-methyl-glucuronide
Formula: $C_{26}H_{39}O_8Cl$
Exact mass: 514.2334

Figure 1: Chemical structure, formula and exact atomic mass of the long-term metabolite DHCMT-M3 (m) and its two possible glucuronide conjugates DHCMT-M3-3-glucuronide (l) and DHCMT-M3-17-methyl-glucuronide (r)

If this assumption is true and conjugation takes place at these two sites, two positional isomers DHCMT-M3-3-glucuronide and DHCMT-M3-17-methyl-glucuronide have to be expected. We combined different analytical techniques such as LC-MSMS, GC-MSMS, fractionation and

derivatization experiments to verify the presence of these two DHCTM-M3 glucuronides in positive human urine samples and to tentatively identify the correct sites of the glucuronic acid conjugates.

Experimental

Chemicals, reagents and solutions

Methanol (MeOH) and water used for HPLC analysis (HPLC grade) were purchased from Biosolve Chimie (Dieuze, France). Formic acid (FA) for HPLC, potassium dihydrogen phosphate, disodium hydrogen phosphate dehydrate, potassium hydrogen carbonate and potassium carbonate were bought from Merck (Darmstadt, Germany). Water (MQ) for sample dilution was obtained by a Milli-Q water purification system (Millipore, Reference A+). Trityl chloride, methyl-t-butyl-ether (TBME), ammonium iodide (NH4I), ethanthiol (97%) and dimethylformamide (DMF) were purchased from Sigma Aldrich (St. Louis, Missouri, USA). Triethyl amine (TEA) was purchased from Acros Organics (Fair Lawn, New Jersey, USA). MeOH for standard solutions was supplied by Chem-Lab (Zedelgem, Belgien). β-Glucuronidase (*E. coli*) for enzymatic hydrolysis was supplied by Roche (Mannheim, Germany). N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) was purchased from Macherey-Nagel (Düren, Germany).

For silylation, a derivatization stock solution was prepared by dissolving 200 mg of NH_4I in a mixture of 10 mL MSTFA and 600 µL ethanthiol. A derivatization working solution was prepared by mixing 3 mL of the stock solution with 9 mL of MSTFA directly before sample preparation.

The internal standard (IS) 16,16,17α-d3-testosterone-glucuronide was purchased from the National Measurement Institute Australia (Sydney). IS solution was prepared by dissolving 1 µg standard substance in 1 ml MeOH (1 µg/ml). Solution was stored at -20°C.

Urine samples

All positive urine samples used in this work were collected by accredited sample collection authorities in compliance with WADA's collection guidelines[34]. The samples were received, analyzed and subsequently provided by the WADA accredited anti-doping laboratory Seibersdorf Labor GmbH (Seibersdorf, Austria). Before the analysis, athletes gave permission to use urine samples for research purposes. This is in accordance with the International Standard for Laboratories (ISL)[35]. Additionally, a quality assurance program sample provided by the World Association of Anti-doping Scientists (WAADS) was used. This sample, which contains pooled DHCMT excretion study samples, had already been used in a previous study to confirm the structure of the unconjugated DHCMT metabolite M3[32]. Blank urine samples were collected from healthy female and male volunteers. All urine samples were stored frozen at -20°C until analysis.

Online – SPE-LC-HRMSMS

A previously established online solid-phase extraction (SPE) method that showed excellent results for the analysis of steroid glucuronides was used in the present work[18]. This automated approach required only straightforward sample preparation: 250 µl of urine was diluted with 250 µl MQ and 15 µl IS solution was added. Afterwards, samples were vortexed for 10 seconds.

The measurements were performed on a Vanquish Horizon UHPLC$^+$ System coupled to a Q-Exactive Orbitrap high-resolution mass spectrometer (Thermo Fisher, Austin, Texas, USA). Analytes extraction was carried out fully automatically using an Accucore Phenyl-Hexyl, 10 x 3 mm column with 2.6 µm

particle- and 80 Å pore size (Fischer Scientific, Loughborough, UK) as extraction column. As an analytical column, a Kinetex EVO C-18, 100 x 2.1 mm column with 2.6 µm particle- and 100 Å pore size was used (Phenomenex, Aschaffenburg, Germany). Chromatography was carried out with mobile phase containing water with 0.2% v/v FA (solvent A) and methanol with 0.1% v/v FA (solvent B). The separation was performed with a constant flow of 0.4 ml/min and constant temperature at 25°C. After loading and washing the pre-column with 10% solvent B for 2 minutes, the solvent gradient continues as follows: start with 10% solvent B up to 100% over 7 minutes, hold 100% B for 2 minutes and again 10% B for 2 minutes to flush and re-equilibrate the system. The sample injection volume was 25 µl.

High-resolution mass spectrometry was carried out in positive and negative electrospray ionization mode (ESI+/-) using the following settings. The spray voltage was 3.8kV and the capillary temperature was set to 320°C. Nitrogen was used as sheath gas (pressure 25 units) as well as auxiliary gas (pressure 8 units) and the auxiliary gas heater temperature was set to 310°C. The mass resolution was set to 70 000 at m/z 200 and automatic gain control (AGC) to 2×10^5 ions. Internal calibration with the lock-mass m/z 391.28429 (di-isooctyl phthalate) was used. Full scanning in the range of m/z 300 – 600 and parallel reaction monitoring (PRM) were performed. Collision energies (CE) were optimized to get the most abundant signal intensities. Extracted ion chromatograms (XIC) with an extraction range of 5 ppm and isolation windows of ±1 m/z were generated by choosing the most specific product ions. All systems were controlled with the software Xcalibur 4.0 (Thermo Fischer). Data procession and calculation of monoisotopic masses was performed with the software Xcalibur Qual Browser 4.1.45 (Thermo Fischer).

Sample concentration

In order to increase yields, urine samples were alternatively concentrated using the following protocol. An Oasis HLB, 6 ml, 500 mg cartridge and a vacuum ejector-driven glass chamber were utilized for sample extraction (Waters Corporation, Milford, MA, USA). The cartridge was conditioned with 5 ml MeOH and washed with 5ml MQ. After loading 5 ml of urine, the sample was washed with 2 x 5 ml MQ, dried for 5 min and eluted with 2 ml MeOH. Subsequently, samples were evaporated to dryness using nitrogen stream and reconstituted in 500 µl MQ.

Fractionation

After identifying potential metabolites by LC-HRMSMS, the next step was to confirm whether the signals found actually correspond to the DHCMT metabolite M3. The idea was to fractionate each peak, collect the separated molecules and confirm their structure doing a standard GC-MSMS analysis. However, since the concentration levels of these metabolites are quite low, in the lower ng/ml range, and the peaks to be separated are quite narrow to each other, conventional HPLC fractionation via UV/VIS detection was not possible. In order to solve this problem we used the above described online – SPE-LC-HRMS system and installed a fractionation arrangement positioned between the analytical column and the ESI-source. This system consisted of a simple T-piece and three HPLC capillaries: one leading from the analytical column to the T-piece, one from the T-piece to the ion source and one capillary leading from the T-piece to an open-end used to collect the fractions. Consequently the stream was divided into two flows, one went to the mass spectrometer and the other was used for fractionation. In order to get the same retention times for both measurement and sample collection, both capillary pathways had the same length. With this approach, real-time monitoring of the fractionation procedure was possible. Ten runs of 50 µl injected sample each were performed and three fractions were collected at three different retention time windows, 7.75 – 8.10 min, 8.10 – 8.45

min and 8.45 – 8.80 min. Afterwards, collected and pooled samples were evaporated, dried and subsequently reconstituted in 500 µl MQ. In order to check the successful separation, samples were analyzed with the above described LC-HRMSMS method. Concentration and fractionation were performed twice and the fractions were pooled to obtain the necessary sample volume for the following GC-MSMS analysis and derivatization experiment.

GC-MSMS

GC-MSMS analysis was carried out according to the standard protocol as accredited by WADA[36]. In brief, 500 µl of each fractionated sample, 1 ml of blank urine sample, 1 ml of the original WAADS sample and 1 ml of blank urine spiked with 1 ng/ml DHCMT M3 reference standard were diluted with 1 ml 0.8 M phosphate buffer (pH 7), 25 µl β-glucuronidase and 50 µl IS solution were added and then samples were heated 2 hours at 50°C to perform enzymatic hydrolysis. Subsequently, 1 mL of 20% potassium carbonate buffer (pH 9.0) and 5 mL of MTBE were added to perform a liquid-liquid (l/l) extraction by shaking samples for 10 minutes. After centrifugation for 5 minutes at 2100 rpm, the organic layer was separated by freezing samples in a cooled ethanol bath at -30°C. Subsequently, samples were evaporated to dryness and dried for 15 minutes in a heated vacuum chamber. As the last step of sample preparation, 80 µl derivatization working solution was added and samples were heated for 20 min at 60°C to perform silylation.

GC-MSMS analysis was carried out on a Trace-1300 gas chromatograph coupled to a TSQ-8000 Evo triple quadrupole mass spectrometer and a TriPlus-100 autosampler (Thermo Fisher, Austin, TX, USA) using an optimized method designed for metabolite M3 confirmation purposes. For separation, a RTX-1MS fused silica capillary column, 15 m × 0.25 mm ID, 0.11 µL film thickness (Restek, CP-Analytica, Mistelbach, Austria) was used. Injections with 2 µl volume were performed in splitless mode at 270°C injector temperature. The following temperature program for the GC was carried out: 150°C initial temperature, 25°C/min to 310°C, final temperature held for 2 minutes. High-purity helium with a constant pressure of 90 kPa was used as carrier gas. Transfer line and ion source were heated to 270°C. Electron ionization (EI) mode with electron energy of 70 eV was carried out and data were acquired with selected reaction monitoring (SRM) mode. Following ion transitions were selected for the DHCMT metabolite M3: m/z 379 → 253 (8eV), m/z 381 → 253 (8eV), and m/z 381 → 343 (8eV) and IS d3-testosterone: 435 → 209 (12eV).

Derivatization

The aim of this derivatization experiment was to determine the respective positions of the glucuronic acids on three potential glucuronide metabolites. Two variants of glucuronic acid conjugation, shown in Figure 1, differ in the steric hindrance of the underlying hydroxyl group. The hydroxyl group on position 3 is a secondary alcohol while the 17-hydroxymethyl group is a primary alcohol. The property of trityl chloride to selectively protect primary alcohols in the presence of secondary alcohols was used to distinguish between these two groups[37]. Only the metabolite with the glucuronide conjugation on position 3 is expected to be etherified with trityl chloride (Figure 2), as the primary alcohol on positions 17-methyl remains free, and the metabolite with the 17-methyl glucuronide conjugation is expected to remain unchanged. The reaction should therefore exclusively lead to the formation of DHCMT-M3-3-glucuronide-17-methyl-O-trityl, as illustrated in Figure 2.

Primary alcohol

TEA, DMF
room temperature

DHCMT-M3-3-glucuronide **DHCMT-M3-3-glucuronide-17-methyl-O-trityl**

Secondary alcohol

TEA, DMF
room temperature

DHCMT-M3-17-methyl-glucuronide **DHCMT-M3-17-methyl-glucuronide-3-O-trityl**

Figure 2: Reaction scheme of the derivatization experiment of DHCMT-M3-3-glucuronide and DHCMT-M3-17-methyl-glucuronide with trityl chloride

The derivatization with trityl chloride was performed as follows: chemicals were used without further purification as received from the suppliers. All reactions were performed under argon atmosphere. The tritylation agent was prepared by dissolving trityl chloride (1400 mg, 5 mmol) in 5 ml dry DMF to give a 1 N solution. Triethylamine (0.5 ml, 1.3 eq) was added and the solution stirred for 5 min. Portions of 500 µl of the three sample fractionations and of the concentrated WAADS sample were evaporated and subsequently set under argon atmosphere, 5 ml tritylation agent was added and samples were stirred at room temperature. Samples of 500 µl were taken in regular intervals (up to 6 days reaction time) and quenched by the addition of 250 µl of sat. aq. $NaHCO_3$. After stirring for 1 h, water and DMF were removed under high vacuum to give a brown-yellowish solid residue. These residues were then dissolved in 500 µl MQ by vortexing for 5 min and subsequently centrifuged for 8 minutes at 8000 rpm. The supernatant (≈250µl) was transferred into an LC-vial and 15 µl IS solution was added. The samples were analyzed with the above described LC-HRMS method.

Results and discussion

Identification of M3 glucuronides

The first step of identifying new phase-II metabolites was to analyze several positive urine samples with LC-HRMSMS using different analytical settings. It turned out that for the analysis of DHCMT M3 glucuronides, the negative ionization mode (ESI-) is most sensitive mode to find potential signals. After identifying some promising peaks with negative full-scans analysis, negative PRM experiments were performed. Precursor ion was set to m/z = 513.2255, which correspond to theoretical species [DHCMT-M3-mono-glucuronide – H]$^-$. After optimizing collision energies and chromatographic conditions, XICs (m/z = 513.2255 → 301.2168, 35 eV) as shown in Figure 3A were achieved for most positive samples. In all samples, 1 to 3 prominent peaks (**I-III**) were visible, with varying intensity, probably depending

on the metabolic status. In blank urine samples, no signals at all have been observed by using this mass transition. The WAADS-excretion sample shown in this figure showed the best signals for all three peaks and was therefore used for further investigations.

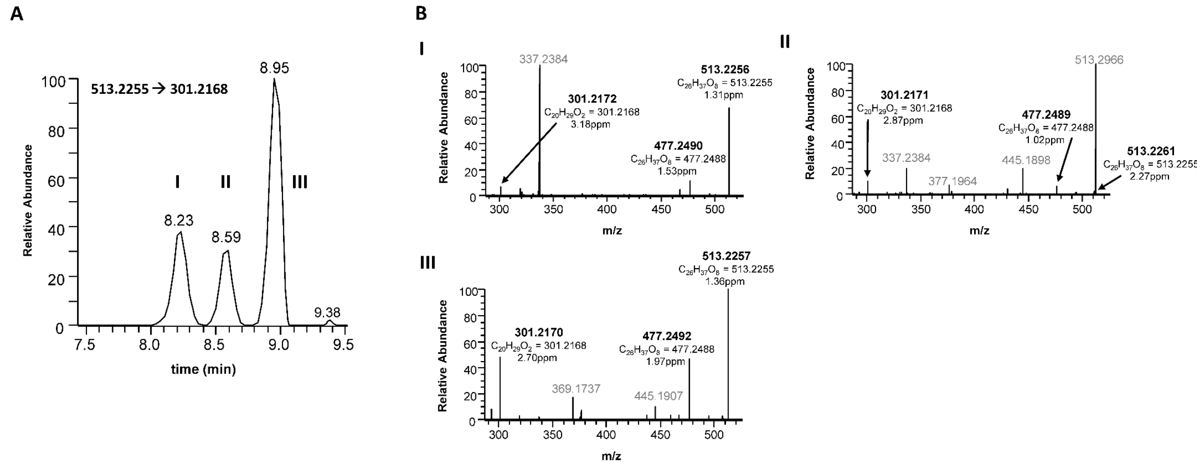
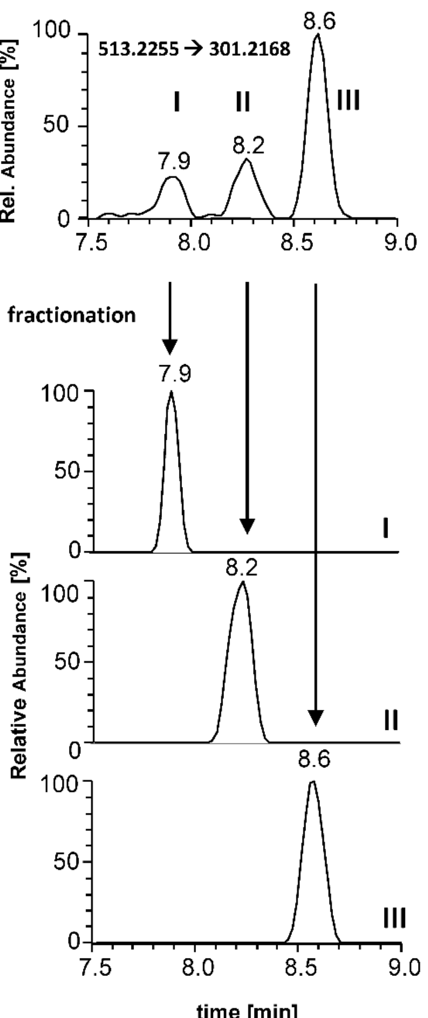


Figure 3: Results of PRM measurements; **A:** XIC of excretion urine sample; m/z 513.2255 -> 301.2168 (35eV), ESI-, 5ppm mass tolerance; **B:** corresponding PRM spectra of 3 potential peaks **I-III**; Two most specific fragment and parent substance signals are highlighted

As shown in 3B, all three peaks yielded different mass spectrometric patterns. Several fragments were formed during the collision-induced dissociation with 35 eV. However, the two most specific product ions, m/z = 477.2488, which is created by the loss of hydrogen chloride (HCl) and m/z = 301.2168, which is formed by the cleavage of the glucuronic acid and the loss of HCl, were generated in all three peaks. The parent molecule with a mass of m/z = 513.2255 was also detectable in all three signals. The deviations between theoretical mass and experimental mass were below 3.2 ppm for both fragments and the parent molecule in all three peaks. These data gave us the first indication of the existence of DHCMT M3 glucuronides and prompted us to proceed with follow-up experiments.

Fractionation



In Figure 4, the results of the separation and collection procedure of the three individual peaks are illustrated. At the top the XIC with transition m/z = 513.2255 → 301.2168 (35eV) of the concentrated excretion urine sample before HPLC separation is shown and below the XICs of the pooled fractionated and reconstituted signals. As can be seen in the figure, all signals were adequately separated. As a positive side effect of this approach, the analytes were additionally purified. By comparing signal intensities a recovery of about 50 % was roughly estimated.

Figure 4: Results of fractionation; XIC m/z 513.2255 -> 301.2168 (35eV), ESI-, 5ppm mass tolerance; **On top:** Concentrated urine sample before fractionation; **Below:** Individual measurements of signals **I-III** after fractionation

Confirmation of M3 glucuronides

In Figure 5, the results of the GC-MSMS analysis of the three fractionation samples, the original WAADS excretion samples and DHCMT M3 reference standard is shown. On the left side the LC-HRMS spectra of the fractionated peaks is illustrated and on the right the corresponding GC-MSMS results for the most abundant transition m/z = 379 → 253 is shown. Below, the original WAADS excretion sample and

a blank urine sample spiked with the standard substance of DHCMT M3 (1 ng/ml) are presented as reference samples.

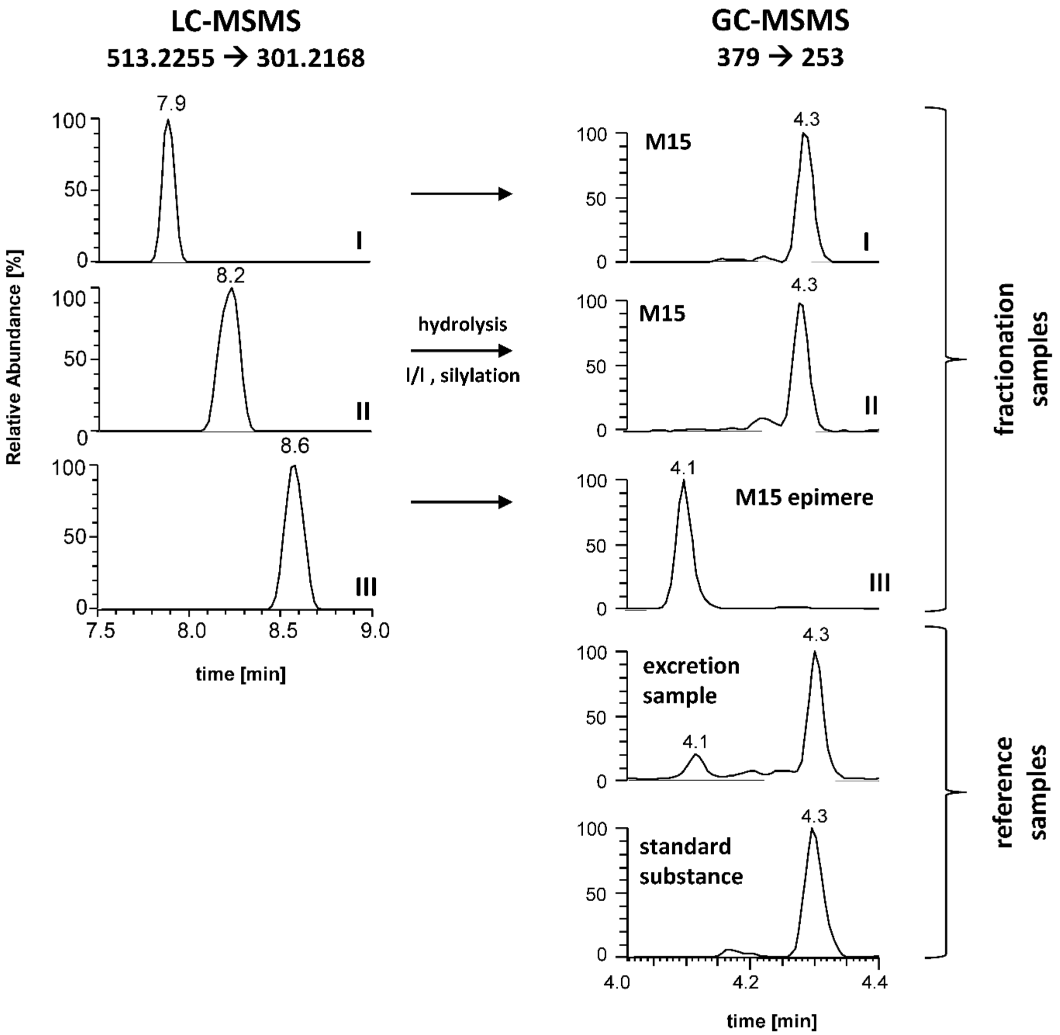


Figure 5: Confirmation of fractionated peaks with GC-MSMS; **Left:** LC-MSMS XIC of fractions **I - III**, m/z 513.2255 -> 301.2168 (35eV), ESI-, 5ppm mass tolerance; **Right:** GC-MSMS SRM chromatogram; On top: Fraction **I – III**, below: positive urine sample and blank urine sample spiked synthesized reference standard (1 ng/ml); m/z 379 → 253 (8eV), EI

All fractions showed signals after enzymatic cleavage of the glucuronic acid. In order to fulfill WADA identification criteria, a comparison of retention times and at least two MS/MS transitions of the targeted analyte in a positive sample and a reference sample is requested[38]. In this work, the relative abundance of three diagnostic ions determined from peak areas was used. Both fraction **I** and **II** showed perfect matching retention times (4.3) with the unconjugated reference standard of DHCMT M3, as illustrated in Figure 5. Transition abundance ratios for three product ions compared with the reference standard are shown in Table 1.

All differences between sample and reference abundance of all fragments for both fractions were quite low and within the maximum tolerance ranges. These data confirm unambiguously that both peak **I** and peak **II** correspond to glucuronic acid conjugates of the DHCMT long-term metabolite M3.

These findings are supported by the fact that M3 has two different hydroxyl sites where conjugation can occur (Figure 1). In order to complete the characterization of peaks **I** and **II**, the position of the glucuronic acid on each of these molecules was determined by the derivatization experiment as discussed below.

Table 1: Comparison of relative abundances of three mass transitions for fraction **I** and **II** with a reference standard for DH*C*MT M3 and relative abundances of fragment **III**; ***** Maximum tolerance windows were calculated according to WADA Technical Document – TD2021IDCR[36]

Substance	**Transitions** [m/z]	**Relative abundance** sample	reference	**Difference**	**Maximum tolerance window***
Fraction I	379 --> 253	100%	100%	0.00%	90 - 110%
	381 --> 253	34.30%	33.50%	0.90%	27.5 - 41.2%
	381 --> 343	10.90%	8.50%	2.50%	5.9 -15.9%
Fraction II	379 --> 253	100%	100%	0.00%	90 - 110%
	381 --> 253	30.00%	33.50%	4.00%	24.0 - 36.0%
	381 --> 343	6.00%	8.50%	2.50%	1.0 -10%
Fraction III	379 --> 253	100%	-	-	-
	381 --> 253	34.10%	-	-	-
	381 --> 343	10.30%	-	-	-

Fraction **III** matches with a different signal with the retention time 4.1 min measured in the WAADS excretion samples. This finding and the very similar fragmentation pattern of **III** compared to metabolite M15 (Table 1) suggest that peak **III** is an isomeric variation of metabolite M15. Peak **III** thus appears to be a glucuronic acid conjugate of an M3-epimer of unknown structure. It is conceivable that it is the 17α-epimer of M3 that Sobolevsky already mentioned in his work from 2012[31]. However, there are no reference materials currently available for this metabolite to confirm this assumption.

Determination of glucuronic acid conjugation sites

To visualize the successful derivatization, the three sample fractions and the concentrated WAADS sample were measured before and after the derivatization reaction with trityl chloride with the LC-HRMSMS method described above. In Figure 6 the results are summarized. Best results were obtained after a reaction time of 48 hours. Again XICs with the transition m/z = 513.2250 → 301.2162 (35eV) are presented before and after the derivatization reaction. On top of Figure 6 the concentrated excretion study sample and below the three fractionated samples are illustrated.

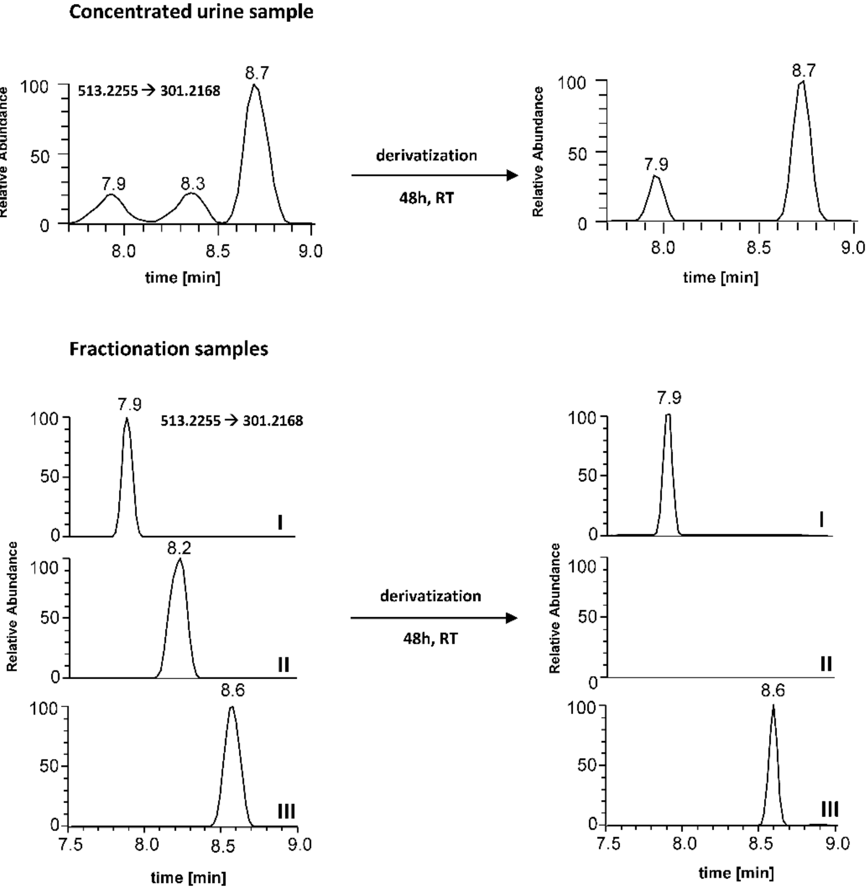


Figure 6: Results of derivatization experiment with trityl chloride; LC-MSMS XIC, m/z 513.2255 -> 301.2168 (35eV), ESI-, 5ppm mass tolerance; **Left:** Chromatograms of the concentrated urine sample and fractionation samples (**I** – **III**) before derivatization; **Right:** Chromatograms of the concentrated urine sample and fractionation samples after derivatization; RT = room temperature

In both cases, peak **II** disappeared entirely after the derivatization. Peak **I** and **III**, on the other hand, remained utterly unharmed. If our assumption is correct, this is a clear sign that selective derivatization of signal **II** has occurred and conversely, **I** and **III** remained unchanged in this reaction. Considering these findings and the theoretical structures of these metabolites allows the conclusion to be drawn that peak **I** represents DHCMT-M3-17-hydroxymethyl-glucuronide and peak **II** represents DHCMT-M3-3-glucuronide (Figure 1). Peak **III** also appears to have a glucuronic acid conjugation at the 17-hydroxymethyl position, but the correct isomerism of the phase-I metabolite is not known.

Conclusion

In the presented work we introduced a new approach for the detection of DHCMT abuse. Instead of the comparably time and resource consuming GC-MSMS approach, a simple previously developed LC-HRMSMS method is applied for the direct analysis of DHCMT phase-II glucuronides. Using a combination of LC-MSMS analysis, fractionation and GC-MSMS analysis, we found strong evidence for the presence of two distinct glucuronide conjugates of the important DHCMT long-term metabolite M3 (4-chloro-18-nor-17β-hydroxymethyl-17α-methyl-5β-androst-13-en-3α-ol) in positive human urine samples. The presented approach for the analysis of these metabolites is particularly interesting

for routine confirmation analysis due to the small sample volume required and the very short analysis time. Even though the established GC-MSMS method still seems to be more sensitive, the approach presented in this work provides satisfying detection limits. Simple dilution experiments showed that detection of the M3-glucuronides up to approximately 100 pg/ml was possible. Nevertheless, a suitable reference sample for comparison is needed for unambiguous detection as long as no synthesized standards are available.

With the derivatization experiment we demonstrated a comparatively simple method for distinguishing between a 3- and a 17-hydroxymethyl-conjugation site of glucuronic acid on a steroid molecule. However, the synthesis of high-quality reference standards of the different DHCMT M3 glucuronides is highly recommended to confirm the presented results.

This work is another step on the path of shifting AAS analysis more and more from GC-MSMS to LC-MSMS, by direct analysis of steroidal phase-II metabolites, leading to more resource and time-saving fight against doping abuse.

Acknowledgement

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References

[1] A. Ljungqvist, Brief History of Anti-Doping, *Med. Sport Sci.* 62 (2017) 1–10. https://doi.org/10.1159/000460680.

[2] World Anti-Doping Agency, Prohibited List Wada 2021. https://www.wada-ama.org/sites/default/files/resources/files/2021list_en.pdf (accessed 16 August 2021).

[3] M. Thevis, T. Kuuranne, H. Geyer, Annual banned-substance review: Analytical approaches in human sports drug testing, *Drug Test. Anal.* 10 (2018) 9–27. https://doi.org/10.1002/dta.2336.

[4] World Anti-Doping Agency, Anti-Doping Testing Figures 2019. https://www.wada-ama.org/sites/default/files/resources/files/2019_anti-doping_testing_figures_en.pdf (accessed 16 August 2021).

[5] O. J. Pozo, N. De Brabanter, A. Fabregat, J. Segura, R. Ventura, P. Van Eenoo, K. Deventer, Current status and bioanalytical challenges in the detection of unknown anabolic androgenic steroids in doping control analysis, *Bioanalysis* 5 (2013) 2661–2677. https://doi.org/10.4155/bio.13.242.

[6] M.K. Parr, W. Schänzer, Detection of the misuse of steroids in doping control, *J. Steroid Biochem. Mol. Biol.* 121 (2010) 528–537. https://doi.org/10.1016/j.jsbmb.2009.12.008.

[7] W. Abushareeda, A. Fragkaki, A. Vonaparti, Y. Angelis, M. Tsivou, K. Saad, S. Kraiem, E. Lyris, M. Alsayrafi, C. Georgakopoulos, Advances in the detection of designer steroids in anti-doping, *Bioanalysis* 6 (2014) 881–896. https://doi.org/10.4155/bio.14.9.

[8] C. Gómez, O.J. Pozo, J. Marcos, J. Segura, R. Ventura, Alternative long-Term markers for the detection of methyltestosterone misuse, *Steroids* 78 (2013) 44–52. https://doi.org/10.1016/j.steroids.2012.10.008.

[9] A.G. Fragkaki, Y.S. Angelis, P. Kiousi, C.G. Georgakopoulos, E. Lyris, Comparison of sulfo-conjugated and gluco-conjugated urinary metabolites for detection of methenolone misuse in doping control by LC-HRMS, GC-MS and GC-HRMS, *J. Mass Spectrom.* 50 (2015) 740–748. https://doi.org/10.1002/jms.3583.

[10] G. Balcells, X. Matabosch, R. Ventura, Detection of stanozolol O- and N-sulfate metabolites and their evaluation as additional markers in doping control, *Drug Test. Anal.* 9 (2017) 1001–1010. https://doi.org/10.1002/dta.2107.

[11] A. Kotronoulas, J. Marcos, J. Segura, R. Ventura, J. Joglar, O.J. Pozo, Ultra high performance liquid chromatography tandem mass spectrometric detection of glucuronides resistant to enzymatic hydrolysis: Implications to doping control analysis, *Anal. Chim. Acta.* 895 (2015) 35–44. https://doi.org/10.1016/j.aca.2015.08.043.

[12] A. Fabregat, O.J. Pozo, J. Marcos, J. Segura, R. Ventura, Use of LC-MS/MS for the open detection of steroid metabolites conjugated with glucuronic acid, *Anal. Chem.* 85 (2013) 5005–5014. https://doi.org/10.1021/ac4001749.

[13] R.L. Gomes, W. Meredith, C.E. Snape, M.A. Sephton, Conjugated steroids: Analytical approaches and applications, *Anal. Bioanal. Chem.* 393 (2009) 453–458. https://doi.org/10.1007/s00216-008-2451-8.

[14] C. Gomez, A. Fabregat, O.J. Pozo, J. Marcos, J. Segura, R. Ventura, Analytical strategies based on mass spectrometric techniques for the study of steroid metabolism, *TrAC - Trends Anal. Chem.* 53 (2014) 106–116. https://doi.org/10.1016/j.trac.2013.08.010.

[15] Z. Wang, X. Zhou, X. Liu, Y. Dong, J. Zhang, A novel HPLC-MRM strategy to discover unknown and long-term metabolites of stanozolol for expanding analytical possibilities in doping-control, *J. Chromatogr. B* 1040 (2017) 250–259. https://doi.org/10.1016/j.jchromb.2016.11.006.

[16] G. Balcells, O.J. Pozo, A. Esquivel, A. Kotronoulas, J. Joglar, J. Segura, R. Ventura, Screening for anabolic steroids in sports: Analytical strategy based on the detection of phase I and phase II intact urinary metabolites by liquid chromatography tandem mass spectrometry, *J. Chromatogr. A* 1389 (2015) 65–75. https://doi.org/10.1016/j.chroma.2015.02.022.

[17] E. Tudela, K. Deventer, P. Van Eenoo, Sensitive detection of 3’-hydroxy-stanozolol glucuronide by liquid chromatography-tandem mass spectrometry, *J. Chromatogr. A* 1292 (2013) 195–200. https://doi.org/10.1016/j.chroma.2013.01.001.

[18] L. Göschl, G. Gmeiner, V. Enev, N. Kratena, P. Gärtner, G. Forsdahl, Development and validation of a simple online-SPE method coupled to high-resolution mass spectrometry for the analysis of stanozolol-N-glucuronides in urine samples, *Drug Test. Anal.* 12 (2020) 1031–1040. https://doi.org/10.1002/dta.2805.

[19] W. Schänzer, S. Guddat, A. Thomas, G. Opfermann, H. Geyer, M. Thevis, Expanding analytical possibilities concerning the detection of stanozolol misuse by means of high resolution/high accuracy mass spectrometric detection of stanozolol glucuronides in human sports drug testing, *Drug Test. Anal.* 5 (2013) 810–818. https://doi.org/10.1002/dta.1516.

[20] M. Thevis, J. Dib, A. Thomas, S. Höppner, A. Lagojda, D. Kuehne, M. Sander, G. Opfermann, W.

Schänzer, Complementing the characterization of in vivo generated N-glucuronic acid conjugates of stanozolol by collision cross section computation and analysis, *Drug Test. Anal.* 7 (2015) 1050–1056. https://doi.org/10.1002/dta.1907.

[21] L. Göschl, G. Gmeiner, P. Gärtner, G. Stadler, V. Enev, M. Thevis, W. Schänzer, S. Guddat, G. Forsdahl, Stanozolol-N-glucuronide metabolites in human urine samples as suitable targets in terms of routine anti-doping analysis, *Drug Test. Anal.* (2021). https://doi.org/10.1002/dta.3109.

[22] R. Hobkirk, Steroid sulfotransferases and steroid sulfate sulfatases: characteristics and biological roles, *Can. J. Biochem. Cell Biol.* 63 (2009) 1127–1144. https://doi.org/10.1139/o85-141.

[23] C.D. King, G.R. Rios, M.D. Green, T.R. Tephly, UDP-Glucuronosyltransferases, *Current Drug Metabolism* 1 (2000) 143–161. DOI: 10.2174/1389200003339171.

[24] J. Paxton, TOPICS ON DRUG METABOLISM Edited by James Paxton, IntechOpen, London United Kingdom, 2012. DOI: 10.5772/1180.

[25] T. Kuuranne, Phase-II Metabolism of Androgens and Its Relevance for Doping Control Analysis, *Handbook of Experimental Pharmacology* 195 (2009) 65-75. https://doi.org/10.1007/978-3-540-79088-4.

[26] Schubert K, Wehrberger K. Metabolism of steroid drugs. II. Isolation and identification of metabolites of 4-chlor-17 alpha-methyl-17 beta- hydroxy-1,4-androstadien-3-one. *Endokrinologie* 55 (1970) 257-269.

[27] Schubert K, Schumann G. Metabolism of steroid drugs. IV. Isolation and identification of a dihydroxylated metabolite of 4-chlorine-17 alpha-methyl-17 beta-hydroxy-1,4-androstadien-3-on. *Endokrinologie* 56 (1970) 172-177.

[28] H.W. Durbeck, I. Buker, B. Scheulen, B. Telin, GC and Capillary Column GC / MS Determination of Synthetic Anabolic Steroids (Oral Turinabol) and Its Metabolites, *J. Chromatogr. Sci.* 21 (1983) 405–410. DOI: 10.1093/chromsci/21.9.405.

[29] S. Horning, G. Opfermann, M. Donike, W. Schänzer, Gas Chromatography/Mass Spectrometry Identification of Long-term Excreted Metabolites of the Anabolic Steroid 4-Chloro-1,2-dehydro-17alpha-methyltestosterone in Humans, *J. Steroid Biochem. Molec. Biol.* 57 (1996) 363–376. DOI: 10.1016/0960-0760(95)00276-6.

[30] M.K. Parr, G. Fußhöller, M. Gütschow, C. Hess, W. Schänzer, GC-MS(/MS) investigations on long-term metabolites of 17-methyl steroids, *Recent Advances in Doping Analysis* 18 (2010) 64–73.

[31] T. Sobolevsky, G. Rodchenkov, Detection and mass spectrometric characterization of novel long-term dehydrochloromethyltestosterone metabolites in human urine, *J. Steroid Biochem. Mol. Biol.* 128 (2012) 121–127. https://doi.org/10.1016/j.jsbmb.2011.11.004.

[32] G. Forsdahl, T. Geisendorfer, L. Göschl, S. Pfeffer, P. Gärtner, M. Thevis, G. Gmeiner, Unambiguous identification and characterization of a long-term human metabolite of dehydrochloromethyltestosterone, *Drug Test. Anal.* 10 (2018) 1244–1250. https://doi.org/10.1002/dta.2385.

[33] M. Fernández-Álvarez, J. Lu, D. Cuervo, Y. Xu, J. Munoz-Guerra, R. Aguilera, Detection of new Oral-Turinabol metabolites by LC-QToF, *Recent Advances in Doping Analysis* 22 (2014) 182–

187.

[34] World Anti-Doping Agency, Guidelines for Sample Collection 2021. https://www.wada-ama.org/sites/default/files/resources/files/isti_sample_collection_guidelines_en_final_2_feb_2021_0.pdf (accessed 16 August 2021).

[35] World Anti-Doping Agency, International Standard for Laboratories 2021. https://www.wada-ama.org/sites/default/files/resources/files/isl_2021.pdf (accessed 16 August 2021).

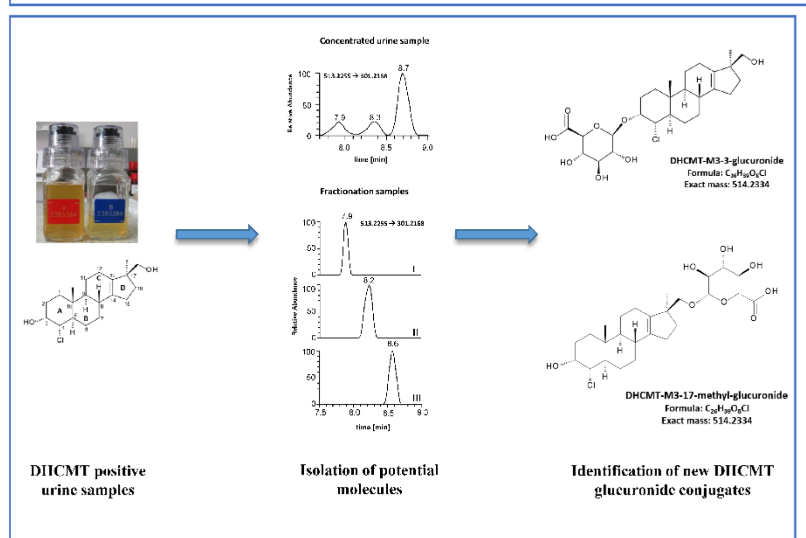
[36] World Anti-Doping Agency, Technical Document - TD2021EAAS. Measurement and Reporting of Endogenous Anabolic Androgenic Steroid (EAAS) Markers of the Urinary Steroid Profile 2021. https://www.wada-ama.org/sites/default/files/resources/files/td2021eaas_final_eng_v_2.0.pdf (accessed 16 August 2021).

[37] J.G. Hendrickson, Determination of Primary Alcohol Groups in Polyglycols Using Triphenylchloromethane, *Anal. Chem.* 36 (1964) 126–128. https://doi.org/10.1021/ac60207a039.

[38] World Anti-Doping Agency, Technical Document – TD2021IDCR MINIMUM CRITERIA FOR CHROMATOGRAPHIC-MASS SPECTROMETRIC CONFIRMATION OF THE IDENTITY OF ANALYTES FOR DOPING CONTROL PURPOSES 2021. https://www.wada-ama.org/sites/default/files/resources/files/td2021idcr_final_eng_0.pdf (accessed 16 August 2021).

Graphical abstract:

Detection of DHCMT long-term metabolite glucuronides with LC-MSMS as an alternative approach to conventional GC-MSMS analysis



Background:
Dehydrochloromethyltestosterone (DHCMT) is one of the most detected illicit used anabolic-androgenic steroids in professional sports. The aim of this work was to implement a new time- and resource-saving LC-MSMS method for the analysis of this drug.

Method:
LC-HRMSMS, GC-MSMS, fractionation and derivatization experiments were combined to find new potential metabolite markers for DHCMT suitable for LC-MSMS analysis, with a focus on phase-II metabolites.

Results:
A new approach for simple detection with LC-MSMS was developed. The method is based on the direct analysis of two newly described phase-II metabolites of the DHCMT long-term metabolite 4-chloro-18-nor-17β-hydroxymethyl-17α-methyl-5β-androst-13-en-3α-ol.