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Screening of Doping Substances in Human Urine with Gas and Liquid Chromatography Coupled to High-Resolution Mass Spectrometry

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Screening of Doping Substances in Human Urine with Gas and Liquid Chromatography Coupled to High- Resolution Mass Spectrometry

PhD thesis

Wadha Abushareeda

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Screening of Doping Substances in Human Urine with Gas and Liquid Chromatography Coupled to High- Resolution Mass Spectrometry

PhD thesis

to obtain the degree of PhD at the
University of Groningen
on the authority of the
Rector Magnificus Prof. C. Wijmenga
and in accordance with
the decision by the College of Deans.

This thesis will be defended in public on

Monday 7 September 2020 at 9.00 hours

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This Thesis is dedicated to my husband

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List of abbreviations

5AAdiol	5A-androstan-3A,17B-diol
5Badiol	5B-androstan-3A,17B-diol
5αDHTS	5α-dihydrotestosterone sulfate
A	Androsterone
AAF	Adverse Analytical Finding
AAS	Anabolic androgenic steroids
ABP	Athlete Biological Passport
ADLQ	Anti-doping laboratory Qatar
AGC	Automatic gain control
APCI	Atmospheric pressure chemical ionization
AR	Androgen receptor
AS	Androsterone sulfate
BALCO	Bay Area Laboratory Co-operative
CRMs	Certified reference materials
D&S	Dilute and shoot
DBS	Dried blot spots
DDR	German Democratic Republic
DHEAS	Dehydroepiandrosterone sulfate
E	Epitestosterone
<i>E. coli</i>	<i>Escherichia coli</i>
EAAS	Endogenous anabolic androgenic steroids
EAAS-S	Sulfo-conjugate endogenous anabolic androgenic steroids
EDR	Extended dynamic range
EI	Electron ionization
EIC	Extracted ion chromatogram
ELISAs	Enzyme-Linked Immunosorbent Assays
ES	Epitestosterone sulfate

ESI	ElectroSpray Ionization
Etio	Etiocholanolone
ETIOS	Etiocholanolone sulfate
FS	Full-scan
FS/HR	Full scan high-resolution
GC	Gas chromatography
GC/MS	Chromatography/Mass Spectrometry
GC/Q-Orbitrap	Gas Chromatography quadrupole Orbitrap technology
GC/QQQ	GC triple quadrupole technology
GC/Q-TOF	Gas Chromatography quadrupole time-of-flight
HPLC	High Performance Liquid Chromatography
HR	High resolution
IAAF	International Association Athletics Federation
IC	Identification Capability
IFPMA	The International Federation of Pharmaceutical Manufacturers & Associations
IOC	International Olympic Committee
IRMS	Isotope Ratio Mass Spectrometry
ISL	International Standard for Laboratories
ISTD	Internal standards
ITP	Initial testing procedure
LC	Liquid chromatography
LC/HRMS	LC high-resolution MS
LC/MS	Liquid Chromatography/Mass Spectrometry
LOD	Limit of Detection
LOQ	Limit of Quantification
LTM	Long-term metabolites
m/z	Mass-to-charge ratio
MRM	Multiple Reaction Monitoring
MRPL	Minimum Required Performance Levels
MS	Mass spectrometry
MSD	Mass Selective Detector

MSTFA	N-methyl-N-(trimethylsilyl) trifluoroacetamide
MU	Measurement uncertainty
NADOs	National Anti-doping Organizations
NARL	National Analytical Reference Laboratory
PFTBA	Perfluorotributylamine
QC	Quality control
QQQ	Triple quadrupole analyser
RBA	Relative binding affinity
RMs	Certify appropriate reference materials
RT	Retention time
SIM	Selected Ion Monitoring
SP	Steroid profile
SPE	Solid Phase Extraction
T	Testosterone
TD	Technical document
THG	Tetrahydrogestrinone
TMS	Trimethylsilyl
TOF	Time- of- flight
TS	Testosterone sulfate
UGT	Uridine diphosphoglucuronosyl-transferase
USADA	United States Anti-doping Agency
WAADS	World Association of Anti-Doping Scientists
WADA	World Anti-doping Agency
WPL	World Anti-doping Agency Prohibited List

Chapter 1

General Introduction and Outline of The Thesis

1.1 General Introduction

Enhancing athletic performance by administrating substances is forbidden in human sports. The global fight against doping in sports is supervised by the World Anti-Doping Agency (WADA). WADA was founded in 1999 as an organization supported by sport federations and governmental organizations worldwide to fight against doping in sports. In 2004, WADA published its first list of prohibited substances and methods [1]. The WADA Prohibited List (WPL) includes substances and methods that are prohibited as doping according to the WADA codes [2] due to their potential of enhancing performance or masking drug abuse in sports. The substances and methods that are prohibited by WADA can be divided in different groups or classes. Some of them are prohibited continuously (in- and out-of-competition), for example, anabolic agents, hormones, β -2-agonists, diuretics, etc., while others are prohibited only in competition, such as stimulants, narcotics, cannabinoids and glucocorticosteroids. Other substances, such as β -blockers, are prohibited only in particular sports. The WPL comprises forbidden pharmacological effects and respective drug categories; therefore, it does not consist of an exhaustive list of prohibited compounds. This is reflected by the addition of the following phrase to the WPL: “and other substances with a similar chemical structure or similar biological effect(s)” [1]. The meaning of this phrase is that prohibited substances are not only those referred explicitly in the WPL as examples but also any other molecule, known, secreted or designed in the future, legally marketed or not, with or without clinical studies, having the same pharmacological effect.

Currently, LC/MS and GC/MS screening by WADA accredited laboratories is applied for the detection of small molecules included and defined in the WPL. For LC/MS, the laboratories use LC triple quadrupole MS (LC/QQQ) [3-5] or high- resolution LC MS (HR-LC/MS) equipped with Orbitrap or TOF mass analyzers [6,7]. On the other hand, regarding GC/MS screening, only low-resolution GC triple quadrupole (GC/QQQ) technology is used currently in routine analysis [8,9]. The detection and reporting of prohibited substances are based on specific criteria described in the WADA Technical Document for Identification Criteria for Qualitative Assays [10]. According to this document, report a violation of the WPL, laboratories must detect compounds by strictly matching the chromatographic retention times and m/z ranges specific for the compounds of interest, both in the athlete’s sample and in a sample prepared from an excretion study or a

synthetic reference material, which are analyzed simultaneously. The reporting of a prohibited substance included in the WPL in an anti-doping sample is impossible if the reference materials are not available. As a result, there is a motivation by illegal laboratories for the design and production of new molecules unknown to the anti-doping community called designer drugs. Designer drugs are structurally modified analogues or derivatives of known substances, which were never approved for human use in the past or were never produced by pharmaceutical companies, and thus, these drugs may lack toxicological and clinical studies. They are used by cheating athletes to avoid detection by WADA laboratories. Designer drugs induce less, similar or better pharmacological effects and can be purchased on the Internet as nutritional supplements. Another motivation of illegal laboratories to produce designer drugs is to avoid legislative limitations imposed on known molecules because of public health issues and to avoid costly toxicological, pharmacological and clinical assessment.

AAS is a group of natural and synthetic compounds that are chemically similar and mimic the action of endogenous testosterone in term of anabolic activity and that may possess an enhanced activity. In addition to their medical use, AAS is the most misused class of drugs in sports today [11] by a wide variety of athletes in the hope of improving their training endurance performance. AAS abuse has also become increasingly prevalent outside of sports. The misuse of AAS in sports has led to ban their use for sport performance enhancement by the International Olympic Committee (IOC) since 1974 and by the WADA since 2003. WADA encourages antidoping laboratories to develop screening methods that combine the detection of AAS in urine with other pharmacological drug classes. Detecting AAS is considered a challenge due to the presence of numerous different endogenous, natural and synthetic steroids with similar chemical structures and their extensive metabolism in the body, which results in low concentrations of diverse urine metabolites [12].

As a result of analytical technological development, especially mass spectrometry and chromatographic separation, the improvement of knowledge and increasing the understanding of drug and endogenous compound metabolism, new slowly excreted metabolites of known synthetic anabolic androgenic steroids (AAS) have been discovered; compared to the previously known metabolites, these long-term metabolites (LTMs) prolong the detection of steroid abuse after discontinuing steroids administration. The first LTM discovered was of the widely used AAS

methandienone [13]. The same metabolic pathway provided LTM for oxandrolone [14], dehydrochloromethyltestosterone [15], desoxymethyltestosterone [16], and oxymetholone [16]. Accordingly, when new pharmaceutical and metabolic knowledge is available, the retesting of previously collected and stored negative samples is considered as an important resource for anti-doping screening. However, retesting of stored samples creates a logistics problem due to limitations in the available sample volume, the human, reagent and instrumental resources needed for sample handling, preparation and reanalysis in WADA accredited laboratories [17,18]. The advantage of having a modern mass spectrometer equipped with a high-resolution full scan (FS/HR) acquisition mode and fast scanning speed allows the re-processing of already acquired data files without the need to re-test samples, which saves the urine sample volume and saves laboratory resources. Nevertheless, fast re-processing of thousands of data files is also challenging due to the large size of high-resolution LC/GC/MS data files, which involve the use of high-performance storage and computing infrastructure. Moreover, the manual processing of thousands of data files with currently used manual processing procedures requires extensive human resources to generate and manually evaluate extracted ion chromatograms. A solution to alleviate the data processing bottleneck is to use algorithms such as those implemented in MetAlign software, which reduce the dataset by removing noise and keeping the signals related to compounds detected by the LC/GC/MS platform [19-21].

Endogenous anabolic androgenic steroids (EAAS) are precursors or metabolites of naturally occurring steroid such as testosterone (T), which is considered as the most important androgenic steroid. The use of synthetic forms of EAAS is one of the most serious violations of anti-doping rules in sports, and the most challenging to detect. EAAS are misused by athletes to avoid their detection in urine samples, because it is difficult to distinguish between the endogenously produced and the externally administered T by conventional mass spectrometry. However, the intake of endogenous steroids alters the concentration of one or more components of the urinary steroid profile (SP) [22]. The present SP evaluation is personalized for each athlete and uses athletes' population reference ranges by using a Bayesian statistical model developed by Sottas [23-24]. This statistical model is based on the urine SP of each athlete collected over a long time period and is registered in the Athlete Biological Passport (ABP) [25,26]. Currently, the steroidal module of the ABP contains the total concentration of the free and glucuronide conjugated forms of the following urinary EAAS: T, epitestosterone (E), androsterone (A), etiocholanolone (Etio)

5A-androstan-3A,17B-diol (5AAAdiol), and 5B-androstan-3A,17B-diol (5BAAdiol), as well as the ratios T/E, A/Etio, 5AAAdiol/5BAAdiol, A/T, 5AAAdiol/E. All the previously mentioned EAAS are analyzed by GC/MS [22]. The steroidal ABP data of all the athlete' samples, over the entire careers, are introduced in the statistical model, and deviations from the population and intra-athlete accepted ranges are estimated. The monitoring of sulfate steroid conjugates in addition to glucuronide conjugates as biomarkers could be an important complement and improvement for the ABP, as proposed by several studies [25-32].

A combination of LC and GC systems with FS/HR-MS acquisition is an ideal combination for the practical high-throughput detection and identification of any small molecule that can be extracted from urine with the simultaneous determination of the steroid profile according to the WADA Code specifications and detection of intact sulfo-conjugated metabolites of phase II metabolism. Sulfo-conjugated AAS metabolites are considered an important class of compound for the detection of exogenous AAS abuse and an important additional class of biomarkers that should be added to the steroidal ABP to improve antidoping screening efficiency of the steroidal ABP. In addition, the joint application of FS/HR GC/MS and LC/MS platforms should allow the retrospective detection of designer drugs currently unknown to WADA [33,34] and the detection of new metabolites of exogenous AAS, which following their discovery, could prolong the detection of steroid abuse, even after their use is discontinued by cheating athletes. FS/HR GC/MS and LC/MS platforms will therefore contribute to identifying cheating athletes, which have so far been undetected due to the use of targeted analysis and the processing of collected low resolution data GC/MS and LC/MS data.

My PhD dissertation aims to develop novel antidoping analytical approaches to screen for prohibited substances using FS/HR LC/MS and GC/MS instruments, including WADA steroidal ABP parameters, and to develop and assess a GC/MS and LC/MS pre-processing method using MetAlign for the retrospective reprocessing of acquired data [19]. The FS/HR LC/MS analytical approach developed in this thesis consists of amongst others a generic sample preparation and MS acquisition, endogenous steroid profile quantitation of intact phase II sulfate metabolites, which methods may contribute to the improvement of the WADA steroidal ABP biomarkers repertoire. The methods developed within this PhD Thesis will provide novel analytical tools for WADA to identify and monitor doping activities of athletes and will contribute to improve the fairness of sport competitions and athlete wellbeing.

1.2 Thesis Outline

This Thesis has the following outline:

Chapter 1 (current chapter) is a general introduction to the scope of the Thesis.

Chapter 2 provides a literature review on the natural forms of AAS and their already known structural modifications used to create new designer molecules. The review also discusses the progress in the detection of designer AAS using mass spectrometry and bioassays; it presents how already collected LC/MS data can be re-processed to identify unknown designer AAS and how these designer AAS are synthesized. Finally, this chapter discusses the regulations of sport's authorities as preventive measures aimed for the long-term storage of samples and potential re-processing of the acquired digital LC/MS data initially reported as negatives to identify retrospectively designer prohibited substances not known at the time of the measurement.

Chapter 3 presents the development and validation of a quantitative and qualitative detection method for the sulfo-conjugated endogenous steroids, the same analytical method used for the detection of the rest of prohibited small molecules substances, according to the WADA Technical Documents specifications. The presented method is aimed to expand the WADA ABP profile with this new class of endogenous steroids. Currently, only the gluco-conjugated endogenous steroids are included in the ABP. This method allows the simultaneous measurement of the SP according to WADA Code specifications and detection of intact sulfo-conjugated metabolites of phase II metabolism by using one measurement with FS/HR -GC and another one with FS/HR-LC platforms. Orbitrap LC/MS operated in FS/HR with polarity switching allows for the simultaneous detection of synthetic doping compounds as well endogenous AAS and their metabolites with a high sensitivity and specificity.

Chapter 4 presents the development and validation of a quantitative and qualitative GC/MS profiling method by using FS/HR acquisition of small molecules. This chapter discusses the use of FS/HR gas chromatographic quadrupole Time-of-Flight mass spectrometry (GC/Q-TOF) to identify compounds included in the WPL not analyzed by LC/MS and profiling of endogenous steroids included in the current form of the WADA ABP. The presented method is developed as a complement for the currently used Orbitrap LC/MS screening method, which is described in **Chapter 3**.

Chapter 5 compares the performance of the GC/Q-TOF and GC/Q-Orbitrap methods to detect and quantify exogenous and endogenous AAS in the same quality control urine samples. The study includes a limited number of exogenous AAS metabolites and the endogenous AAS of the steroidal ABP. The data proved that both platforms are fit-for-purpose for antidoping screening. The mass accuracies are excellent in both instruments, but the GC/Q-Orbitrap is superior to GC/Q-TOF, because the resolution of GC/Q-Orbitrap is higher than that of GC/Q-TOF

Chapter 6 presents the performance of the MetAlign software for data reduction and searching processing to accurately identify compounds by retroactive reprocessing existing HR/FS LC/MS data acquired during routine antidoping screening. MetAlign/HR_MS_Search software is an LC/MS data pre-processing tool, that can be used for the retroactive reprocessing of already measured data of antidoping samples acquired in HR/FS mode using Orbitrap LC/MS. The compound identification performance was evaluated using false negatives and false positives as the main criteria. This chapter examines the speed and the ease-of-use of the FS/HR -LC/MS data search module and demonstrates how to minimize the initial testing procedure (ITP) of false negatives and false positives using automatic compound identification. Practically, this chapter discusses the performance and limitations of MetAlign as a retroactive re-processing tool to identify doping cases in the ITP and to be used routinely in antidoping activity of WADA.

Chapter 7 describes a pilot comparative study, as a continuation of **Chapter 5**, and assesses the performance and efficiency of the MetAlign software to reduce the large volume of HR/FS GC/MS datafiles and search the m/z of target compounds in the data generated in **Chapter 5**. The assessment was performed using the same parameter used for the MetAlign performance, as described in **Chapter 6**, and using data acquired by GC/MS TOF and Orbitrap mass analyzers, as presented in **Chapter 5**. This chapter concludes that MetAlign software is appropriate for FS/HR GC/MS data reduction and searching for the presence or absence of a particular compound, with an efficiency similar to that of LC/MS. However, extended validation data must be collected before the method can be considered validated for the automatic detection of doping compounds.

Finally, **Chapter 8** summarizes the research work described in this PhD Thesis on the potential application of FS/HR-GC/MS and FS/HR-LC/MS platforms and the use of MetAlign pre-processing software to identify known and unknown doping substances for the antidoping screening of urine samples. Moreover, this chapter summarizes and provides a future outlook for

the use of the sulfo-conjugated endogenous AAS LC/MS profiles for antidoping screening and the potential application of sulfo-conjugated endogenous AAS as additional biomarkers to be included in the steroidal ABP module.

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Chapter 2

Advances in the Detection of Designer Steroids in the Anti-Doping

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Abstract

The abuse of unknown designer androgenic anabolic steroid (AAS) molecules is considered to be a problem of significant importance, as the AAS consist of the doping mean of preference according to the World Anti-doping Agency (WADA) statistics and since the WADA mass spectrometric identification criteria cannot be applied to unknown molecules. Consequently, cheating athletes have a strong motivation to use designer AAS to achieve performance enhancement and to escape from testing being positive in anti-doping tests. To face the problem, a synergy is required between the antidoping analytical science and the sports antidoping regulations. This review examines various aspects of the designer AAS. First, the structural modifications of the already known AAS to create new designer molecules are examined, which is followed by the discussion of the current WPL of designer synthetic and endogenous AAS. Second, the progress in the detection of designer AAS using (i) mass spectrometry and bioassays, (ii) analytical data processing of the unknown designer AAS, (iii) metabolite synthesis as well as (iv) long-term storage of urine and blood samples are described. Finally, the introduction of regulations from sports authorities as preventive measures for long-term storage and reprocessing of samples, initially reported as negatives, is discussed.

2.1 Introduction

The World Anti-Doping Agency (WADA) [1], which is considered to be the accepted organization by sports and governmental organizations worldwide to combat doping in sports, revises and publishes at least once per year the “WADA Prohibited List” (WPL) as an International Standard [2]. The WPL identifies substances and methods that are - according to the WADA Code [3] - prohibited as doping, because of their potential of either enhancing performance or masking drug abuse. The substances of the WPL are claimed to induce pharmacological effects on the cell, the tissues and the organism. Anabolic Agents constitute Class S1 of the WPL and they comprise the following drug categories with anabolic action: exogenous (synthetic) and endogenous anabolic androgenic steroids (AAS), as well as other anabolic agents such as selective androgen receptor modulators (SARMs). Examples of drugs and medicines that fall under the Class S1 are the synthetic AAS stanozolol, methandienone, oxandrolone, tetrahydrogestrinone, oral turinabol, SARMs, zeranol etc. However, drug interaction with cells to induce a certain pharmacological effect can be achieved by several structural features of the drug molecule, which practically creates an unlimited combination of the molecular features that could provide the particular performance enhancing effect. Since the WPL comprises prohibited pharmacological effects and respective drug categories, it is not possible to be exhaustive, hence, the following phrase has been added: “and other substances with a similar chemical structure or similar biological effect(s)” [2]. The meaning of the last phrase is that prohibited substances are not only those referred to as examples in the WPL, but also any other molecule, known, secreted or designed in the future, legally marketed or not, with or without clinical studies, having the same pharmacological effect.

The WADA Accredited Laboratories [4] perform the analysis mainly in urine samples, detecting small drugs contained in the WPL by using explicitly mass spectrometry (MS), either coupled to gas (GC) or liquid chromatography (LC). Detection and reporting of prohibited substances is based on specific criteria described in the WADA Technical Document for Identification Criteria for Qualitative Assays [5]. According to this document, to report for a violation of the WPL, antidoping laboratories must match in strict ranges chromatographic retention times and abundances of ions specific for the compounds of interest, both in the athlete’s sample and in a sample originating from an excretion study or a synthetic reference material analyzed contemporaneously. Without the existence of the reference material, the reporting of a prohibited

substance of the WPL in an anti-doping sample is impossible. As a result, there is a motive for the unethical scientists to create new molecules unknown to the anti-doping community, the designer drugs. The designer drugs are structurally modified analogues or derivatives of known substances, which were never approved for human use in the past or never made it to production by pharmaceutical companies. They are used by cheating athletes to avoid detection by the accredited WADA laboratories. Designer drugs induce less, similar or better pharmacological effect and usually circulate in the market without following formal regulations (labelling, approval, clinical studies) or via Internet as food supplements. Another motive for illegal laboratories to produce designer drugs is to avoid legislative limitations imposed to known molecules because of public health issues.

The current review presents several aspects of the designer AAS in sports doping. The idea of designer AAS has been around for quite a while and elements of their history as well as the current situation are of great importance for both the anti-doping science and public health in general. Since the financial interest to produce new designer AAS is substantial, the rationale behind the molecular changes of the already existing AAS to create new designer molecules is explained later in the chapter. The problem of the production and circulation of illegal molecules is known to the sports and public authorities and certain measures have been taken against illegal laboratories. A list of the designer synthetic AAS is presented in “The chemistry” section. The use of designer AAS does not only appear in human sports, but also in animal racing samples as well. The antidoping Laboratories have made progress for the detection of designer AAS using MS and bioassays. Anti-doping laboratories, guided by the need of elucidating the metabolism of the designer AAS, have adopted sample preparation techniques and performed synthesis of designer AAS metabolites. However, *in silico* predicted analytical data related to designer AAS have also been used. In addition, the sports authorities have introduced the element of time in the fight against cheating athletes: “I cannot catch you now; I’ll catch you later, when I know more about the designer drugs you are using”. As a result, accredited laboratories have made relevant adaptations in their procedures such as long-term storage of samples and data reprocessing of already analyzed samples that were initially reported as negatives.

2.2 The Past and Present of The Designer AAS

Since the 1970s, sports authorities have banned the use of AAS and other performance-enhancing drugs. Nonetheless, since 1966, in East Germany, the German Democratic Republic (DDR) government and its state security “Stasi” coordinated the development of new synthetic AAS to enhance sports performance [6]. No further anti-doping regulations from official authorities had been established until then thus, no doping rules’ violation existed. A typical example DDR synthetic AAS is the famous oral turinabol (or dehydrochormethyltestosterone) [7].

After 1982, the DDR regime also created endogenous designer AAS to escape the anti-doping tests for testosterone (T) abuse, which were organized by the International Olympic Committee (IOC), the International Association Athletics Federation (IAAF) and the anti-doping laboratory of Cologne, West Germany [8]. Epitestosterone (E) and androstenedione were also included in the synthesized endogenous steroids of that time period. The rationale behind the creation of designer endogenous AAS, takes into consideration the fact that athletes trying to avoid the detection of synthetic AAS, were interrupting the relevant therapy close to the competition periods, changing to T esters intake. Exogenous T was mixed with endogenous, making its direct urinary detection impossible, due to the fact that the mass spectra of the endogenous and the exogenous preparations are identical. Its indirect detection is based on the measurement of the ratio T to E (T/E) [8]. Epitestosterone is the inactive isomeric molecule of T and its biosynthesis is inhibited after T intake. The mean human population statistic for the urinary T/E is close to unity and the threshold ratio chosen to be the limit for doping purposes was set to 6:1 by both the IOC and the IAAF. To circumvent the anti-doping controls after the abuse of testosterone esters, DDR sports medicine administered athletes with T and E esters produced by the state pharmaceutical manufacturer Jenapharm [6]. Since 2005, the WADA has changed the reporting threshold for T/E from 6:1 to 4:1 to improve the sensitivity for the detection of T misuse [8] (see also the “Endogenous designer AAS” section).

Nowadays, two trends for the circulation of designer AAS exist: the first trend comprises the creation of novel molecules in order to be used by cheating athletes without failing doping tests. Since the 1980’s, the MS detection of synthetic AAS has being improved, altogether with the improvement of the anti-doping system regulations after WADA’s activation in 2004. As a result, the cheating athletes switched to the abuse of designer AAS. The most striking example was the

BALCO case [9]. BALCO (Bay Area Laboratory Co-operative) was a San Francisco Bay laboratory, which was supplying steroids to athletes. BALCO was initially known as a vitamin and mineral shop, which was later transformed to a laboratory that black-marketed, illegally produced steroids to elite athletes of baseball, American football and athletics. The “products” of BALCO comprised the designer AAS norbolethone [10], the tetrahydrogestrinone (THG) [11] and the “cream”, a salve containing mixture of T and E. Norbolethone is a synthetic AAS that was available as a pharmaceutical in the 1960’s; however, it was never marketed due to its toxicity. THG is also a designer AAS. The “cream” was widely used by athletes because it gave normal T/E ratios following administration. Another famous synthetic AAS, seized by Canadian customs in 2004, is MADOL (desoxymethyltestosterone or DMT) [12, 13] that was initially detected by the US Accredited Laboratory of University of California, Los Angeles (UCLA) [12]. It is worth mentioning that no Adverse Analytical Finding (AAF) for elite athletes is related to MADOL. It is probable that the UCLA and the Canadian Accredited Laboratories [12, 13] timely communicated the detection data to all WADA Accredited Laboratories and in this way MADOL was no longer a tempting substance for cheating athletes. The “cream” is another illegal preparation for avoiding detection of T abuse, though less effective than T injections. In 2008 the Cologne Accredited Laboratory (Germany) revealed an important case of the designer synthetic AAS methyltrienolone abuse, involving 11 Greek weightlifters [14]. The origin of the methyltrienolone synthesis is not known, but athletes sanctioned claimed the use a Chinese food supplements.

The second trend for the circulation of designer AAS is the food supplement market. Several countries, like USA, have introduced legislations to restrict the production and circulation of food supplements based on AAS, such as the US Anabolic Steroids Control Act, 2004 [15]. Food supplements circulate through the Internet, in shops, the gyms, etc. Non-hormonal supplements such as vitamins and amino acids may contain designer AAS not declared on the labels of the products [16]. Unfortunately, several reports have been published relating these food supplements with AAF cases in doping controls [e.g. 17, 18]. A thorough review was recently published by Teale et al. [19], describing the phenomenon of designer drugs for the entire spectrum of the prohibited drug classes for doping control.

2.3 Authorities Against Illegal Laboratories

In May 2011, WADA circulated guidelines with the title “Coordinating Investigations and Sharing Anti-Doping Information and Evidence” [20]. In this document, WADA recognizes the crucial role of the National Anti-doping Organizations (NADOs) to expand the fight against doping, apart from their existing anti-doping programs, with further measures to be taken against illegal laboratories and illegal substances trafficking networks. As expected, the BALCO case is referred in the document. BALCO’s activities were revealed with the involvement of the United States Anti-doping Agency (USADA) [9]. Another important investigation against illegal laboratories held in USA in 2007, the Operation Raw Deal, is mentioned [20]. New elements of the fight against doping are described in this report [20]: (a) the concept of ‘Non-analytical’ anti-doping rule violations, (b) perpetrators falling outside sport’s authorization, (c) activating the public authorities in the fight against doping in sport and finally, (d) strengthening relationships between NADOs and public authorities. The Memorandum of Understanding between WADA and Interpol is also published, showing the importance of police authority involvement in the fight against doping [20]. Three other reports [21-23] also associate the fight against doping with the reinforcement of national legislations. The first report [21] deals with the illegal drugs trafficking in various countries. Another report studies the Italian situation of doping in sports [22]. This report, which can be considered as indicative for many other countries, examines Italy’s anti-doping criminal law experience with a two-fold purpose: 1) to analyze the production and distribution of doping products and 2) to give evidence of how anti-doping criminal provisions and their enforcement can contribute to improve the fight against doping, both within and outside the sports community. The multilateral use of legislation to control the production, movement, importation, distribution and supply of performance-enhancing drugs in sport (PEDS) by several countries is the subject of a report written by Houlihan and Garcia in 2012 [23]. Furthermore, The Australian Crime Commission conducted an investigation and published in 2013 a report examining the extent by which the organized crime is related to illicit drug markets [24].

The aforementioned reports make several references to the role of the Chinese pharmaceuticals industries in the production of raw materials for prohibited drugs. Aligned to these references, WADA’s General Director made a statement in February 2013:” Ninety-nine percent of the raw materials that are used through the Internet to make up in your kitchen or your backyard laboratory

are emanating from China” [25]. However, the head of Chinese NADO J. Zhixue replied immediately, [26] asking for evidence concerning the alleged “Ninety-nine percent”, albeit admitting anti-doping problems in China.

The International Federation of Pharmaceutical Manufacturers & Associations (IFPMA) and the WADA have collaborated to combat the latest doping techniques [27] performing the following declaration: “The Joint Declaration on Cooperation in the Fight against Doping in Sports facilitates voluntary cooperation between IFPMA member companies and WADA to identify medicinal compounds with doping potential, minimize misuse of medicines still in development, improve the flow of relevant information, and facilitate development of detection methods.” The WADA report on the “Lack of (In) Effectiveness of Testing Programs” published in May 2013 [28] completes a thorough description of the problem of illegal drugs’ circulation in sports.

2.4 Designer Synthetic AAS–The Chemistry

Designer AAS are substances with sufficient chemical diversity from known AAS, developed either in the past for clinical practice, or to evade doping control from official doping authorities. These designer AAS pose a serious health risk to consumers due to limited available pharmacological and toxicological data. The male hormone T (Figure 1) is the basic steroidal structure upon which a considerable number of modifications can be applied, to achieve the design of novel molecules with enhanced anabolic potency and reduced androgenic effect.

Figure 1. Testosterone molecule, a representative steroidal structure indicating carbon numbering.

Androgens mediate their action through their binding to the androgen receptor (AR) [29, 30]. Besides natural androgens, AR binds a variety of synthetic molecules with different affinities. AR ligands are classified as steroidal or non-steroidal based on their structure, or as agonist or antagonist based on their ability to activate or inhibit transcription of AR target genes. The strength

of the interaction between a ligand and a receptor is difficult to predict, since AAS with similar structures can possess different affinities for a given receptor, while structurally different ligands may show similar affinities [31]. Relative binding affinity (RBA) has been used as a term for the quantitative estimation of the receptor-ligand interaction. Methyltrienolone binds AR so strongly that is used in studies as a reference substance to estimate the RBAs of other steroids, which are characterized as strong (19-nortestosterone, methenolone) or weak ligands (stanozolol, methandienone). Other compounds show RBAs too low to be determined (oxymetholone, ethylestrenol). A possible explanation for steroids with anabolic-androgenic activity *in vivo* but with no binding to AR, is the existence of an indirect mechanism of action, e.g. via biotransformation to active compounds [32, 33]. Structure-activity studies have revealed that the most important structural elements of a steroidal structure for effective binding to the AR are:

- the 3-keto group in the A-ring [31]. The reduction of this 3-keto group to an alcohol (either to α or β isomers) does not favour binding [34].
- the 17 β -hydroxyl in the D-ring [31]. Any modification or elimination of the 17 β -hydroxyl group reduces the AR binding affinity. A reduction in binding affinity was also occurred by esterifying e.g. the 17 β -hydroxyl in T [34]. The 17 α -hydroxyl group is not favourable to binding, either.
- the 5 α -steroidal framework [34].
- a small steric substitution at the 7 α -position, but large substituents reduce affinity. It has been shown that in 17 β -hydroxy-4-androstenes the combined removal of the 19-methyl group and 7 α -methylation can enhance binding to the AR [35].

Other studies demonstrated that key structural characteristics of a steroidal structure that affect either anabolic or androgenic activities of a given steroid are:

- the 17 α -alkylation. 17 α -Alkylation contributes to the prolongation of the anabolic effect. The oral effectiveness of 17 α -alkylated androgens is due to lower hepatic inactivation; the intracellular metabolism is limited and transformation of this part of the molecule does not occur leading to liver disturbances [36, 37]. 17 α -Alkylation also prevents aromatization of A-ring to estrogens [38].
- the 17 β -hydroxyl group. Its esterification (propionate, enanthate, cypionate, decanoate and undecanoate esters) induces enhancement of anabolic activity and its prolongation due to

the reduction in the elimination rate as a result of the slow release of the parent non-esterified molecule. The absence of a 17-hydroxyl group induces the complete loss of the androgenic activity [39], while due to the oxidation to 17-keto steroids, the androgenic activity is significantly reduced or disappears [40].

- the C-4,5 double bond. Its presence seems to cause an increase in activity.
- the 3-keto group. It is necessary for androgen activity but has no effect on anabolic activity [40, 41]. However, 3-deoxy steroids, in presence of the C-4,5 double bond, were found to be compatible with high anabolic to androgenic activities (e.g. ethylestrenol).
- the removal of the 19-methyl group. This structural change offers, partially, dissociation of the androgenic and anabolic activities for a given molecule [42].
- the modification of ring A, either by the junction with another ring (e.g. a pyrazol ring, as in stanozolol), or by the introduction of an oxygen atom (e.g. oxandrolone), leads to a considerable increase in the anabolic activity.

The structural characteristics mentioned above, inspired research teams to the synthesis of a vast number of designer steroids (even for ethical purposes to synthesize standards), retaining one or more of the above modifications while modifying further the structure of known anabolic steroids, at positions where no significant reduction to AR binding or biological activity (either anabolic or androgenic) is induced. These further modifications (and/or their combinations) include:

- Alkylation at different positions in the steroidal structure, such as methylation at C-1 (e.g. mesterolone), C-2 (e.g. drostanolone), C-6 (e.g. 6-methyltestosterone), C-7 (e.g. bolasterone), C-17 (also, ethylation or ethynylation, e.g. methandienone, norethandrolone and danazol, respectively) and C-18.
- Introduction of a double bond at different positions in the steroidal structure, such as at C-1,2 (e.g. 1-testosterone), C-2,3 (e.g. desoxymethyltestosterone) [12, 43], C-5,6 [44] and C-5,10 (e.g. tibolone). In addition, many compounds with conjugated double bonds extending from ring A and B to C have been synthesized (e.g. methyltrienolone, methyldienolone) [41, 45, 46].
- Addition of heteroatoms, either to replace a carbon atom of the steroidal structure (e.g. with an oxygen atom [47, 48] at C-2 as in oxandrolone, C-3, C-4, C-7, C-11 [49] or with a sulfur atom [50, 51], or with a nitrogen atom [52]), or as a substituent (e.g. a chlorine at

C-4 as in dehydrochlormethyltestosterone or at C-7 [53], or a fluorine at C-2, at C-6 [54], at C-7 [55] or at C-9 as in fluoxymesterone).

- Hydroxylation, such as at position C-4 (oxymesterone, oxabolone) or at C-11 (fluoxymesterone).
- Fusion of heterocyclic rings to the A-ring of the steroidal structure, such as of a pyrazole ring (stanozolol), an isoxazole ring (danazol) or a furazan ring (furazabol).

Table 1 summarizes designer AAS found in literature circulated either in the black market or in food supplements.

Table 1. Designer AAS from literature.

Entry	Substance	Reference
1	1-androstenediol	56
2	1-androstenedione	56
3	dehydrochlormethyltestosterone	57
4	desoxymethyltestosterone	9
5	methasterone	58
6	methylnortestosterone	58
7	methyldienolone	16
8	methyl-1-testosterone	59
9	metribolone	16
10	norboletone	10
11	norclostebol	60
12	prostanazol	61
13	1-testosterone	62
14	tetrahydrogestrinone	11
15	methylstenbolone	63
16	2 α ,3 α epithio17 α methylandrostande 17 β ol	64
17	2 β ,3 β epithio17 α methylandrostande 17 β ol	64
18	5 β -Mestanolone	61
19	methylclostebol	65
20	promagnon	65
21	17-hydroxyandrost-3,5-diene	66
22	Δ 6-methyltestosterone	67
23	17 β -hydroxyandrostanol[3,2-d]isoxazole	68
24	17 β -hydroxyandrostanol[3,2-c]isoxazole	68
25	6 α -Methylandrostenedione	69
26	estra-4,9-diene-3,17-dion	70

Entry	Substance	Reference
27	androsta-1,4,6-triene-3,17-dione	71
28	4-androstene-3,6,17 trione	72
29	1-adrosterone	73
30	Methyl drostanolone	74
31	7 α -methyl nortestosterone	75
32	17 α -methyl nortestosterone	75
33	18-methyl nortestosterone	75
34	Halodrol	75
35	4-hydroxytestosterone	75

2.5 Endogenous Designer AAS

The use of preparations containing T and E as endogenous designer AAS to escape doping tests has been described in the previous sections. Two cases of preparations have become known: the case of Jenapharm [6] and the case of BALCO [9]. In urine, a T/E ratio greater than 4.0 triggers follow-up tests to investigate whether the elevated T/E ratio is of natural or exogenous origin [8]. The anti-doping analytical technology has incorporated the use of the Isotope Ratio Mass Spectrometry (IRMS) to enable the differentiation between endogenously produced and exogenous T. The reader can be directed to a thorough review [76] for more information on this technology. Briefly, pharmaceutical preparations of T are synthesized by plants' extracts, whereas human endogenous T is originated from the endocrine system. T contains 19 carbon atoms (Figure 1). The most abundant carbon isotope is ^{12}C , approximately 99 % in nature and the less abundant carbon isotope is ^{13}C , approximately 1 %. Due to the differences in the synthetic routes, the endogenous T contains more ^{13}C atoms among the 19 C atoms of the T molecule, compared to the pharmaceutical preparations. This difference in ^{13}C content between endogenous and exogenous T is measurable for the T molecule and its urine metabolites by IRMS. Doped athletes using pharmaceutical T, excrete T and metabolites in urine with less ^{13}C atoms compared to the endogenous, because exogenous T inhibits the production of the endogenous one. Many manufacturers of references material produce ^{13}C labelled T for the analytical and pharmaceutical industries. In these reference materials, ^{13}C atoms replace ^{12}C in the positions mainly 2, 3, and 4 (Figure 1). Unpublished data presented in the 29th Cologne Workshop on Dope Analysis (13-18/2/2011) by L. Bowers and D. Eichner of USADA [77], raised suspicion that athletes already

use pharmaceutical T preparations mixed with ^{13}C labelled T, in order to create a T cocktail with ^{13}C content similar to the endogenous, with the purpose of misleading IRMS tests.

2.6 Detection of Designer AAS

Chromatographic techniques combined with MS, such as GC/MS or LC/MS are the first approach of the anti-doping laboratories for the detection of AAS. Commonly used instrumentation such as the Mass Selective Detector (MSD) with a single quadrupole mass analyser or the magnetic sector high-resolution mass spectrometer, operating in Selected Ion Monitoring (SIM) mode, combine high sensitivity and specificity. These analytical instruments have been used for years for the detection of targeted anabolic steroids and their metabolites in the required low concentrations in urine. As an alternative to the GC/MS, the combination of LC/MS instrumentation with Electrospray Ionization (ESI) has been introduced the last decades, for the detection of known steroids operating in Multiple Reaction Monitoring (MRM) mode (for triple quadrupole analysers) or product ion scan mode (for ion trap mass analysers). All the above detection techniques allow efficient detection of known anabolic steroids that are included in the WPL of screened substances. Unknown designer AAS can be detected only by coincidence in the cases that they share the same precursor and product ions with the targeted compounds and they are eluted in a close chromatographic time inside the defined time window that is selected for the printout of the chromatograms. The preventive detection of unknown designer AAS requires a generic screening protocol, which combines a generic sample preparation with a sensitive high-resolution full scan MS analysis [77-82]. Regarding sample preparation, the unification of different extraction/derivatization procedures applied for different classes of substances to a single extraction step, which will be able to isolate the unconjugated and conjugated (after enzymatic hydrolysis) low molecular weight substances, has been an important issue for the anti-doping laboratories. The analysis of this extract is performed by GC/MS (following a generic derivatization procedure) and/or LC/MS analytical systems that can acquire high-resolution full scan accurate mass spectrometric data, which allow for the detection of an unlimited number of known and unknown substances. Such analytical systems include GC/TOF and GC/Q-TOF (TOF stands for Time-Of-Flight) and the combination of mass spectrometers with TOF, Q-TOF or orbitrap mass analysers with HPLC or UHPLC (Ultra High-Performance LC) systems. In addition, with the use of mass analysers that can perform fast scan to scan polarity switching, as the recently

introduced benchtop orbitrap mass spectrometer, the intact sulfoconjugated molecules of the designer steroids can also be detected as deprotonated molecules. The generic screening approach described above, contributes to the enhancement of the preventive role of the anti-doping system against the use of designer drugs, especially if combined with the long-term storage of the samples. The acquisition of full scan data enables the retrospective analysis of samples for the presence of designer drugs or new metabolites, without the need of reanalysing the samples, by simply reprocessing already acquired LC/MS and/or GC/MS data files. Important information, such as the molecular weight of the unknown and the elemental composition, can be obtained by accurate mass full scan mode analysis, while the appearance of a combination of adduct ions can provide additional valuable information about the steroid structure.

Another approach for the detection and identification of unknown steroids, is the development of methods based on precursor ion scan and neutral loss scan using triple quadrupole or Q-TOF LC/MS/MS instruments, as steroids with common structural features under collision-induced dissociation (CID) or collisionally activated dissociation (CAD) can share common fragmentation patterns. The common characteristic product ions or neutral losses can be used as markers to identify unknown compounds. Published research describe protocols that can be used as complementary approaches to the existing analytical screening procedures of the laboratory [83-89], especially in cases of suppressed steroid profile as measured by GC/MS. In these protocols, product ion scan LC/MS analyses of known steroids were conducted and with the use of deuterium derivatives or modified structurally related synthetic analogues, characteristic fragmentation pathways are proposed, that provide classification of the steroids by the generated characteristic product ions. For example, precursor ion scans of ions at m/z 97 and 109, indicate steroids with a 3-keto-4-ene structure and the detection of abundant product ions at m/z 241 and 199 or 227 and 199 indicate a 4,6,11-triene steroid with ethyl or methyl group at C-13. In a similar way, neutral loss scan can be used for the detection of unknown steroids with a particular structure. Some of the common losses observed in steroids are lacking specificity (e.g. loss of water (18 amu) or acetone (58 amu), while others are considered more specific (e.g. 84 amu and 30 amu) and they can be used as a diagnostic tool for the detection and characterisation of unknown steroids. As suggested by Pozo et al. [90], the integrated use of the four different types of scan modes (neutral loss and precursor ion scan followed by full scan and product ion scans) can be the most powerful tool for the detection and characterization of a designer steroid.

MS based techniques are used as the standard highly sensitive routine screening methods for the known AAS. However, they are depended on the known chemical structures. This led to the development of *in-vitro* androgen bioassays, for the screening of designer AAS based on androgen receptor activation instead of knowing the chemical structure. Androgen bioassays are not depended on specific chemical structures. An approach based on the combination of LC separation – androgen bioactivity testing and Q-TOF-MS identification was developed by Nielen *et al.* [91-92]. According to this protocol, urine samples after enzymatic hydrolysis and generic SPE are analyzed using gradient LC and a dual 96-well fraction collector, where one plate is used for androgenic bioactivity detection by yeast-based reporter gene bioassay. In case that a well is found suspect, the duplicate plate is subjected to high-resolution LC/Q-TOF-MS analysis, leading to elemental composition calculations of the designer steroids, search to electronic databases and structural elucidation. This approach was recently also applied to detect and identify unknown androgens in herbal samples and sport supplements. Radioimmunoassays and Enzyme-Linked Immunosorbent Assays (ELISAs) have been used in the past, showing good sensitivity for the screening of AAS, with the disadvantage of limited specificity due to antibody cross reactivity profiles [93]. Recently, a multi-analyte ELISA protocol based on site encoded ELISA microplate has been reported, that allows the simultaneous detection of up to eleven AAS in human serum samples in concentrations below MRPL. This protocol enables the development of multiplexed immunoassays performed in a microarray format [94].

A thorough review on the androgen bioassays has been recently published [95], where the various types of this approach have been described. In the next lines, some studies on bioassays of AAS in biological matrices and dietary supplements are presented. Nielen *et al.* [96] had developed a simple yeast-based reporter gene bioassay for trace analysis of estrogens, characterized by direct measurement of yeast enhanced green fluorescent protein for the detection of estrogen activity in dietary supplements. It was shown that bioassays play a valuable role in the fight against doping as compared to a LC/MS/MS screening method alone. As a test to examine its efficiency, 18 dietary supplements were analyzed and shown negative in LC/MS/MS, while two of them screened positive by androgen yeast bioassay. The applicability of a yeast androgen and estrogen bioassay, in the detection of steroid esters in hair samples of animals treated with a hormone ester cocktail, was also shown [97]. Another approach for the advantage of a yeast androgen screening was studied by Wolf *et al.* [98]. The long-term detection of methyltestosterone abuse by a yeast

transactivation system has been successfully validated. For the purpose of that study, a human volunteer was orally administered a single dose of 5 mg methyltestosterone and urine samples were collected after different time periods (0-307 h). The samples were analyzed in the yeast androgen screen and in parallel GC/MS. The results demonstrate that the yeast androgen receptor was able to detect methyltestosterone abuse for a longer period of time in comparison with classical GC/MS. It was found that bioassay was able to trace methyltestosterone in urine samples for at least 14 days while the GC/MS method was able to detect it up to the sixth day from the intake. The result of this study demonstrated that the yeast reporter gene system could detect the activity of anabolic steroids such as methyltestosterone with high sensitivity even in urine, providing further evidence for the high potential of yeast androgen screening as a pre-screening tool for doping analysis. Even though promising, this approach has been criticised at the following points: a) metabolites of many AAS may be inactive and do not show androgenic activity, b) the background activity from endogenous sources reduces specificity and c) its applicability is limited due to reduced sensitivity, mostly in out of competition collected anti-doping samples, where the AAS analytes would be more easily detected due to higher concentrations in urine.

In addition, a promising strategy of screening methods for the misuse of designer steroids by their physiological effects is the use of omics technologies [99-102]:

- a) Transcriptomics for finding gene expression biomarkers, with *in vivo* studies in showing alteration of gene expression in human blood cells caused by steroid hormones.
- b) Proteomics for investigating changes in protein expression or excretion caused by AAS, with a few publications available showing that different lipoproteins or apolipoproteins, propeptide of type III procollagen, apoptotic factors, pro- and anti- inflammatory factors can be promising biomarker candidates.
- c) Metabolomics for detecting perturbations in the metabolic profile after administration of AAS, with creatine, creatine kinase and plasma urea levels being potential biomarkers. Recently, Dervilly-Pinel et al. [100] published two protocols based on LC/HR-MS fingerprinting and multivariate data analysis, to investigate metabolome modifications upon steroid administration in calves, showing urine profiles discrimination of the treated animals from the control ones; the results showed that the protocols need to be applied to a larger population of treated and control animals in order to describe generic, reliable and

robust biomarkers. An untargeted steroidomic approach was proposed for the discovery of new biomarkers for the detection of T intake, by applying UHPLC/Q-TOF-MS urine sample analysis and chemometric tools, showing the pertinence to monitor both glucuronide and sulfate conjugates as well as a number of promising biomarkers that can be also related to the administration of other AAS.

Recently, in 2009, WADA introduced the term “athlete biological passport” (ABP) in the WADA Code [103] as an additional indirect tool to detect athletes manipulating their physiological steroid and haematological variables, without detecting a particular prohibited substance or method. The ABP does not replace the routine methods, but rather complements analytical methods. Although there has already been some longitudinal profiling of markers of steroid doping [8], the ABP now introduces a standardized approach to determine steroid abuse through urine sampling. The ABP regulations are based on the innovative approach developed by the Swiss WADA Accredited Laboratory of Lausanne [for example 104, 105].

2.7 Data processing

Methods based on mass spectrometry produce data for known, unknown, targeted, untargeted and endogenous substances of biological samples. Specific software extracts MS information from analyzed urine samples, eliminates interferences, and identifies metabolites in a series of samples from excretion studies, using data from MS libraries of known substances, spectra and accurate masses databases. Several tools for processing MS data have been proposed in the literature and are available, e.g. MetaboLynx of Waters, Sieve of ThermoFischer Scientific and MetAlign of RIKILT. The MetAlign [79] is an interface-driven tool for full scan MS-data processing. The main purpose of this software is the automated processing of MS-based metabolomics data with baseline correction, accurate mass calculation, smoothing and noise reduction and alignment between chromatograms. By comparing data after pre-processing with MetAlign, it was noted that besides the chromatogram baseline line correction, there were better defined peaks which improved peak picking for the identification of targeted and untargeted compounds [79]. For identification of untargeted peaks, an accurate-mass database was constructed containing approximately 40,000 pharmacologically relevant and existing compounds extracted from Internet-accessible database PubChem [106]. Calculation of the exact mass of each protonated and deprotonated molecule, the isotope ratio and an estimate of the retention time was also performed.

Peters et al. [79] have modified MetaboLynx for the determination of *in silico* predicted metabolites of glucocorticosteroids and designer modifications of anabolic steroids in human urine. It was successfully used for the detection of THG [107]. Synergetic methods for the prediction of AAS metabolites, retention times and MS fragmentation have been proposed by Fragkaki et al. [108]. A method predicts the Phase I metabolites of AAS [108]. The statistical tool of principle components analysis (PCA) was used to classify the parent AAS into different classes, based on their structure's similarities. Another method [109] was used for the prediction of MS fragmentation of AAS, including designer compounds. The results derived from the previous two studies were combined with the study of Quantitative Structure Retention Relationships (QSRR) prediction of retention times [110]. xlogP molecular descriptors have also been used [79] for retention times predictions of PubChem database compounds. Finally, an LC/MS library searching method for the identification of AAS in dietary supplements has been developed [111].

2.8 RNA-sequencing

A recent study [112] opened new frontiers in the detection of designer AAS, even though it was applied in meat production animals (boars and calves). Changes in the molecular level caused by the administration of AAS were quantified by a new high-throughput and sensitive technology for holistic gene expression analysis, RNA sequencing. The results demonstrated the potential of the new technology for the screening of highly regulated genes that can act as biomarker candidates for the detection of the misuse of anabolic substances in farm animals. This novel approach can be evolved as an alternative indirect detection method of designer and known AAS in human sports in the future.

2.9 Synthesis of Metabolites of Designer AAS

The *in vivo* production of reference substances of AAS metabolites in humans suffers from ethical, as well as, practical problems associated with the implementation of clinical studies and the isolation of pure metabolites from urine. To overcome these problems, several methods of synthesis of metabolites have been developed. The enzyme-assisted synthesis catalyzed with microsomal uridine diphosphoglucuronosyl-transferase (UGT) enzymes has been developed, offering the main advantage of the stereospecificity of the enzymes, which allows synthesis of stereospecifically pure conjugates. Moreover, enzyme-assisted synthesis is used for the rapid

production of small amounts of glucuronides when needed, e.g. in the build-up of an analytical method. Using the enzyme-assisted synthesis, the preparation of glucuronide conjugate standards of eleven AAS and their metabolites which can be detected in human urine after dosing of exogenous anabolic steroids (e.g. methandienone, metenolone, methyltestosterone, nandrolone and T) has been described [113]. In another study, microsomal and S9 fraction of human liver preparations were used as source of metabolizing enzymes, and the co-substrates of the synthesis mixture were selected to favour phase-I metabolic reactions and phase-II conjugation reactions of relatively new AAS [114,115]. Equine liver microsomes and S9 *in vitro* fractions were also found to generate all the major phase-I metabolites observed, following *in vivo* administrations of stanozolol in the equine [116].

Chemical synthesis methods have been also developed as an alternative for the synthesis and identification of AAS and/or their metabolites, as occurred in a study for 4-hydroxytestosterone [117], madol [12], tetrahydrogestrinone [11] and other AAS [13, 118, 119]. The approach to synthesize, characterize and certify appropriate reference materials (RMs) and certified reference materials (CRMs) from the National Analytical Reference Laboratory (NARL), which are fit-for-purpose for the current requirements of sports testing laboratories, has been described [120].

The identification of AAS metabolic pathways have been also successfully conducted through either animal experiments, as for madol [12] or using cryopreserved human hepatocytes, as for drostanolone and 17-methyl drostanolone [121] and other AAS [122, 123]. The results of the *in vitro* experiments carried out using homogenized horse liver for five anabolic steroids (turinabol, methenolone acetate, androst-4-3,6,17-dione, T and E) have also been presented [124], as an alternative for AAS metabolism studies.

The chimeric uPA^{+/+}-SCID mouse model, transplanted with human hepatocytes, has been also used to study *in vivo* the human steroid metabolism, as occurred for methasterone, promagnon, methylclostebol [65], 4-androstene-3,17-dione [125] and methandienone [126].

Recently, another strategy for synthesis of the methandienone long-term metabolite, 17 β -hydroxymethyl-17 α -methyl-18-norandrost-1,4,13-trien-3-one, was reported [127]. According to this, eleven recombinant strains of the fission yeast *Schizosaccharomyces pombe*, expressing different human hepatic or steroidogenic cytochrome P450 enzymes, were screened for production of this metabolite in a whole-cell biotransformation reaction. 17,17-Dimethyl-18-norandrost-

1,4,13-triene-3-one, chemically derived from methandienone, was used as substrate for the biotransformation reaction, as it was converted to the final product in a single hydroxylation step. The metabolism of methyl-1-testosterone has also been studied according to this strategy [128].

2.10 Animal Doping with Designer AAS

In several publications related to AAS screening in animal sports, designer AAS have been introduced to the protocols, such as in [129], proving that the problem has been inherited from the human sports to the animal ones. Several animal racing anti-doping laboratories have conducted studies on the metabolism of designer AAS [130-132]. The *in vitro* metabolism of a designer steroid featuring in 2010 in a large number of marketed products on the Internet, *estra-4,9-diene-3,17-dione*, was studied in equine, canine and human species with the major metabolites identified for target testing in sports doping control [130]. In another study [131], the equine *in vitro* metabolism of seven steroids available for purchase on the Internet, including *androsta-1,4,6-triene-3,17-dione*, *4-chloro,17 α -methyl-androsta-1,4-diene-3,17 β -diol*, *estra-4,9-diene-3,17-dione*, *4-hydroxyandrostenedione*, *20-hydroxyecdysone*, *11-keto-androstenedione*, *17 α -methylrostanolone* was reported. Initiated by the doping scandals in human sports [11], the pharmacokinetics of THG in equine [132] and its *in vitro* metabolism [131] were also studied.

2.11 Anti-Doping Samples Preservation: Urine Stabilization, Blood Spots

The designers AAS molecules and their metabolism are unknown to the anti-doping laboratories at the time of their first circulation. WADA Code [3] has introduced the dimension of time in the anti-doping system, allowing laboratories to organize their detection of the designers with knowledge in the structures, the metabolism and the synthesis of reference materials. The dimension of time in the anti-doping system is practically applied with samples long-term storage. Two methodologies have been developed to facilitate the urine and blood stability over time: urine samples stabilization and the dried blood spots.

Retrospective analysis can only be conducted if urine samples quality is not undermined over time due to reactions enhanced by the presence of microorganisms or proteolytic enzymes in urine. Doping control urine samples are collected and stored in Doping Control Stations and WADA accredited laboratories, in a way that protects their identity, integrity and security [106], which is

of particular importance in case that the already analyzed samples are eventually submitted to retrospective analysis [3]. What if sample delivery to the WADA accredited laboratories is not immediate? Hydrolysis of steroid conjugates followed by modifications of the steroids' structure by oxidoreductive reactions may take place due to the occurrence of microorganisms that can be found in the human body or the surrounding environment, especially during sample transportation in the warm periods of the year [133-138]. The best practice to ensure that samples' integrity is maintained for possible reanalysis would be to store samples frozen as well as stabilized. Up to present, no preservative is added to sport urine samples [139]. The implementation of a specially designed sample collection container, incorporating a generic sample stabilization mixture consisting mainly of antibiotic, antimycotic substances and protease inhibitors has been proposed [140]. The purpose of an on-going project funded by WADA, is the investigation of the efficiency of the in-house chemical stabilization mixture in spray-coated form with simultaneous minimization of analytical interferences. Preliminary results demonstrate that the cell growth of five representative microorganisms (*Escherichia coli*, *Nocardioideis simplex*, *Aspergillus flavus*, *Candida albicans*, *Enterococcus faecalis*) is completely inhibited after a 7-day incubation period at 37 °C in those urine samples which were stored in spray coated stabilized containers. Moreover, the degradation of steroid glucuronides is prevented in the stabilized urine samples (unpublished data). Evaluation of analytical interferences is still under way [141]. The implementation of specially designed plastic urine collection containers, spray-coated in their interior surface with the stabilization mixture is currently more realistic than it was a few years ago. If this preventive approach is applied in the doping control sampling procedure, it would be a major step towards the preservation of urine samples for long term storage and eventual retrospective analysis.

In the context of long-term storage of samples for retrospective analysis, the dried blot spots (DBS) technique is gaining increasing importance in the doping control field. It involves collection of small volumes (10 to 30 μ L) of whole blood obtained from heel or finger pricks, drying it on a piece of filter paper, extracting and subsequently analysing it by liquid chromatography-mass spectrometry (LC/MS). DBS offers numerous advantages over conventional whole blood, plasma, or serum analysis, like ease of collection (even in remote control stations), minimal potential of sample manipulation, cost-effectiveness, less invasiveness, simplified storage and transport of DBS samples – absence of refrigeration –, enhanced stability described for many analytes on the cellulose sampling paper [142-145]. An apparent limitation of the DBS method is the small blood

volume collected, thus representing a challenge for the sensitive determination of some analytes in elite sports like anabolic steroids at sub ng/mL levels. In addition, a new plasma screening method has been developed for the retrospective reanalysis of stored samples for new xenobiotic drugs at low ng/mL levels [146]. It is based on protein depletion, U-HPLC-based liquid chromatographic separation and detection by means of high-resolution/high accuracy mass spectrometry. The use of either DBS or plasma samples cannot replace (at least for the time being) the conventional urine analysis procedure, however, they are both attractive alternatives and can enable the retrospective qualitative data evaluation for known and unknown xenobiotics.

2.12 Conclusion

Designer AAS represent a dark and dangerous side of drug abuse in sports. AAS remain the prevalent drug-class according to the WADA statistics [147]. The borderline between the use of novel substances as new therapeutics or as potential doping agents is often a challenge for cheating athletes to overstep. Control laboratories and regulatory authorities are aware of analytical advancements and legislation improvements for successful detection and prevention of AAS. This review presented main issues concerning AAS, such as their scientific background, progresses in their analytical detection and the preventive anti-doping which is intended to reveal positive analytical findings in samples initially reported as negatives, as occurred in the reanalysis of stored anti-doping samples of the Olympic Games, 2004, [148] and the World Championships in Athletics, 2005, [149].

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Chapter 3

High-Resolution Full Scan Liquid Chromatography Mass Spectrometry Comprehensive Screening in Sports Antidoping Urine Analysis

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Abstract

The aim of this study is to present the development and validation of a high-resolution full scan (FS/HR) electrospray ionization (ESI) liquid chromatography coupled to quadrupole Orbitrap mass spectrometer (LC/Q-Orbitrap MS) platform for the screening of prohibited substances in human urine according to World Antidoping Agency (WADA) requirements. The method was also validated for quantitative analysis of six endogenous steroids (epitestosterone, testosterone, 5 α -dihydrotestosterone, dehydroepiandrosterone, androsterone and etiocholanolone) in their intact sulfates form. The sample preparation comprised a combination of a hydrolysed urine liquid-liquid extraction and the dilute & shoot addition of original urine in the extracted aliquot. The FS/HR-MS acquisition mode with Polarity Switching was applied in combination of the Quadrupole-Orbitrap mass filter. The FS/HR acquisition of analytical signal, for known and unknown small molecules, allows the inclusion of all analytes detectable with LC/MS for antidoping investigations to identify the use of known or novel prohibited substances and metabolites after electronic data files' reprocessing. The method has been validated to be fit-for-purpose for the antidoping analysis.

3.1 Introduction

The World Antidoping Agency (WADA) is an international agency, which has the task to prevent and monitor any doping in sport. Sports movements and governments around the world collaborate with WADA to prevent, deter and define the rules with which such doping practices and duplicitous behaviour of athletes using doping substances in sports can be challenged and fought on a united and global basis. The WADA prohibited List (WPL) [1] includes among other substances pharmacological classes of drugs, either exogenous substances or endogenous substances administered exogenously, which define doping. The WADA antidoping control activity is based on the WADA accredited laboratories operating under the specifications of the WADA International Standard of Laboratories (ISL) [2]. In addition to these criteria, WADA publishes technical documents which provide analytical requirements to be fulfilled by laboratories. Examples are the Technical documents for Minimum required performance levels (MRPL) [3] which describes the main specifications for the analysis and detection of exogenous substances in human urine. Another example is the WADA Endogenous Anabolic Androgenic Steroids technical document TD2016EAAS [4] that provides the specifications for the analysis of the endogenous profile of small molecules present in urine, such as testosterone (T) and testosterone-like substances used for doping. The evaluation of the steroidal profile is done by the adoptive module of Urinary Athlete Biological Passport and a thorough review of the steroidal ABP has been published [5].

The doping control laboratories implement different analytical techniques to be able to detect a large variety of classes of prohibited substances. For that purpose, a Mass spectrometer (MS) coupled either with Gas Chromatography (GC/MS) or Liquid Chromatography (LC/MS), is the most standard technology used to ensure compliance with [1-4] in the analysis of urine samples. The use of GC/MS and LC/MS is complementary for the specifications [1-4]. The GC/MS is used as standard technique for small molecules, in particular for anabolic steroids, because these compounds provide a weak ionization yield when the atmospheric LC/MS interfaces like the electrospray ionization (ESI) is used [6].

Several studies have been conducted by LC/MS concerning the screening for small molecules following the WADA's technical documents. The urine sample preparation can either be applied as direct urine LC/MS analysis using dilute &shoot (D&S) approach [7, 8] or following urine

extraction procedures such as [9] applied during the Olympics games in 2012 in the London Olympic Laboratory. The LC/MS systems are operated either in tandem MS/MS mode [7] or FS/HR acquisition that either with Quadrupole/Time-Of-Flight (LC/Q-TOF/MS) [10] or Orbitrap (LC/Q-Orbitrap/MS) [9] instruments.

The WPL [1] includes prohibited pharmacological classes and the therein particular molecules are referred as examples, however it is open for other substances with a similar chemical structure or similar biological effect. Consequently, the screening procedure should be generic and comprehensive for all compounds detectable by the molecular profiling platform such as LC/MS; this should include the sample preparation and FS/HR-MS acquisition of known and unknown molecules. Moreover, FS/HR-MS acquisition and polarity switching for positive and negative ionized molecules are important parameters to be considered [11] to obtain comprehensive molecular profile detectable by an LC/MS system.

The limitations of the steroid profiles in anti-doping applications have been addressed for several years [5] and studies have been conducted to improve its effectiveness as a forensic biomarker tool to monitor and identify testosterone (T) and analogs abuse [12]. In 1996, a study conducted by Dehennin *et.al* [13] proposed the introduction of epitestosterone sulfate (ES) in the athlete's steroid profile as a ratio of T glucuronide to the total E fractions. In another study, several additional endogenous steroid forms from those referred in [4] have been identified [14, 15]. Untargeted metabolomics studies for the detection of steroid biomarkers have been performed as well [16-18]. Complementary to these studies [13], the sulfate fraction of endogenous steroid profile, in addition to the free and glucuronide fractions of [4], has been studied by several research groups in relation to the genotype for the UGT2B17 enzyme, where it is proposed that in case of exogenous T intake, the ratio between androsterone glucuronide and E glucuronide as a complement to T and E glucuronide ratio can be used especially in UGT2B17 *del/del* individuals. In addition, etiocholanolone sulfate was excreted at significantly higher levels in those individuals [19-22].

The detection and quantification of the intact conjugated endogenous and exogenous steroids by LC/MS has been studied and reviewed by several groups since '90s [23] and [24]. The quantification of glucuronidated and sulfated endogenous steroids by LC/MS has been reported earlier [25]. A similar approach has been followed in other studies for the analysis of intact conjugated steroids of endogenous and exogenous origin [26-28].

This article focuses on the application and validation of an LC/MS screening for small molecules using FS/HR acquisition with polarity switching. The method has been developed using an LC/Q-Orbitrap MS with a combination of D&S and a solvent extraction procedure for urine sample preparation. The method is intended to be generic in order to allow the detection of known designer drug urine metabolites [29] together with quantification of six intact endogenous sulfate steroids: epitestosterone (ES), testosterone (TS), 5 α -dihydrotestosterone (5 α DHTS), dehydroepiandrosterone (DHEAS), androsterone (AS) and etiocholanolone (ETIOS) using negative ionization mode, with possibility to detect unknown exogenous substances. This method is developed as a complementary for a previously published GC/Q-TOF/MS screening method [30]. Accordingly, the antidoping laboratories will have a completed GC and LC FS/HR screening method with simultaneous measurement of steroid profile according to WADA Code specifications [4] and detection of intact sulfo-conjugated metabolites of Phase II metabolism.

3.2. Materials and Methods

3.2.1 Reagents

β -Glucuronidase from *Escherichia Coli* (*E. coli*), for the enzymatic hydrolysis, was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Methanol (HPLC grade), di-potassium hydrogen phosphate trihydrate ($K_2HPO_4 \cdot 3H_2O$), potassium dihydrogen phosphate (KH_2PO_4), sodium bicarbonate ($NaHCO_3$), sodium carbonate (Na_2CO_3) and acetonitrile were supplied by Sigma Aldrich (Darmstadt, Germany). Ethylacetate was supplied by Merck (Darmstadt, Germany). Formic acid ($HCOOH$) and 5 M ammonium formate ($HCOONH_4$) were from Agilent Technology.

3.2.2 Reference Materials

The Reference Materials used in the current study were from Sigma Aldrich (Darmstadt, Germany), Steraloids (Newport, USA), LGC (Wesel, Germany), Toronto Research Chemicals TRC (Toronto, Canada), Cerilliant (Round Rock, USA), Lipomed (Arlesheim, Switzerland), Cayman (USA), NMI Australian Government National Measurement Institute (Pymble, Australia). Reference compounds that are not commercially available were provided by WADA Accredited Laboratories of Cologne (Germany), Ghent (Belgium), Rome (Italy), Athens (Greece),

Barcelona (Spain) and Sydney (Australia), and the World Association of Antidoping Scientists (WAADS).

The deuterated internal standards of morphine 3- β -D-glucuronide-d3 and mefruside-d3 used for qualitative screening were obtained from TRC (Toronto, Canada). The deuterated internal standards used for quantitative screening were: 5 α -Dihydrotestosterone Sulfate-d3 (5 α -DHTS-d3), Testosterone sulfate-d3 (TS-d3) and Androsterone sulfate-d4 (AS-d4) were all purchased from NMI (Pymble, Australia).

Stock standard solutions of the standard analytes were individually prepared in methanol. For validation purposes, working standard solution containing the standard analytes was prepared in methanol by subsequent dilutions of the stock solutions. The sulfo-conjugated steroids analytes were included in a different working solution. All solutions were stored at -20°C in amber vials until use.

3.2.3 Sample Preparation

To 5 mL urine, 1 mL phosphate buffer at pH 7 (Na₂HPO₄ 0.8 M and NaH₂PO₄ 0.4 M), 100 μ L of β -Glucuronidase from *E. coli* and 50 μ L of a methanolic solution of mefruside-d3 (10 μ g/mL) morphine-3 β -D-glucuronide-d3 (5 μ g·mL⁻¹) were used as internal standards (ISTDs). After addition of the internal standards to the sample, the mixture was incubated for 1.5 h at 50 °C. After hydrolysis, the pH was adjusted to 9–10 with a mixture of sodium hydrogen carbonate and sodium carbonate (10:1) (w/w). A liquid–liquid extraction with 5 mL of ethylacetate was performed using anhydrous sodium sulfate as salting-out agent. After centrifugation, the organic layer was separated from the aqueous phase by freezing the sample at -80 °C. The organic phase was then acidified with 200 μ L of 3 M acetic acid in ethylacetate and evaporated under nitrogen stream at 50 °C. The remaining residue was reconstituted with 200 μ L of 80:20 LC mobile phase A/B (v/v) (see description of eluent at section 2.1.4.1). 20 μ L of the original urine were mixed with the reconstituted extract and 5 μ L of the mixture were injected to the LC/MS system for analysis.

3.2.4 Instrumentation

3.2.1.4.1 Chromatographic Conditions

A Dionex UHPLC system (Thermo Scientific, Bremen, Germany) was used for the chromatographic separation. The system consisted of a vacuum degasser, a high-pressure binary

pump, an autosampler with a temperature-controlled sample tray set at 7°C and a column oven set at 30°C. Chromatographic separation was performed at 30°C using a Zorbax Eclipse Plus C18 column (100 × 2.1 mm i.d., 1.8 µm particle size; Agilent Technologies). The mobile phase consisted of 5 mM ammonium formate in 0.02% formic acid (solvent A) and a mixture of acetonitrile/water (90:10 v/v) containing 5 mM ammonium formate and 0.01% formic acid (solvent B). A gradient elution program was employed at a constant flow rate of 0.2 mL/min with solvent B starting at 5% for 1 min, increasing to 32 % in 2.5 mins and staying constant at 32% for 13 mins and then, set back to 100% within 8 mins, where the eluent composition was held for 2.5 min before returning to 5% within 1 min. The analysis run time was 28 min and the post-run equilibrium time was 4 min. The injection volume was 5 µL.

3.2.4.2 Mass Spectrometric Conditions

The mass spectrometer was a QExactive benchtop Orbitrap-based mass spectrometer (Thermo Scientific, Bremen, Germany) operated in the positive–negative polarity switching modes and equipped with a heated electrospray ionization (HESI) source. Source parameters were: sheath gas (nitrogen) flow rate, auxiliary gas (nitrogen) flow rate and sweep gas flow rate: 40, 10 and 1 arbitrary units respectively, capillary temperature: 300°C, heater temperature: 30°C, spray voltage: +4.0 kV (positive polarity) and –3.8 kV (negative polarity). The instrument operated in FS mode from m/z 100 to 1000 at 17,500 resolving power and duty cycle of 100 ms for both polarities and in MS/MS mode from m/z 100 to 1000 at 17,500 resolving power and duty cycle of 62 ms (product ion mode) for the analytes that showed matrix interfering peaks in the FS mode. The automatic gain control (AGC) was to 10⁶. The mass calibration of the Orbitrap instrument was evaluated in both positive and negative modes daily and external calibration was performed prior to use following the manufacturer’s calibration protocol.

3.2.5. Method Validation

3.2.5.1. Qualitative Method Validation

The following validation parameters have been included in the experimental protocol and have been determined for all substances of interest. The identification capability is the ability of the method to detect the substance at the 50% of the Minimum Required Performance Levels (MRPL) concentration [3] in ten (10) different urine routine antidoping negative samples. In this study, 10

different urine matrices were spiked with the standard multicomponent solutions at concentrations equal to the 50% of MRPL or below (See Table 1). The specificity of the method was determined using the same experimental data obtained to assess the identification capability after checking the detection of substance of interest in the selected ion chromatographic time window obtained at specific m/z value. The absence of matrix interferences was evaluated by the analysis of the urine samples (blank sample) used also for the identification capability without being spiked. The Limit of detection (LOD) was determined as the lowest detectable concentration per substance with subsequent dilution cycles up to 1% of the concentration of the respective MRPL concentration. Sample carry over was evaluated by the detection of substances in blank urine samples injected after the injection of a urine sample spiked with the analytes at a concentration 20 times higher than the respective MRPL level. The target analyte recovery was determined for some selected analytes listed in Table 1 by comparing of the MS signal of the standards at 50% MRPL concentration spiked in urine sample prior sample preparation to the MS signal in urine sample spiked after sample preparation and prior evaporation step. Similarly, the matrix effects were determined for the same analytes by comparing the MS signal at the 50% MRPL concentration urine sample spiked after the extraction to the MS signal of the analytes spiked in the reconstitution solvent at equal concentrations. Matrix effects and extraction recovery were evaluated in 5 different urine matrices. The mass accuracy has been calculated in one urine sample used for identification capability experiment to assess eventual mass bias in the entire range of the full scan MS acquisition, for both ionization polarities.

3.2.5.2. Quantitative Validation

For the endogenous steroids' quantitative validation, the following validation parameters have been determined, in five experimental days. The linearity range of the calibration curves for each sulfate steroid in various concentrations has been evaluated. Seven points' calibration curves for quantification purposes were generated from spiking standards in steroid depleted urine samples. The depleted urine sample was prepared as follow: blank urine was collected from female children and was depleted from endogenous steroids using C18 SPE extraction. The linearity of the calibration curves was assessed in the concentration range of 1-200 ng/mL for TS, ES and 5 α DHTS; 10-2000 ng/mL for AS and EtioS and DHEAS. The calibration curves were built from the peak area ratio of sulfo-conjugates steroids and TS-d3 for TS and ES, AS-d4 for AS, EtioS and

DHEAS and TS-d3 for 5 α DHTS. The intermediate precision and the accuracy (% bias) for each sulfate steroid has been determined by the analysis of the spiked quality control (QC) samples at two concentration levels (low and high), which were prepared during 5 different experimental days and injected twice for each calibration curves. Both the intermediate precision and bias from the QC samples were used to estimate the combined Measurement Uncertainty (MU) for each steroid according to the following equation:

$$\text{Combined } MU = \sqrt{\text{intermediate precision}^2 + \text{Bias}^2}$$

3.3 Result and discussion

3.3.1 Method Development and Optimization

The method development was oriented to create a comprehensive analytical procedure to cover as many different small molecules as possible. Liquid-liquid extraction by ethylacetate as organic solvent was selected in order to achieve recovery of the sulfates phase II metabolites that remain unaffected after the enzymatic hydrolysis by the β -Glucuronidase from *E. Coli*. The extracted and reconstituted urine aliquot was enriched by 10% (v/v) original urine sample as part of D&S approach. The combined sample was analyzed by LC/MS. The D&S part of the analyzed sample is important for small molecules that are not extracted in the extraction process, such as melidonium, ethylglycuronide, FG4592, AICAR, finasteride carboxylic acid metabolite and ritalinic acid. The use of the ethylacetate as extraction solvent and the D&S contribution of the analyzed sample do not contaminate the LC/MS system and do not change the maintenance schedule of the LC/MS system compared with an LC/MS system used to analyze extracted aliquots only. Moreover, the liquid chromatographic column was replaced after approximately 1200 urine sample injections.

One of the main aspects during method development was to optimize the liquid chromatographic separation of AS to ETIOS. To achieve this goal in the current instrumental configuration and settings, several isocratic sections as part of the overall eluent gradient program, were tested to achieve the liquid chromatographic method described in the experimental part 2.1.4.1.

The MS acquisition was performed using an Orbitrap mass analyzer in order to maximize the advantages from simultaneous conditions of polarity switching, full scan in high-resolution mode and tandem MS acquisition, which provide the best performance for the selected substance classes that should be analyzed in complex sample with important matrix interferences. The MS resolution of the acquisition method defined as full width at half maximum (FWHM) at 200 m/z was of 70,000 and resulted in chromatographic peaks with 4 acquisition points for each polarity mode. Under those conditions, the introduction of simultaneous tandem MS acquisition would create long duty cycle leading to low sensitivity due to low number of sampling point of a chromatographic peak. In the current application, however the FWHM was set to 17,500, which resulted in 8-9 acquisition points for an average chromatographic peak in polarity switching mode, while addition of the acquisition up to three MS/MS resulted in 5-6 acquisition points per chromatographic peak. Respectively, a mass window of ± 5 ppm was applied for extraction of the ion chromatograms for all analytes of interest, except for stimulants and narcotics that are eluted at an early retention time where window of the extracted ion chromatogram window was set at ± 10 ppm.

3.3.2 Validation Results

3.3.2.1 Qualitative Validation Results

The identification capability, specificity, matrix interferences, LOD, recovery and matrix effects results for the validated target substances are shown in Table 1. Substances ionizable by ESI with difficulties to meet the validation specifications were analyzed by the GC/MS screening, like aminoglutethimide, 4-androstene-3,6,17-trione, HU-210 and norfenephine. Table 1 summarizes the results of qualitative validation and represents all classes of 304 prohibited substances that were detected with score 10/10 in the different urine matrices tested at concentrations equal to or below 50% of the MRPL. Our approach was able to detect even substances showing ion suppression more than 70% or extraction recovery less than 20%. For most of the substances the LOD are in the level of 10% MRPL or lower. Carry over between injections was not observed at injected concentration of 20 times higher than the MRPL levels [3].

Table 1: Theoretical and experimental data used for qualitative validation of 304 analytes.

Compound	Spiked Conc. (ng/mL)	RT ^a (min)	Main Ion	Ion Mass (m/z)	Secondary Ion	Ion Mass (m/z)	LOD ^a (ng/mL)	% Matrix Effect ^{a,b} (SD)	% Extraction Recovery ^{a,b} (SD)
<i>Diuretics and Masking Agents</i>									
Acetazolamide	100	5.1	[M-H] ⁻	220.9809	[M+H] ⁺	222.9954	2		
Althiazide	25	13.9	[M-H] ⁻	381.9762	[M+NH4] ⁺	401.0168	5		
Amiloride	100	5.0	[M-H] ⁻	230.0552	[M+H] ⁺	228.0406	2		
Asozamide	50	15.3	[M-H] ⁻	369.0001			1	-19.3 (7.2)	82.5 (4.8)
Bendroflumethiazide	100	21.4	[M-H] ⁻	420.0305	[M+NH4] ⁺	439.0711	2		
Benzthiazide	100	19.4	[M-H] ⁻	429.9762	[M+H] ⁺	431.9908	2		
Brinzolamide	50	6.45	[M+H] ⁺	384.0716			2.5	-29.5 (8.3)	93.3 (6.3)
Bumetanide	100	21.9	[M+H] ⁺	365.1166	[M-H] ⁻	363.1020	2		
Buthiazide	100	15.1	[M-H] ⁻	352.0198	[M+NH4] ⁺	371.0604	2		
Canrenone	100	22.8	[M+H] ⁺	341.2111			2		
Chlorothiazide	100	5.7	[M-H] ⁻	293.9415			2		
Chlorthalidone	100	7.6	[M-H] ⁻	337.0055	[M+H] ⁺	339.0201	2		
Cloпамide	100	9.0	[M+HCOO] ⁻	390.0896	[M+H] ⁺	346.0987	2		
Conivaptan	50	19.2	[M+H] ⁺	499.2129			1	-20 (21)	91.6 (6.5)
Cyclopenthiazide	100	21.5	[M-H] ⁻	378.0355			2	-25.1 (4.8)	28.3 (9.3)
Cyclothiazide	100	20.5	[M-H] ⁻	388.0198			10	-21.5 (3.8)	26.8 (9.4)
Dichlorophenamide	100	7.8	[M-H] ⁻	302.9073			2		
Dorzolamide	100	5.3	[M+H] ⁺	325.0345	[M-H] ⁻	323.0199	2	-48 (12)	84 (14)
Epitizide	100	16.5	[M-H] ⁻	423.9480			2	-2.5 (7.6)	35.1 (8.8)
Eplerenone	50	14.5	[M+H] ⁺	415.2115			1	5 (18)	101 (12)
Ethacrynic acid	100	20.9	[M-H] ⁻	301.0040	[M+H] ⁺	303.0185	2		
Furosemide	100	11.8	[M-H] ⁻	329.0004			2		
Hydrochlorothiazide	100	6.0	[M-H] ⁻	295.9570	[M+NH4] ⁺	314.9978	2		
Hydroflumethiazide	100	7.0	[M-H] ⁻	329.9836			1		
Lixivaptan	50	24.2	[M-H] ⁻	472.1234			5		
Mebutizide	50	22.0	[M-H] ⁻	380.0511			5		
Methazolamide	50	6.2	[M+H] ⁺	237.0111			1	5 (15)	42.2 (7.4)
Methylchlorothiazide	25	11.0	[M-H] ⁻	357.9495	[M+NH4] ⁺	376.9901	2		
Metolazone	100	12.8	[M+H] ⁺	366.0674	[M-H] ⁻	364.0528	2		

Compound	Spiked Conc. (ng/mL)	RT ^g (min)	Main Ion	Ion Mass (m/z)	Secondary Ion	Ion Mass (m/z)	LOD ^a (ng/mL)	% Matrix Effect ^{a,b} (SD)	% Extraction Recovery ^{a,b} (SD)
Indapamide	100	16.4	[M+H] ⁺	366.0674	[M-H] ⁻	364.0528	2		
Mozavaptan	50	9.6	[M+H] ⁺	428.2333			1	-12 (12)	92.8 (5.6)
Piretanide	100	21.0	[M+H] ⁺	363.1006	[M-H] ⁻	361.0864	2		
Polythiazide	100	21.4	[M-H] ⁻	437.9636	[M+NH4] ⁺	457.0042	2		
Probenecid	25	21.7	[M-H] ⁻	284.0962			1		
Quinethazone	50	6.3	[M+H] ⁺	290.0361			1	23.2 (7.2)	95.2 (5.1)
Relcovaptan	50	22.1	[M+H] ⁺	637.1285			1	21.5 (7.1)	96.1 (4.6)
Thiazide Artifact ACB ^e	100	5.4	[M-H] ⁻	283.9572			2		
Thiazide Artifact A TPB ^f	100	6.7	[M-H] ⁻	317.9835			2		
Tolvaptan	100	23.0	[M+H] ⁺	449.1626	[M-H] ⁻	447.1481	2	-44.2 (6.8)	94 (10)
Torsemide	100	8.65	[M+H] ⁺	349.1330	[M-H] ⁻	347.1183	2	-45.8 (9.9)	78.1 (6.3)
Triamterene	100	6.0	[M+H] ⁺	254.1147			2		
Trichlormethiazide	25	9.9	[M-H] ⁻	377.8949	[M-H] ^{-c}	379.892	1		
Xipamide	20	21.6	[M-H] ⁻	353.0368	[M+H] ⁺	355.0510	1		
Anabolic Agents									
16β-Hydroxyprostanazol	2.5	11.5	[M+H] ⁺	329.2224			1.25		
3-Hydroxyprostanazol	2.5	15.2	[M+H] ⁺	329.2224			0.6		
4-Hydroxyprostanazol	2.5	16.9	[M+H] ⁺	329.2224			0.6		
6β-Hydroxyfluoxymesterone	2.5	7.6	[M-H] ⁻	397.2032			0.25		
Boldenone	2.5	19.7	MSMS (+)	287.2 > 121.0650			0.25		
Methyldienolone	2.5	20.5	MSMS (+)	287.2 > 135.0806			0.6	-63.2 (6.4)	86.7 (6.8)
Boldione	2.5	21.2	[M+H] ⁺	285.1849			0.25		
Bolasterone	2.5	22.7	[M+H] ⁺	317.2475			0.25		
Calusterone	2.5	23.0	[M+H] ⁺	317.2475			0.25		
9α-fluoro-18-nor-17,17-dimethyl-4,13-diene-11β-ol-3-one (Fluoxymesterone met)	2.5	23.8	[M+H] ⁺	319.2068	[M+HCOO] ⁻	363.1977			
Fluoxymesterone	2.5	15.4	[M+H] ⁺	337.2173	[M+HCOO] ⁻	381.2083	0.5		
11α-Hydroxymethyltestosterone (Formebolone met)	2.5	11.1	[M+H] ⁺	319.2268			0.5		

Compound	Spiked Conc. (ng/mL)	RT* (min)	Main Ion	Ion Mass (m/z)	Secondary Ion	Ion Mass (m/z)	LOD ^a (ng/mL)	% Matrix Effect ^{a,b} (SD)	% Extraction Recovery ^{a,b} (SD)
2-hydroxymethyl-11 α ,17 β -dihydroxy-17 α -methylandroster-1,4-diene-3-one (Formebolone met)	2.5	8.3	MSMS (+)	347.2 >147.0804	347.2 >281.1898		0.25		
Gestrinone	2.5	22.3	[M+H] ⁺	309.1849			0.05		
16 β -Hydroxystanozolol	1	16.0	[M+H] ⁺	345.2537			1		
4 β -Hydroxystanozolol	1	15.1	[M+H] ⁺	345.2537			0.5		
3 [*] -Hydroxystanozolol	1	14.2	[M+H] ⁺	345.2537			1		
18-nor-17 β -hydroxymethyl,17 α -methylandroster-1,4,13-trien-3-one (Methandienone long term-met)	1	21.9	MSMS (+)	299.2 >147.0804	299.2 >269.1894		0.1	-71.2 (9.4)	98 (15)
Methyltrienolone	2.5	20.4	MSMS (+)	285.2 >227.1430	285.2 >159.0804		0.1	-67.1 (7.9)	90.3 (4.8)
Oxandrolone "night watch" met	*	22.1/22.7	MSMS (+)	305.2 >275.2006	305.2 >257.1900				
Stanozolol	1	22.3	[M+H] ⁺	329.2584			1		
THG	2.5	23.3	[M+H] ⁺	313.2162			0.25		
Trenbolone	2.5	17.2	[M+H] ⁺	271.1693			0.05		
17-Epi trenbolone	2.5	19.8	[M+H] ⁺	271.1693			0.05		
Mesterolone sulfate met	*	16.9	[M-H] ⁻	383.1898					
Mesterolone hydroxy sulfate met	*	8.5	[M-H] ⁻	399.1847					
Ractopamine	10	6.2	[M+H] ⁺	302.1751	[M-H] ⁻	300.1605	0.5		
LGD4033	1	23.7	[M+HCOO] ⁻	383.0836			0.1	-28.4 (4.9)	89.6 (5.4)
S1	2.5	23.9	[M-H] ⁻	401.0766			0.1		
S4 (Andarine)	2.5	21.9	[M-H] ⁻	440.1075	[M+H] ⁺	442.1219	0.05	-13.1 (5.4)	92.9 (9.1)
O-dephenyl andarine (Andarine met)	2.5	14.9	[M-H] ⁻	307.0547			0.025		
S9	2.5	24.4	[M-H] ⁻	417.0473			0.1		
S22 (Ostarine)	2.5	23.0	[M-H] ⁻	388.0915	[M+NH4] ⁺	407.1320	0.05	-14 (11)	88 (13)
O-dephenyl ostarine (Ostarine met)	2.5	11.5	[M-H] ⁻	287.0647			0.025		
Zilpatrol	2.5	4.7	[M+H] ⁺	217.0972			0.25		
BETA-2 Agonists									
Bambuterol	10	7.1	[M+H] ⁺	368.2182			0.2		
Brombuterol	5	7.2	[M+H] ⁺	366.9838			0.1	-15 (13)	88.7 (7.1)

Compound	Spiked Conc. (ng/mL)	RT ^g (min)	Main Ion	Ion Mass (m/z)	Secondary Ion	Ion Mass (m/z)	LOD ^a (ng/mL)	% Matrix Effect ^{a,b} (SD)	% Extraction Recovery ^{a,b} (SD)
Cimaterol	5	4.8	[M+H] ⁺	220.1444	[M+H-H ₂ O] ⁺	202.1338	0.1	+13 (13)	85.4 (4.1)
Cimbuterol	5	5.4	[M+H] ⁺	234.1601					
Clenpenetrol	5	7.5	[M+H] ⁺	291.1025			0.1	-23 (17)	86.2 (6.3)
Clenproperol	5	6.3	[M+H] ⁺	263.0712			0.1	-25 (16)	87.1 (3.8)
Fenoterol	10	5.5	[M+H] ⁺	304.1543					
Fenoterol Artifact		5.7	[M+H] ⁺	316.1543					
Formoterol	20	6.6	[M+H] ⁺	345.1809	[M-H] ⁻	343.1663	0.4		
Higenamine	10	5.3	[M+H] ⁺	272.1272			0.5		
Coclaurine	10	5.8	[M+H] ⁺	286.1438			0.5		
Indacaterol	5	12.2	[M+H] ⁺	393.2173			0.5	-35 (19)	77 (11)
Mabuterol	5	7.7	[M+H] ⁺	311.1133			0.1	-21 (11)	87.8 (5.2)
Mapenterol	5	9.0	[M+H] ⁺	325.1289			1.0	-30 (16)	85.5 (8.1)
Olodaterol	5	7.0	[M+H] ⁺	387.1914			0.1	-24 (17)	88.3 (5.8)
Pirbuterol	5	4.6	[M+H] ⁺	241.1547			2.0		
Procaterol	5	5.1	[M+H] ⁺	291.1703			0.5		
Reproterol Artifact	5	5.3	[M+H] ⁺	402.1772			0.5		
Ritodrine	10	5.7	[M+H] ⁺	288.1594			0.5		
Salbutamol	100	4.7	[M+H] ⁺	240.1594	[M-H] ⁻	238.1449	1.0		
Salmeterol	10	20.8	[M+H] ⁺	416.2795	[M-H] ⁻	414.2650	0.2		
Terbutaline	10	4.7	[M+H] ⁺	226.1438	[M-H] ⁻	224.1292	0.2		
Tulobuterol	5	6.6	[M+H] ⁺	228.1147			0.1	-24 (14)	86.1 (4.3)
Vilanterol	5	18.6	[M+H] ⁺	486.1809			0.1	-31 (16)	82 (13)
HORMONE AND METABOLIC MODULATORS									
6 α -Hydroxytestosterone (6-OXO met)	10	8.2	[M+H] ⁺	305.2111			0.5	-50 (12)	88.7 (4.2)
Anastrozole	10	16.0	[M+H] ⁺	294.1713			0.2		
Androsta-1,4,6-triene-3,17-dione (ATD)	8	21.0	[M+H] ⁺	283.1693			0.2		
17 β -Hydroxyandrost-1,4,6-triene-3-one (ATD met)	10	18.3	[M+H] ⁺	285.1849			0.2		91.5 (6.0)
Clomiphene 4-hydroxy met	*	21.7	[M+H] ⁺	422.1881					

Compound	Spiked Conc. (ng/mL)	RT ^g (min)	Main Ion	Ion Mass (m/z)	Secondary Ion	Ion Mass (m/z)	LOD ^a (ng/mL)	% Matrix Effect ^{a,b} (SD)	% Extraction Recovery ^{a,b} (SD)
Clomiphene-hydroxy-methoxy met	*	21.8/22.1	[M+H] ⁺	452.1987					
Clomiphene-N-desethyl-hydroxy met	*	21.3	[M+H] ⁺	394.1568					
Exemestane	10	22.5	[M+H] ⁺	297.1849			1		
17β-Hydroxyexemestane (Exemestane met)	10	21.9	[M+H] ⁺	299.2006	MSMS 299.2 > 135.0804		0.2	-62 (7.4)	91.9 (5.6)
GW1516	10	24.5	[M-H] ⁻	452.0607					
GW1516 sulfoxide		21.8	[M-H] ⁻	468.0557					
Bis(4-cyanophenyl)methanol (Letrozole met)	10	17.9	[M-H] ⁻	233.0720	[M+HCOO] ⁻	279.0775	1		
Melidonium	50	1.15	MSMS 147.11 > 58.0659				12.5		
Raloxifene	10	11.5	[M+H] ⁺	474.1734			1		
Tamoxifene 3-hydroxy-4-methoxy	10	22.2	[M+H] ⁺	418.2377			1		
Testolactone	10	12.6	[M+H] ⁺	301.1798			0.2		
Tetrahydrotestolactone	*	21.4	[M+H] ⁺	305.2111					
Toremifene	10	23.1	[M+H] ⁺	406.1932					
Toremifene-carboxy metabolite	*	13.8	[M+H] ⁺	402.2064					
Tamoxifene-carboxy metabolite									
Trimetazidine	10	5.6	[M+H] ⁺	267.1703			0.4	-43 (18)	68 (11)
AICAR	4700	1.5	[M+H] ⁺	259.1037	[M-H] ⁻	257.0891			
STIMULANTS									
1-Benzylpiperazine	50	5.1	[M+H] ⁺	177.1386			1		
1-(3-Chloro)phenyl-Piperazine	25	6.8	[M+H] ⁺	197.0840			1.25	-61.9 (7.7)	87 (15)
2-Amino-N-Ethyl-Phenylbutane	25	6.7	[M+H] ⁺	178.1590			1.25	-57 (12)	91.2 (5.0)
4-Ethylphenidine	50	6.8	[M+H] ⁺	194.1539	[M+H-H ₂ O] ⁺	176.1434	2.5	-38.6 (9.5)	88.8 (5.5)
4-Methylhexanamine	50	5.9	[M+H] ⁺	116.1434			1		
Tuaminoheptane	50	6.2	[M+H] ⁺	116.1434			1		
1,4-Dimethylpentylamine	50	6.0	[M+H] ⁺	116.1434					
1,3-Dimethylbutylamine	50	5.1	[M+H] ⁺	102.1277			1	+11 (26)	65 (12)
6-Hydroxybromantane	10	23.9	[M+H] ⁺	322.0801	[M+H] ⁺ ^d	324.0781	1		
Adrafinil	50	9.0	C ₁₃ H ₁₀	167.0855	[M-H] ⁻	288.0700	1		

Compound	Spiked Conc. (ng/mL)	RT ^g (min)	Main Ion	Ion Mass (m/z)	Secondary Ion	Ion Mass (m/z)	LOD ^a (ng/mL)	% Matrix Effect ^{a,b} (SD)	% Extraction Recovery ^{a,b} (SD)
Amiphenazole	50	5.1	[M+H] ⁺	192.0590			5		
Amphetamine	50	6.0	[M+H] ⁺	206.1539			1		
Amphetamine	50	5.6	[M+H] ⁺	136.1121	[M+H-NH ₃] ⁺	119.0855	1		
β-Methylphenethylamine	25	5.7	[M+H] ⁺	136.1121	[M+H-NH ₃] ⁺	119.0855	1.25	-42.0 (9.2)	79.6 (4.5)
Benfluorex	50	20.8	[M+H] ⁺	352.1519			1		
Benzylecgonine	50	6.0	[M+H] ⁺	290.1387			20		
Benzphetamine	50	9.4	[M+H] ⁺	240.1747			1		
Carphedone	50	6.8		219.1128		174.0913	1		
Cathine	50	5.0	[M+H-H ₂ O] ⁺	134.0964	[M+H] ⁺	152.1070	1		
Cathinone	25	5.1	[M+H] ⁺	150.0913			6.25		
Clobenzorex	50	10.8	[M+H] ⁺	260.1201			1		
Cropropamide	50	10.4	[M+H- C ₂ H ₅ NH ₂] ⁺	196.1332	[M+H] ⁺	241.1911	1		
Crotethamide	50	7.9	[M+H- C ₂ H ₅ NH ₂] ⁺	182.1176	[M+H] ⁺	227.1754	1		
Cyclazodone	50	8.2	[M+H] ⁺	215.0826		217.0972	1		
DiMethylamphetamine	50	6.0	[M+H] ⁺	164.1434			1		
Ethylamphetamine	50	6.2	[M+H] ⁺	164.1434			1		
Mephentermine	50	6.2	[M+H] ⁺	164.1434			1		
Dobutamine	50	6.2	[M+H] ⁺	302.1751	[M-H] ⁻	300.1605	5		
Ecgonine methyl ester	10	1.3	[M+H] ⁺	200.1281			1		
Ephedrine/Pseudoephedrine	50	5.3	[M+H] ⁺	166.1226	[M+H-H ₂ O] ⁺	148.1121	1		
Etafedrine	50	5.8	[M+H] ⁺	194.1539			1		
Ethamivan	50	8.1	[M+H] ⁺	224.1281			1		
Etilefrine	50	3.1	[M+H] ⁺	182.1176			20		
Etilefrine-Sulfate	*	3.0	[M-H] ⁻	260.0598	[M+H] ⁺	262.0744			
Famprofazone	50	23.5	[M+H] ⁺	378.2540	[2M+H] ⁺	755.5007	1		
Fenbutrazate	50	23.3	[M+H] ⁺	368.2220			1		
Fencamfamine	50	8.1	[M+H] ⁺	216.1747			1		
Fencamine	50	6.3	[M+H] ⁺	385.2346	[M-H] ⁻	383.2201	1		
Fenethyliline	50	6.6	[M+H] ⁺	342.1925			1		
Fenfluramine	50	8.6	[M+H] ⁺	232.1308			1		

Compound	Spiked Conc. (ng/mL)	RT ^g (min)	Main Ion	Ion Mass (m/z)	Secondary Ion	Ion Mass (m/z)	LOD ^a (ng/mL)	% Matrix Effect ^{a,b} (SD)	% Extraction Recovery ^{a,b} (SD)
Fenproporex	50	6.1	[M+H] ⁺	189.1386			1		
Flephedrone	50	5.6	[M+H] ⁺	182.0976			1	-38 (12)	42 (16)
Furfenorex	50	7.6	[M+H] ⁺	230.1539			1		
HMA	50	4.8	[M+H] ⁺	182.1176	[M+H-NH ₃] ⁺	165.0910	10	-35 (15)	55 (10)
HMA	50	5.0	[M+H] ⁺	196.1332	[M-CH ₃ NH] ⁺	165.0910	5	-18 (12)	71.7 (9.0)
Heptaminol	50	4.1	[M+H] ⁺	146.1539	[M+H-H ₂ O] ⁺	128.1434	1		
Isomethoprene	50	6.6	[M+H] ⁺	142.1590			1		
MDA	50	5.8	[M+H-NH ₃] ⁺	163.0754	[C ₈ H ₆ O ₂] ⁺	135.0440	1		
MDMA	50	6.0	[M+H] ⁺	194.1176	[M-CH ₃ NH] ⁺	163.0754	1		
Mefenorex	50	7.4	[M+H] ⁺	212.1201			0.2		
Mephedrone	10	6.1	[M+H] ⁺	178.1226	[M+H-H ₂ O] ⁺	160.1121	1		
Methedrone	25	6.0	[M+H] ⁺	194.1176			1.25	-42.4 (8.0)	78.9 (8.1)
Methoxyphenamine	50	6.3	[M+H] ⁺	180.1383	[M-CH ₃ NH] ⁺	149.0961	1		
Methylephedrine	50	5.5	[M+H] ⁺	180.1383	[M+H-H ₂ O] ⁺	162.1277	1		
Methylphenitate	50	6.7	[M+H] ⁺	234.1489			1		
Modafinil	50	9.7	C ₁₃ H ₁₀	167.0855			1		
Modafinil carboxylate met	50	8.0	C ₁₃ H ₁₀	167.0855			5	-46 (10)	3.1 (0.8)
Morazone	50	7.4	[M+H] ⁺	378.2176			1	-11 (13)	77.6 (4.0)
N,N-dimethylphenethylamine	25	5.5	[M+H] ⁺	150.1277			1.25	-44 (13)	77 (13)
Nikethamide	50	6.4	[M+H] ⁺	179.1179			1		
Norfenefrine-Sulfate	*	1.5	[M-H] ⁻	232.0285	[M+H] ⁺	234.0431			
Norfenfluramine	10	7.4	[M+H] ⁺	204.0995			0.25		
Octopamine	500	1.3	[M+H-H ₂ O] ⁺	136.0757			50		
Octopamine_sulfate	*	1.3	[M-H] ⁻	232.0285	[M+H] ⁺	234.0431			
Oxilofrine	50	2.0	[M+H] ⁺	182.1176			5		
Oxilofrine sulfate	*	2.0	[M+H] ⁺	262.0757	[M-H] ⁻	164.1070			
PEA	500	5.0	[M+H] ⁺	122.0969		260.0598		79.4 (9.9)	62.2 (6.4)
PVP	50	7.0	[M+H] ⁺	232.1696			2.5	60 (10)	94 (10)
Pemoline	50	6.1	[M+H] ⁺	177.0659			1		
Pentetrazol	50	6.0	[M+H] ⁺	139.0978			1		
Phendimetrazine	10	5.8	[M+H] ⁺	192.1383			0.2		

Compound	Spiked Conc. (ng/mL)	RT ^g (min)	Main Ion	Ion Mass (m/z)	Secondary Ion	Ion Mass (m/z)	LOD ^a (ng/mL)	% Matrix Effect ^{a,b} (SD)	% Extraction Recovery ^{a,b} (SD)
Phenmetrazine	50	5.8	[M+H] ⁺	178.1226			1		
Pholedrine	50	4.3	[M+H] ⁺	166.1226	[M-CH ₃ NH] ⁺	135.0804	1		
Prenylamine	50	22.0	[M+H] ⁺	330.2216			1		
Prolintane	50	8.4	[M+H] ⁺	218.1903			1		
Propylhexedrine	50	7.3	[M+H] ⁺	156.1747			1		
Ritalinic acid	50	5.9	[M+H] ⁺	220.1332			12.5	96.6 (6.9)	2.1 (0.3)
D-methamphetamine	50	5.7	[M+H] ⁺	150.1277			1		
Phenpromethamine	50	5.8	[M+H] ⁺	150.1277			1		
Ortetamine	50	6.2	[M+H] ⁺	150.1277			1		
Phentermine	50	6.0	[M+H] ⁺	150.1277			1		
p-Methylamphetamine	50	6.4	[M+H] ⁺	150.1277			1		
Selegiline	50	6.5	[M+H] ⁺	188.1434			1		
Selegiline desmethyl met	50	6.6	[M+H] ⁺	174.1280					
Sibutramine	50	20.9	[M+H] ⁺	280.1827			1		
Sibutramine desmethyl met	50	20.4	[M+H] ⁺	266.1670			2.5	40.9 (8.8)	89.9 (9.6)
Strychnine	50	6.0	[M+H] ⁺	335.1754			1		
Sydnocarb	50	22.6	[M+H] ⁺	323.1502			1		
p-Hydroxysydnocarb	10	16.4	[M+H] ⁺	339.1452			0.5	34.0 (8.8)	93.3 (5.5)
p-Hydroxyamphetamine	50	3.9	[M+H] ⁺	152.1077	[M+H-NH ₃] ⁺	135.0802	5		
NARCOTICS									
3-Methylfentanyl	1	12.3	[M+H] ⁺	351.2431			0.1		
6-Acetylmorphine	25	5.7	[M+H] ⁺	328.1543			0.5		
Buprenorphine	2.5	12.1	[M+H] ⁺	468.3108			0.05		
Norbuprenorphine	2.5	7.6	[M+H] ⁺	414.2638			0.25		
Methadone	5	19.8	[M+H] ⁺	310.2165			0.1		
EDDP (Methadone met)	25	13.2	[M+H] ⁺	278.1903			0.5	43.3 (5.4)	85.5 (8.7)
Fentanyl	1	9.9	[M+H] ⁺	337.2274			0.1		
Norfentanyl	1	6.2	[M+H] ⁺	233.1648			0.1		
Hydromorphone	25	4.8	[M+H] ⁺	286.1438			1		
Morphine	25	3.4	[M+H] ⁺	286.1438			5		
Oxycodone	25	5.7	[M+H] ⁺	316.1543			0.5		

Compound	Spiked Conc. (ng/mL)	RT ^g (min)	Main Ion	Ion Mass (m/z)	Secondary Ion	Ion Mass (m/z)	LOD ^a (ng/mL)	% Matrix Effect ^{a,b} (SD)	% Extraction Recovery ^{a,b} (SD)
Oxymorphone	25	4.3	[M+H] ⁺	302.1378	[M+Na] ⁺	324.1206	0.5		
Pentazocine	25	8.2	[M+H] ⁺	286.2165			0.5		
Pethidine	25	7.3	[M+H] ⁺	248.1645			0.5		
Racemoramide	25	19.8	[M+H] ⁺	393.2537			0.5		
Sufentanyl	1	15.7	[M+H] ⁺	387.2101			0.02		
CANNABINOIDS									
Carboxy-Δ9-tetrahydrocannabinol	75	25.0	[M-H] ⁻	343.1915	[M+HCOO] ⁻	389.1970			
JWH-250	0.5	25.8	[M+H] ⁺	336.1958			0.1		
JHW-250 5-hydroxypentyl met	0.5	22.7	[M+H] ⁺	352.1902			0.1	-44.6 (9.0)	87.8 (5.1)
JWH-200	0.5	22.4	[M+H] ⁺	385.1918			0.05		
JWH-122	0.5	27.5	[M+H] ⁺	356.2009			0.2		
JWH-73 N-(4-hydroxybutyl) met	0.5	23.0	[M+H] ⁺	344.1645			0.05		
JWH-73 N-butanolic acid met	0.5	22.9	[M+H] ⁺	358.1438	[M-H] ⁻	356.1292	0.05		
JWH-18 N-(4-hydroxypentyl) met	0.5	23.5	[M+H] ⁺	358.1802			0.2		
JWH-18 pentanoic acid met	0.5	23.2	[M+H] ⁺	372.1594			0.05		
GLUCOCORTICOIDS									
Beclomethasone	15	16.9	[M+H] ⁺	409.1776	[M+HCOO] ⁻	453.1686	1.5		
Betamethasone	15	14.4	[M+H] ⁺	393.2072	[M+HCOO] ⁻	437.1981	0.3		
Dexamethasone	15	14.9	[M+H] ⁺	393.2072	[M+HCOO] ⁻	437.1981	0.3		
6β-Hydroxybudesonide	15	12.5	[M+HCOO] ⁻	491.2287	[M+H] ⁺	447.2377	0.3	-45 (12)	90 (11)
Budesonide	15	22.2	[M+H] ⁺	431.2428	[M+HCOO] ⁻	475.2337	0.3		
Ciclesonide	15	27.7	[M+H] ⁺	541.3160			4		
Clobetasol propionate	15	24.0	[M+H] ⁺	467.1995			1	-65.5 (5.0)	88.5 (7.1)
Deflazacort 6βhydroxy desacetyl met	15	6.3	[M+H] ⁺	416.2068	[M+HCOO] ⁻	460.1970	1.5	-65.1 (7.0)	80 (11)
Deflazacort desacetyl met	15	10.4	[M+H] ⁺	400.2118	[M+HCOO] ⁻	444.2028	0.3		
Desonide	15	17.8	[M+HCOO] ⁻	461.2184	[M+H] ⁺	417.2272	0.3		
Fludrocortisone	15	10.5	[M+H] ⁺	381.2072	[M+HCOO] ⁻	425.1981	0.3		
Flumethasone	15	15.3	[M+HCOO] ⁻	455.1887	[M+H] ⁺	411.1978	0.3		
Fluocortolone	15	20.5	[M+H] ⁺	377.2123	[M+HCOO] ⁻	421.2032	1.5		

Compound	Spiked Conc. (ng/mL)	RT ^g (min)	Main Ion	Ion Mass (m/z)	Secondary Ion	Ion Mass (m/z)	LOD ^a (ng/mL)	% Matrix Effect ^{a,b} (SD)	% Extraction Recovery ^{a,b} (SD)
Fluorometholone	15	20.8	[M+H] ⁺	377.2123	[M+HCOO] ⁻	421.2032	0.75	-27 (11)	90.9 (3.4)
Fluprednisolone	15	10.0	[M+H] ⁺	379.1915	[M+HCOO] ⁻	423.1825	0.3		
Fluticasone 17β-carboxy met	15	15.8	[M+H] ⁺	397.1821	[M-H] ⁻	395.1676	1.5		
Fluticasone prop. 17β-carboxy met	15	21.9	[M+H] ⁺	453.2083	[M-H] ⁻	451.1938	0.3	-60.1 (9.4)	55.0 (5.4)
Methylprednisolone	15	13.3	[M+H] ⁺	375.2166	[M+HCOO] ⁻	419.2075	1.5		
Prednisolone	15	9.8	[M+H] ⁺	361.2009	[M+HCOO] ⁻	405.1919	1.5		
Prednisone	15	10.1	[M+H] ⁺	359.1853	[M+HCOO] ⁻	403.1762	1.5		
Triamcinolone acetonide	15	18.2	[M+H] ⁺	435.2177	[M+HCOO] ⁻	479.2087	0.3		
Flunisolide	15	18.9	[M+H] ⁺	435.2177	[M+HCOO] ⁻	479.2087	0.3		
Triamcinolone	15	6.8	[M+H] ⁺	395.1864	[M+HCOO] ⁻	439.1774	0.3		
BETA-BLOCKERS									
Acebutolol	50	6.4	[M+H] ⁺	337.2122	[M-H] ⁻	335.1976	0.5		
Alprenolol	50	9.3	[M+H] ⁺	250.1802			0.5		
Atenolol	50	4.9	[M+H] ⁺	267.1703			0.5		
Befunolol	25	6.6	[M+H] ⁺	292.1543			0.5	-3.2 (8.6)	91.1 (4.0)
Betaxolol	50	9.6	[M+H] ⁺	308.2220			0.5		
Bisoprolol	50	7.9	[M+H] ⁺	326.2326			0.5		
Bufarolol	25	10.5	[M+H] ⁺	262.1802			0.5	-14 (11)	86.9 (3.9)
Bupranolol	25	9.5	[M+H] ⁺	272.1412			0.5	-13 (12)	86.0 (5.1)
Carteolol	50	5.6	[M+H] ⁺	293.1860			0.5		
Carvedilol	50	15.5	[M+H] ⁺	407.1965	[M-H] ⁻	405.1821	0.5		
Celiprolol	50	7.0	[M+H] ⁺	380.2544	[M-H] ⁻	378.2399	0.5		
Esmolol	25	7.0	[M+H] ⁺	296.1856			1.25	-40 (10)	89.4 (8.1)
Labetalol	50	7.9	[M+H] ⁺	329.1860	[M-H] ⁻	327.1715	0.5		
Levobunolol	50	6.7	[M+H] ⁺	292.1907			0.5		
Metipranolol	25	8.7	[M+H] ⁺	310.2013			1		
Metoprolol	50	6.6	[M+H] ⁺	268.1907			0.5		
Nadolol	50	5.7	[M+H] ⁺	310.2013	[M+HCOO] ⁻	354.1921	0.5		
Nebivolol	25	20.2	[M+H] ⁺	406.1824			0.5	-35 (15)	77.8 (8.8)
Oxprenolol	50	7.5	[M+H] ⁺	266.1751			0.5		
Penbutolol	25	20.5	[M+H] ⁺	292.2271			0.5	-25.5 (7.7)	80.1 (6.2)

Compound	Spiked Conc. (ng/mL)	RT ^g (min)	Main Ion	Ion Mass (m/z)	Secondary Ion	Ion Mass (m/z)	LOD ^a (ng/mL)	% Matrix Effect ^{a,b} (SD)	% Extraction Recovery ^{a,b} (SD)
Pindolol	50	6.0	[M+H] ⁺	249.1598			0.5		
Propranolol	50	9.0	[M+H] ⁺	260.1645			0.5		
Sotalol	50	4.9	[M+H] ⁺	273.1267	[M-H] ⁻	271.1122	0.5		
Timolol	50	6.3	[M+H] ⁺	317.1642			0.5		
CONFOUNDING FACTORS									
Ethyl glucuronide	5000	1.1	MSMS	221.07 >75.0070	[M-H] ⁻	221.0660			
Finasteride carboxy-met	*	10.3	[M+H] ⁺	403.2591	[M-H] ⁻	401.2446			
Fluconazole	50	6.7	[M+H] ⁺	307.1110			1.0	-20 (12)	94.4 (5.1)
Miconazole	50	23.8	[M+H] ⁺	414.9924			1.0		
Ketoconazole	50	20.3	[M+H] ⁺	531.1560			2.5	-63 (14)	82.1 (8.3)
OTHER SUBSTANCES									
Roxadustat (FG4592)	1	23.1	[M+H] ⁺	353.1132			0.25	-17 (10)	54.2 (9.1)
Ibutamoren	1	16.3	[M+H] ⁺	529.2479			0.2		
Efaproxiral	10	22.0	[M-H] ⁻	340.1553	[M+H] ⁺	342.1700	0.2		
2-Hydroxyflutamide	*	19.6	[M-H] ⁻	291.0588					
Hydrocodone	5	5.9	[M+H] ⁺	300.1594					
Codeine	5	5.4	[M+H] ⁺	300.1594			0.1		
Pipradrol	50	7.7	[M+H] ⁺	268.1696	[M+H-H ₂ O] ⁺	250.1590	1		
Telmisartan	25	22.7	[M+H] ⁺	515.2442			0.5	-54 (10)	86 (13)
Tramadol	25	6.7	[M+H] ⁺	264.1958			0.5		
Bupropion	25	7.8	[M+H] ⁺	240.1150			1		
Caffeine		5.7	[M+H] ⁺	195.0877					
Mitraginine	25	13.4	[M+H] ⁺	399.2278					
Tapentadol	25	6.8	[M+H] ⁺	222.1852			0.5	-16 (10)	79.5 (8.3)
Norephedrine	10	4.8	[M+H] ⁺	134.0964	[M+H-H ₂ O] ⁺	152.1070	0.2	-17.3 (7.5)	89.1 (3.2)

*Compound available from excretion urine reference material

^aLOD, Matrix Effects and Extraction Recovery, were estimated according to the main ion intensities.

^b Matrix Effects and Extraction Recovery, were evaluated for a limited number of selective analytes

^c Isotope of ³⁷Cl, ^d Isotope of ⁸¹Br

^e4-Amino-6-chloro-1,3-benzenedisulfonamide

^f4-Amino-6(trifluoromethyl) benzene-1,3-disulfonamide

^gRT referred to retention time.

3.3.2.2 Quantitative Validation Results

The validation data related to the sulfate endogenous steroids are summarized as follow: the representative calibration curves of the 6 steroids are presented in Figure 1; precision, accuracy expressed as % of bias and combined measurement uncertainties of the steroids are shown in Table 2; calibration curves obtained following the protocol described in section 2.1.5.2 were calculated from the peak area ratio of sulfo-conjugates steroids and the deuterated standards except for AS and EtioS, calibration curves for AS and EtioS were calculated from the height ratio of the analytes due to the low chromatographic resolution and considerable overlap of the chromatographic peaks of AS and EtioS at high concentration level. The validation results demonstrated high analytical performance with respect of linearity, accuracy (2.4–17%), intermediate precision (1.2-4.1%), and combined measurement uncertainties (3.2-18%) over the range of 1–200 ng/mL for TS, ES and 5 α DHTS and over the range 10-2000 ng/mL for AS, EtioS and DHEAS. Weighted linear regression was applied to calculate the calibration curves due the wide concentration range of the analytes, which resulted in larger biases at low concentration levels when non-weighted linear regression was used. The analytical results described in this study can be extended to other exogenous and endogenous steroids.

Figure 1. Calibration curves of ES, TS, 5A-DHTS, DHEAS, AS and ETIOS.

Table 2: Summary of the data used for quantitative validation of endogenous sulfate steroids.

Compound name	RT ^a (min)	Ion (m/z)	QC level (ng/ml)		bias%		Intermediate precision (%)		MU%	
			QC1	QC2	QC1	QC2	QC1	QC2	QC1	QC2
TS	10.1	367.1585	2.5	100	17.5	14.0	3.8	4.1	17.9	14.5
ES	10.9	367.1585	2.5	100	14.1	5.7	3.2	3.4	14.4	6.6
5 α DHTS	13.2	369.1741	2.5	100	2.8	7.5	2.3	0.3	3.6	7.5
DHEAS	12.7	367.1585	25	1000	9.9	2.4	2.4	2.2	10.1	3.2
ANDROS	16.3	369.1741	25	1000	17.9	15.7	1.6	1.7	18	15.8
ETIOS	17.2	369.1741	25	1000	10.3	4.8	3.0	1.2	10.7	4.9
TS-d3	10.1	370.1773	Internal standards							
AS-d4	16.2	373.1992								
5 α DHTS -d3	13.0	372.1929								

^aRT referred to retention time

3.3.3 Application to Real Samples

The current method had been applied as screening method to official antidoping samples, among others, reported as Adverse Analytical Findings (AAF, positive case) based on the GC/MS technology detection of AAS abuse. The examples presented herein do not represent an exhaustive study of the sulfate metabolites of the respective AAS, but the detection of sulfate metabolites based on matching the m/z of the sulfate conjugate metabolites published in literature. The first example concerns with a sample contained a metabolite (1 α -methyl-5 α -androstane-3 α -ol-17-one) of the anabolic steroid of mesterolone at the concentration of 3 ng/mL, without the presence of mesterolone parent compound. Our LC/MS method was applied to this sample in order to test and corroborate the results obtained using standard GC/MS approach. Two mesterolone sulfate metabolites with parent ion m/z of 383.1898 and 399.1847 Da, and the non-sulfate metabolite detected by GC/MS with m/z of 488>433 were detected in full scan LC/MS data. Figure 2 shows the extracted ion chromatogram for mesterolone sulfate metabolites in the sample presented together with blank urine sample. These two mesterolone sulfate metabolites are considered to be markers of mesterolone abuse [31]. The second example is related to a positive case reported for the AAS nandrolone based on the GC/MS detection of the metabolites 19-Norandrosterone and 19-Noretiocholanolone from the free and glucuronide urine fraction at a concentration of approximately 50 ng/mL for 19-Norandrosterone. The current LC/MS method analysis revealed two peaks of m/z 355.1585 attributed to 19-Norandrosterone sulfate and 19-Noretiocholanolone

sulfate not present in the LC/MS of negative samples [27] (Figure 3). In a third example, a sample reported for boldenone detected by GC/MS at a concentration of the level of 500 ng/mL analyzed in parallel by the LC/MS method in MS/MS mode showed the peak m/z 365.1428>350.1188 for boldenone sulfate and epiboldenone sulfate [26] (Figure 4).

Figure 2. Extracted ion chromatograms at ± 5 ppm mass window of two mesterolone sulfate metabolites in a urine sample suspected for illegal use of mesterolone anabolic steroid and in a negative urine sample.

Figure 3. Extracted ion chromatograms at ± 5 ppm mass window of 19-norandrosterone sulfate and 19-noretiocolanolone sulfate in a urine sample suspected for use of nandrolone anabolic steroid and a negative sample.

Figure 4. Extracted ion chromatograms at ± 5 ppm mass window of boldenone sulfate and epi-boldenone sulfate in a urine sample suspected for use of nandrolone anabolic steroid and a negative sample.

3.4 Conclusion

The presented LC/MS screening method was developed and validated for the analysis of endogenous and exogenous substances, with aim to expands the analytical repertoire of small molecules to the detection of sulfate Phase II metabolites and allow the quantitative determination of endogenous sulfate steroids in their intact form. This becomes very important, because the sulfoconjugated steroid profile in this project can be analyzed in all samples without any further analysis and without creating any interferences to glucuroconjugated steroid profile required by WADA [4]. The use of the combined ethylacetate liquid-liquid extraction of hydrolysed urine and the D&S urine addition allow to extract both sulfate derivatives and intact steroids and drugs. In future study, we aim to assess the method for analysis of large number of steroid sulfate metabolites. This expansion is important due to fact that several studies [20,22,27] show that sulfate metabolites can be detected much later after steroid abuse due to the longer half-life of sulfate metabolites compared to non-conjugated forms. Besides the advantage of the detection time of sulfoconjugated metabolites of exogenous steroids, they are eliminated in greater quantities in comparison to their glucuronide analogues, resulting in better retrospective detection capabilities [32].

The high-resolution full scan LC/MS data with the polarity switching MS acquisition mode provides the fingerprint of all compounds detected by an LC/MS(/MS) system with several advantages over targeted analysis of compounds. These include the high MS specificity without reduction in the sensitivity of detection, the ability to identify all compounds, which can be detected by an LC/MS system in positive and negative ionization modes, the ability to reprocess the collected data retrospectively, to search for new metabolites, unknown prohibited designer drugs, and to obtain information on the overall metabolomic state of a person, which may reflect illegal use of performance enhancer compounds.

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Chapter 4

Gas Chromatographic Quadrupole Time-of-Flight Full Scan High-Resolution Mass Spectrometric Screening of Human Urine in Antidoping Analysis

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Abstract

This study presents the development and validation of a high-resolution full scan (FS) electron impact ionization (EI) gas chromatography coupled to quadrupole Time-of-Flight mass spectrometry (GC/Q-TOF) platform for screening anabolic androgenic steroids (AAS) in human urine samples. The World Antidoping Agency (WADA) enlists AAS as prohibited doping agents in sports, and our method has been developed to comply with the qualitative specifications of WADA to be applied for the detection of sports antidoping prohibited substances, mainly for AAS. The method also comprises of the quantitative analysis of the WADA's Athlete Biological Passport (ABP) endogenous steroidal parameters. The applied preparation of urine samples includes enzymatic hydrolysis for the cleavage of the Phase II glucuronide conjugates, generic liquid-liquid extraction and trimethylsilyl (TMS) derivatization steps. Tandem mass spectrometry (MS/MS) acquisition was applied on few selected ions to enhance the specificity and sensitivity of GC/TOF signal of few compounds. The full scan high-resolution acquisition of analytical signal, for known and unknown TMS derivatives of AAS provides the antidoping system with a new analytical tool for the detection designer drugs and novel metabolites, which prolongs the AAS detection, after electronic data files' reprocessing. The current method is complementary to the respective liquid chromatography coupled to mass spectrometry (LC/MS) methodology widely used to detect prohibited molecules in sport, which cannot be efficiently ionized with atmospheric pressure ionization interface.

4.1 Introduction

Anabolic Androgenic Steroids (AAS) are the most frequently used class of prohibited substances by athletes [1, 2] to boost their performance in sport activities. The detection of AAS in athletes' urine is a challenge for the doping control laboratories because of a) the low concentrations of the precursors and their metabolites, b) the low Minimum Required Performance Limits (MRPL) requested by the World Anti-Doping Agency (WADA) [3], c) the availability of designer steroids, which have similar activity but different chemical composition from the endogenous counterparts [4, 5], d) the continuous discovery of new long-term metabolites of AAS that extend their retrospectively considerably [6-9], and e) the rumored use of "micro dosing", where athletes are doping with small doses which provide concentration in body fluids sampled for anti-doping below the detection limit.

The doping control laboratories implement different analytical techniques in order to be able to detect a large variety of classes of prohibited substances. Mass spectrometry is the method of choice for the detection of the small molecules present in prohibited list of substances [1] combined either with gas chromatography (GC/MS) or with liquid chromatography (LC/MS). Due to their limited ionization efficiency, AASs are mainly screened by GC/MS [10, 11] and LC/MS are used only for AASs which cannot be efficiently derivatized for GC/MS analysis [12]. Regarding the LC/MS screening, WADA accredited laboratories use either LC triple quadrupole MS (LC/QQQ) [13-15] or LC High-Resolution MS (LC/HRMS) - orbitrap or TOF – mass analyzers [16, 17]. During the Olympic Games, London 2012 and Rio 2016, LC/HRMS technology was used [17]. On the contrary, regarding the GC/MS screening, merely low-resolution GC triple quadrupole technology (GC/QQQ) is used [10,11], while GC/HRMS is used only for special purposes as for the detection of Xenon [18]. To the best of our knowledge, there is only one article regarding the use of full scan (FS) GC/HRMS technology as screening tool in the doping control field [19]. The main advantages of FS/HR-MS compared to triple quadrupole technology are the significantly reduced background noise originating from the urine matrix and the capability to perform reanalysis of the samples by simply reprocessing the stored data files, whenever there is a special request for this such as when a new doping substance or its metabolite is discovered. The second feature (reanalysis) is gaining importance in sports drug testing, due to the impressive results that came out after the retesting of samples from the Beijing 2008 and the London 2012 Olympic

Games some weeks before the Rio 2016 Olympic Games took place. While the percentage of positive cases coming from the original analysis of samples during the Games was far less than 1% (0.13% in Beijing 2008 [20], 0.16% in London 2012 [21]), the reanalysis of samples in 2016 of 1243 samples from Beijing 2008 and London 2012 Games that was reported previously negative, lead to additional identification of 98 positive cases, constituting an astonishing percentage of 8% [22].

In addition to screening for the exogenous compounds, GC/MS screening is used for the quantification of markers of the urinary WADA Athlete Biological Passport (ABP) Steroid Profile (SP) [23]. Currently, the SP consists of Testosterone (T), Epitestosterone (E), Androsterone (A), Etiocholanolone (Etio) 5A-androstan-3A,17B-diol (5AAAdiol), 5B-androstan-3A,17B-diol (5BAAdiol), as well as the ratios T/E, A/Etio, 5AAAdiol/5BAAdiol, A/T, 5AAAdiol/E. The analytical method used should be fit-for-purpose and allow to cover the dynamic concentration range of listed compounds determined in both males and females. This means that the method should be able to quantify concentrations ranging from 2 ng/mL to more than 10 µg/mL in a single aliquot. The implementation of GC/HRMS for quantification of the compounds listed in ABP-SP presents a challenge in terms of the dynamic range of currently available instruments.

In this chapter, we described the use of high-resolution full scan gas chromatographic quadrupole Time-of-Flight mass spectrometry (GC/Q-TOF) to be used as screening platform for doping control purpose using FS and HRMS data. The method is validated for 73 analytes – mainly AAS but other categories of prohibited substances as well – at concentrations levels at or below the WADA MRPL [3]. Furthermore, this method is used for the quantification of the SP in ABP [23]. To assess the performance of FS HRMS GC/Q-TOF approach, we present a comparison between GC/QQQ and GC/Q-TOF profiling of SP measured in the same sample set. The prospect and the feasibility of the implementation of FS obtained with HR-MS as a routine screening method is discussed with aim to substitute the triple quadrupole method for doping control purpose.

4.2. Material and Methods

4.2.1 Reagents

Sodium hydrogen carbonate and diethyl ether were supplied by Merck (Darmstadt, Germany). Methanol (HPLC grade), 2-Propanethiol, di-potassium hydrogen phosphate trihydrate ($K_2HPO_4 \cdot 3H_2O$), potassium dihydrogen phosphate (KH_2PO_4), ammonium iodide (NH_4I), sodium bicarbonate ($NaHCO_3$) and sodium carbonate (Na_2CO_3) were supplied by Sigma Aldrich (Darmstadt, Germany). β -Glucuronidase from *Escherichia Coli* (*E. coli*) was supplied by Roche (Mannheim, Germany). MSTFA (N-Methyl-N-(trimethylsilyl) trifluoroacetamide) was supplied by Chemische Fabrik Karl Bucher (Waldstetten, Germany). Perfluorotributylamine (PFTBA) from Agilent.

4.2.2 Reference Materials

The following internal standards (ISTD) were purchased from LGC (Wesel, Germany): etiocholanolone-D5 (d5 Etio), androsterone glucuronide-D4 (d4A Glu), testosterone-D3 (d3T), epitestosterone-D3 (d3E), 5 β -androstane-3 α -17 β -diol-D5 (D5-5 β Adiol). The remaining reference materials of the study were purchased from LGC (Wesel, Germany), TRC (Toronto, Canada), Sigma Aldrich (Darmstadt, Germany), Steraloids (Newport, USA), and Cerilliant (Round Rock, USA). Stock standard solutions of the analytes were individually prepared in methanol. For validation purposes, working standard solution containing the analytes was prepared in methanol by subsequent dilutions of the stock solutions. All solutions were stored at -20°C in amber vials. The steroid profile analytes were included in a different working solution.

Urine samples from excretion study of dehydrochloromethyltestosterone (oral turinabol), Desoxymethyltestosterone (Madol), Oxymetholone, Methandienone, Oxandrolone were donated by the Doping Control Laboratory of Athens, Greece or provided by the World Association of Antidoping Scientists (WAADS).

4.2.1.3 Sample Preparation

Two and a half (2.5) mL of urine aliquot is hydrolysed by 50 μ L of beta-glucuronidase enzyme from *E. Coli* and incubated for 90 min in 50 °C after the addition of 25 μ L of ISTD mixture (d3T, d3E, d4A Glu, d5 Etio, d5-5 β Adiol) and 1 mL pH 7 phosphate buffer that was prepared by adding

169.8 g of $K_2HPO_4 \cdot 3H_2O$ and 54 g KH_2PO_4 in 1 L of water . After hydrolysis, the urine is buffered by $NaHCO_3$: Na_2CO_3 (10:1) and extracted at pH 9-10 by 5 mL diethyl ether. The sample is centrifuged at 3000 rpm for 12 min and the organic phase is separated from the aqueous phase in frozen conditions at $-80^\circ C$ and evaporated under nitrogen flow at $50^\circ C$. The residue was TMS derivatized by adding 50 μL of derivatization reagent MSTFA/ NH_4I /2-Propanthiol (1000:4:8) and was incubated in $100^\circ C$ for 60 min.

4.2.4 Instrumentation

4.2.4.1 GC/Q-TOF

The GC/MS system used in the current study is an Agilent GC 7890 coupled with an Agilent 7200 QTOF MS (G3850-64101) equipped with 5% Phenyl polysilphenylene-siloxane capillary column (30 m length, 0.25 mm ID, 0.1 μm film thickness, SGE BP X5) and back flush system. The quadrupole device prior the TOF MS analyzer provides the capability of applying MS/MS experiments. Helium was used as carrier gas with a constant flow set at 1.1 mL /min. Two microliters were injected in split mode of 20:1. The injection port and the interface temperatures were set at $280^\circ C$. Initial oven temperature was $160^\circ C$, ramped at $10^\circ C$ /min to $200^\circ C$, then ramped at $2^\circ C$ /min to $220^\circ C$, ramped at $6^\circ C$ /min to $292^\circ C$, $50^\circ C$ /min up to $310^\circ C$ and held for 3 min, total run time 29.36 min. Two (2) GHz extended dynamic range (EDR) acquisition mode was used for TOF data acquisition. The acquisition rate was 5 spectra per sec, 200 msec per spectrum, number of transients per spectrum was 2718. The used GC/MS has the capacity of acquiring MS data in high-resolution FS mode with a mass accuracy <5 ppm mass error in EI mode depending on the concentration of the analytes. The MS range (80-670 m/z) is capable of covering MS acquisition of all small molecules analyzed by the GC/Q-TOF. To correct for an eventual shift in m/z, a mass calibration procedure was introduced in the analysis sequence after every three aliquot injections. The instrument calibrator was Perfluorotributylamine (PFTBA, Agilent).

4.2.4.2 GC/QQQ

Agilent GC 7890 coupled with an Agilent 7000C QQQ MS is the routine GC/MS screening system of Antidoping Lab Qatar and it is equipped with an Agilent 7693 auto sampler with 10 μL syringe, split/splitless system and the same SGE BPX5 column that was used in the GC/Q-TOF system

described in the previous section. The same oven temperature program presented in 2.1.4.1 was followed. Injection volume was 2 μ L in a split ratio of 1:10. Helium was used as carrier gas at 1.1 mL/min flow for GC separation and EI at 70eV was used for compound ionization. Helium was used also as a quench gas at a flow of 2.25 mL/min and nitrogen as a collision gas at a flow of 1.5 mL/min. The data acquisition was performed in multiple reaction monitoring (MRM) acquisition mode with a collision energy ranging between 5 and 35 eV.

4.2.5. Method Validation

4.2.5.1. Qualitative Method Validation

In order to demonstrate the suitability of the FS/HR-MS method a validation process was carried out, where the guidelines of the WADA International Standard for Laboratories (ISL) [24] were followed. For that purpose, urine samples were collected by anonymous consented donors. Analysis of 10 different blank urine samples spiked with the reference material solution mixture of AAS at a concentration level of 50% of MRPL [3] was performed for the evaluation of Limit of Detection (LOD) and Identification Capability (IC) validation parameters. The chromatographic Signal to Noise (S/N) ratio of higher than 3 was used as detection criterion. The specificity of the developed method was evaluated by analyzing 10 different blank urine samples to demonstrate the absence of any interfering peaks at the retention times of the analytes of interest. The specific m/z and retention times were used to identify particular AAS analytes. The test for carryover was performed by analyzing a negative urine sample after the injection of the same urine sample spiked with the reference material solution mixture of AAS substances at a concentration level of 10 times the MRPL. The criterion to detect AAS compounds was used to assess the presence of carryover. The extraction recovery was characterized with percentage expressing the difference between the sample spiked with reference material solution mixture of AAS before the extraction and a sample spiked at the end of the extraction procedure with respect of peak height at a concentration level of 50% of MRPL.

4.2.5.2. Quantitative Validation of the ABP SP

Six points' calibration curves were made by spiking steroid stripped urine; blank urines were collected from female children and were stripped from endogenous steroids after C18 SPE

extraction and collection of the urine eluent. Calibration curves were analyzed in each day when validation was performed (total analysis time was 5 days) in the concentration range of interest per endogenous steroid present in SP of ABP. The calibration curves were established over the concentration ranges of 2-400 ng/mL for T and E; 100-8000 ng/mL for A and Etio; and 4-800 ng/mL for 5 α adiol and 5 β adiol. The calibration curves were built from the peak height ratio of steroids and the above referred standard reference mixture containing deuterated endogenous steroids. The assessment of concentration accuracy of the SP method was performed using two levels of spiked Quality Control (QC) samples, which were prepared and injected twice for each calibration curve. The accuracy of the method was estimated by calculating the (%) relative bias of the experimental concentration with respect of the theoretical concentration in the QC samples. The intermediate precision was determined from the data of the QC sample collected during the 5 different experimental days. Both the intermediate precision and bias from the QC samples were used to estimate the combined Measurement Uncertainty for each steroid. At the Antidoping Lab Qatar, the Agilent 7000C GC/QQQ is used as a routine antidoping screening GC/MS instrument for small molecules, similar to already published methods [10, 11]. For a period of three months, approximately 700 samples analyzed routinely with GC/QQQ in our laboratory were reanalyzed with the HRMS GC/Q-TOF for both qualitative and quantitative analytes.

4.3. Result and Discussion

4.3.1. Method Organization

The sample preparation as described in 2.1.3. based on the generic liquid-liquid extraction with diethyl ether at pH 9-10 and desalting step, which approach is commonly used to extract doping substances from urine matrix. Before the extraction step, the steroids deconjugation of the Phase II glucuronide conjugates was performed by enzymatic hydrolysis using the β -glucuronidase from *E. coli*, as indicated in [24]. The final step of the sample preparation was TMS derivatization of the extracts. Trimethylsilylation was performed by MSTFA/ammonium iodide/propanethiol mixture. Under these conditions, both the hydroxyl and the keto steroidal groups are derivatized [26]. A slow temperature gradient was applied in order to better separate and reduce matrix interferences spreading in a longer period of time in the background matrix and to achieve baseline chromatographic separation of isomers such as androsterone-di-TMS to etiocholanolone-di-TMS

and 5 α -androstane-3 α , 17 β -diol-di-TMS to 5 β -androstane-3 α , 17 β -diol-di-TMS. Apart from some exceptions, the analytes were detected in fortified urine samples at a concentration of 50% of MRPL; i.e. in 2.5 ng/mL for most of the AAS in FS MS mode. The FS-MS acquisition mode, in combination with the slow chromatographic temperature gradient program, allows potentially the detection of unlimited number of new analytes in the repertoire of the screening method without the need to modify the chromatographic conditions and revalidations of the existing method's substances.

Results evaluation comprised tracing the target analytes extracted ion chromatograms. The creation of extracted ion chromatograms m/z windows for proper evaluation of the MRPL and elimination of matrix interferences that may reveal minor chromatographic peaks of the prohibited substances is of utmost importance. The extraction of ion chromatograms was performed by the instrument's software (Agilent Mass Hunter Quantitative Analysis for QTOF version B.07.01). The optimized conditions for the evaluation of extracted ion chromatograms comprised the per-compound compilation [27] at a mass accuracy for each substance of ± 20 ppm. Other mass accuracies for the generation of the extracted ion chromatogram were tested between 20 and 100 ppm. We have found that the peak of interest is lost in extracted window lower than 20 ppm and increasing level of matrix interference was observed at 100 ppm.

4.3.2. Qualitative Validation Results

Table 1 shows the qualitative validation data for LOD, IC and recovery rates of AAS substances of the current method. Apart from a few exceptions, e.g. 1-testosterone metabolite, which has interference at low concentration and 13 β , 17 α -diethyl-3 α ,17 β -dihydroxy-5 α gonane, the LOD of 50% WADA MRPL of 2.5 ng/mL were achieved for the AASs listed in Table 1. Similarly, FS HRMS approach enables specific detection most of the AAS with low LOD. Other prohibited substances than AAS such as stimulants, narcotics, b2-agonists, diuretics, beta-blockers, and other AASs, analyzed by LC/MS, were not included in the current study and therefore not listed in Table 1. Several metabolites are not available as synthesized reference materials, but they are available in excretion urines. For these substances, the LOD and IC cannot be applied and only the specificity in blank urine samples is considered.

Figure 1 shows the typical mass errors of the representative AAS' screening diagnostic ions over the entire mass range at a concentration level of 2.5 ng/mL in urine matrix and FS acquisition mode. At all examples in Figure 1, 70% of the mass errors were lower than 5 ppm, 18% between 5 and 10 ppm and 12% of mass error were higher than 10 ppm. The mass errors were considered sufficient for the conditions of the AASs spiked concentrations, urine matrices and FS acquisition. The acquisition rate presented in 2.1.4.1. provided 30-50 data points which is sufficient to identify and quantify analytes peak in the acquired FS GC/MS data. The mass accuracy and the quantitative analysis were also influenced by the instrument's dynamic concentration range. More specifically, substances injected at concentrations greater than 1000 ng/mL resulted in saturation of the MS detector as indicated by the instrument's software. The signals of Etio and A above the 3rd highest calibration concentration of 1000 ng/mL showed sign of detector saturation. This problem was overcome by the use of the MSMS of EI GC/MS ions instead of FS acquisition and using ion transition of m/z 434.3031 to 419.2796 for Etio and A. On the other hand, in the lower concentration range, the detection of the beta2-agonist clenbuterol at 50% MRPL of 100 pg/mL cannot be achieved by FS mode, but only in MS/MS mode (Table 1, Figure 2). The acquisition for T and E was also performed in MS/MS mode to improve detection at 2 ng/mL concentration level and to differentiate from the close eluting 11- β OH-etiocholanolone.

Table 1 GC/Q-TOF analytes (Substance, Derivative, RT, Theoretical m/z of diagnostic ions, Recovery %, LOD)

Name	Derivative	RT (min)	Ions (m/z)	Recovery %	LOD, (ng/ml)	Detection in 10 urine aliquots
1-5A-Androstenedione	di-OTMS	15.08	430.2718, 415.2483,	82	2.5	
18-normetanol	OTMS	8.7	358.2686, 253.1951 , 216.1873	87	2.5	10
1androstene 3B,17B diol	di-OTMS	14.49	405.2640	117	2.5	9
1-Testosterone	di-OTMS	15.45	432.2874, 194.1121	97	2.5	10
3AOH-Tribolone	di-OTMS	16.31	443.2796	NA	NA	excretion urine
3BOH-Tribolone	di-OTMS	15.19	443.2769, 353.2295	94	2.5	10
17A-methyl-5A-androstane-3A,17B-diol	di-OTMS	15.69	270.2342, 450.3344	130	1	8
17A-methyl-5B-androstane-3A,17B-diol	di-OTMS	15.87	270.2342, 450.3344	94	1	10
5A-androstan-3A,17B-diol	di-OTMS	14.21	256.2186, 241.1951	NA	4 (as LOQ*)	10
5B-androstan-3A,17B-diol	di-OTMS	14.55	256.2186, 241.1951	NA	4 (as LOQ)	10
6-OXO androstenedione	tri-OTMS	18.34	501.2671, 516.2906	123	2.5	10
Androsterone msms	di-OTMS	13.58	434.3031 -> 419.2796	NA	100 (as LOQ)	10
Bolasterone metabolite (7α,17α-dimethyl-5β-androstane-3α,17β- diol)	di-OTMS	17.11	374.2999, 269.2264	99	2.5	9
Bolasterone PC	di-OTMS	18.33	460.3187, 445.2953	97	2.5	10
Boldenone metabolite (5β-androst-1-ene-17β-ol-3-one)	di-OTMS	12.43	194.1121	99	2.5	10
Calusterone met (7β,17α-dimethyl-5β- androstane-3α,17β-diol)	di-OTMS	16.8	284.2499, 374.2999, 269.2264	106	2.5	10
Calusterone PC	di-OTMS	18.56	460.3187, 445.2953	NA	2.5	9
Clenbuterol ms/ms	OTMS, NTMS	6.15	335.0690 -> 300.1001 335.0690 -> 227.0525	66	0.1	10
Clostebol met (4-chloroandro-4-en-3α- ol-17-one)	di-OTMS	17.49	466.2485, 468.2587	110	2.5	10
Cyclofenil ml	tri-TMS	20.24	422.2092	97	10	10

Name	Derivative	RT (min)	Ions (m/z)	Recovery %	LOD, (ng/ml)	Detection in 10 urine aliquots
D3-epitestosterone.	di-OTMS	15.86	435.3063, 420.2828	NA	NA	ISTD
D3-testosterone	di-OTMS	16.67	435.3063, 420.2828	NA	NA	ISTD
Danazol ml (Ethinisterone)	tri-TMS	18.64	456.2874, 441.2640	103	2.5	9
desoxymethyltestosterone I (17A-methyl-5A-androstan-2 ξ ,3A,16 ξ ,17B-tetrol)	tetra-OTMS	21.00	626.4033	NA	NA	excretion urine
desoxymethyltestosterone II (17A-methyl-5A-androstan-2 ξ ,3A,17B-triol)	tri-OTMS	15.15	523.3454	NA	NA	excretion urine
desoxymethyltestosterone M1 (17A-methyl-5A-androstan-2A,3A,17B-triol)	tri-OTMS	17.95	523.3454	NA	NA	excretion urine
desoxymethyltestosterone M2LT (18-nor17,17-dimethyl-5A-androst-13-en-2 ξ ,3A-diol)	di-OTMS	10.85	448.3187	NA	NA	excretion urine
Drostanolone PC	di-OTMS	16.75	448.3187	99	2.5	10
Drostanolone met (Drostanolone 3ol17one)	di-OTMS	14.26	448.3187	106	2.5	10
Epimetendiol	di-OTMS	12.55	358.2686, 448.3187	103	1	10
Epitestosterone	di-OTMS	15.99	417.2640, 432.2874	NA	2(as LOQ)	10
ethylestranol		12.4	270.2342	80	2.5	10
etiocholanolone msms	di-OTMS	14.11	434.3031 $\rightarrow$ 419.2796	NA	100 (as LOQ)	10
Fluxymesterone met(9 α -fluoro-18-nor-17,17-dimethyl-4,13-diene-11 β -ol-3-one)	di-OTMS	15.35	462.2780, 447.2545,	106	2.5	10
Formebolone met (Dealdehyde-formebolone)	tri-TMS	19.14	534.3375	97	2.5	10
Formestane	tri-OTMS	19.25	518.3062, 503.2828	95	20	10
Furazabol	OTMS	21.54	387.2462, 402.2697	94	2.5	10
furazanol met(16 β -hydroxyfurazabol)	di-OTMS	24.35	490.3042, 218.1153	54	2.5	10
Letrozol met	C ₁₇ H ₁₁ N ₅	9.91	217.0760, 291.0917	112	6.25	9
MDA	di-NTMS	5	188.1285	81	20	10
mesterolone met(1 α -methyl-5 α -androstane-3 α -ol-17-one)	di-OTMS	15.47	448.3187, 235.1513	89	2.5	10
Mesterolone PC	di-OTMS	16.3	433.2953, 448.3187	101	2.5	10

Name	Derivative	RT (min)	Ions (m/z)	Recovery %	LOD, (ng/ml)	Detection in 10 urine aliquots
Methasterone met(2A,17A-dimethyl-5A-androstane-3A,17B-diol)	di-OTMS	16.24	449.3266, 374.2999	118	2.5	10
Methasterone PC	di-OTMS	18.3	462.3344, 419.2796, 332.2530, 372.2843	105	2.5	7
Methenolone	di-OTMS	17.24	446.3031, 431.2796, 195.1200, 208.1278	100	2.5	9
Methenolone met (3 α -hydroxy-1-methylen-5 α -androst-17-one)	di-OTMS	15.06	446.3031, 431.2796	97	2.5	10
Methyl-1-testosterone	di-OTMS	17.24	446.3031, 431.2796, 194.1121, 356.2530	100	2.5	9
mibolone	di-OTMS	17.78	446.3031, 431.2796, 301.1982	89	2.5	10
Androsterone mono-TMS	mono-OTMS	13.46	272.2135	NA	NA	10
19-norandrosterone	di-OTMS	11.73	405.2640, 315.2139, 420.2874	116	1	9
19-Noretiocholanolone	di-OTMS	13.13	405.2640, 315.2139, 420.2874	116	2.5	10
Norclostebol	di-OTMS	20.15	452.2328, 417.2640	73	2.5	10
Norethandrolone m1 (17 α -Ethyl-5 α -estrane-3 α ,17 β -diol)	di-OTMS	16.65	241.1951, 331.2452	97	2.5	10
Norethandrolone m2(17 α -Ethyl-5 β -estrane-3 α ,17 β -diol)	di-OTMS	17.52	241.1951, 331.2452	107	2.5	10
Norfeneffrine	di-OTMS, di-NTMS	4.82	174.1129, 426.2131	2	50	10
OT M3(4-chloro-18-nor-17 B-hydroxymethyl,17 A-methyl-5 B-androstan-3 A-ol)	di-OTMS	18.5	379.2218, 343.2452, 253.1982	NA	NA	excretion urine
OT EPIM3(4-chloro-18-nor-17 A-hydroxymethyl,17 B-methyl-5 B-androstan-3 A-ol)	di-OTMS	17.5	379.2218, 343.2452, 253.1982	NA	NA	excretion urine
OT M4(4-chloro-18- nor-17 B-hydroxymethyl,17 A-methyl/andro-4-en-3 A-ol)	di-OTMS	18.4	377.2062, 287.1561	NA	NA	excretion urine
OT EPIM4(4-chloro-18-nor-17 A-hydroxymethyl,17 B-methyl/andro-4-en-3 A-ol)	di-OTMS	17.4	377.2, 287.1561	NA	NA	excretion urine

Name	Derivative	RT (min)	Ions (m/z)	Recovery %	LOD, (ng/ml)	Detection in 10 urine aliquots
OT II (4-chloro-3 A,6 B,17 B-trihydroxy-17 A-methyl-5 B-androst-1-en-16-one)	tetra-OTMS	21.3	656.3330	NA	NA	excretion urine
Oxabolone PC	tri-OTMS	18.67	506.3062	102	2.5	10
Oxymesterone	tri-OTMS	20.81	534.3375, 519.3141, 389.2327	84	2.5	10
stenbolone	di-OTMS	16.23	446.3031, 208.1278	106	2.5	10
Testosterone	di-OTMS	16.83	417.2640, 432.2874	NA	2(as LOQ)	10
Oxandrolone NW 1 (17A-hydroxymethyl-oxandrolone)	Mono-OTMS	18.4	273.1849	NA	NA	excretion urine
Oxandrolone NW 2(17B-hydroxymethyl-oxandrolone)	Mono-OTMS	18.6	273.1849	NA	NA	excretion urine
THCCOOH	di-OTMS	18.68	488.2773, 473.2538, 371.2401	80	5	10
5A-Zearalanol	tri-OTMS	19.3	433.2225	83	2.5	10
5B-Zearalanol	tri-OTMS	19.5	433.2225	97	2.5	10
Oxymethelone M1(18-nor-2ξ,17B-hydroxymethyl-17A-methyl-5A-androst-13-en-3A-ol)	tri-OTMS	19.90	447.3109, 357.2608	NA	NA	excretion urine
Oxymethelone M2 LT (18-nor-17B-hydroxymethyl-17A-methyl-2ξ –methyl-5A-androst-13-en-3A-one)	di-OTMS	17.20	357.2608	NA	NA	excretion urine

*LOQ: limit of quantification for the endogenous steroids.

Figure 1. Mass errors of representative AAS' diagnostic ions over the entire mass range.

Figure 2. Clenbuterol detection at 0.1 ng/ml. (A) blank and spiked urine in full scan mode. (B) blank and spiked urine in MS/MS mode

4.3.3. Quantitative Validation Results

The GC/Q-TOF method was validated also for the quantitative analysis of the six steroids of the steroidal ABP according to the approach described in [24]. Table 2 shows the validation results for the six endogenous steroids characterized by linearity range, correlation coefficient, slope and intercept, bias, intermediate precision and combined uncertainty. The combined uncertainty for the determination of A, Etio, 5 α adiol, 5 β adiol, T and E, as estimated during method validation in order to fulfill the requirements described in [24]. The Limit of Quantitation (LOQ) is considered the lowest concentration in the calibration curve for each steroid [24].

Table 2 Concentrations levels used for the construction of the calibration curves.

Compound name	Calibration range (ng/ml)	r ²	Slope	Intercept	Level* (ng/ml)	Intermediate precision (%)	Biases (%)	MU (%)**
Androsterone (A)	100-8000	0.999	0.00160	-0.18370	400	6.2	8.2	10.4
					2000	4.4	5.4	6.9
Etiocholanolone (ETIO)	100-8000	0.999	0.00140	-0.06240	400	5.2	7.5	9.2
					2000	2.8	5.7	6.3
5 α -androstadiol (5 α adiol)	4-800	0.995	0.01520	-0.26740	40	13.5	15.1	20.2
					200	8.7	8.9	12.5
5 β -androstadiol (5 β adiol)	4-800	0.993	0.00030	-0.00470	40	9.5	11.5	14.8
					200	5.9	7.5	9.5
Testosterone (T)	2-400	0.996	0.00030	-0.00250	20	7.9	11.5	14
					100	11.4	8.6	14.3
Epitestosterone (E)	2-400	0.995	0.00020	-0.00220	20	7.4	17.8	19
					100	9.8	9.8	13.8

*Referred to the level of QCs used.

** combined $MU = \sqrt{\text{intermediate precision}^2 + \text{Bias}^2}$

4.3.4. Analytical Performance Comparison GC/QQQ vs GC/Q-TOF

For a period of 3 months in 2016, approximately 700 aliquots selected from ongoing routine analysis of antidoping samples for screening and confirmatory purpose were analyzed with GC/Q-TOF in parallel to the routine GC/QQQ instrument. During analysis period of 3 months, the GC part of the GC/MS required only maintenance, which consisted of replacement of the liner and septum and cutting the beginning of the column. The criteria of the MS maintenance initiation, except those used to accept in-house quality control samples, is the mass accuracy and the stability of the mass calibration within sequence of multiple aliquots analysis. The, mass accuracy in the

system suitability mass calibration algorithm were kept below 3 ppm error for the PFTBA ions. Similarly, calibration of TOF mass axis ran within the analytical sequence every 3 samples injections, resulted in PFTBA mass errors at the level of 5ppm, which were corrected down to 1 ppm level. The lock mass correction of the mass calibration within the acquisition requires the simultaneous infusion of the calibrant PFTBA during sample analysis. This option was not used in order to avoid detector saturation and subsequent reduction of the instrument's dynamic range. The analytical performance of both GC/QQQ and GC/Q-TOF in terms of analytes qualitative detection was comparable; i.e. all findings as analytes detected in GC/QQQ were also detected in GC/Q-TOF in samples used for screening, confirmation and proficiency testing samples. In relation to the quantitative analysis of the six steroids of the ABP-SP, in Figure 3, the correlation graphs of the GC/Q-TOF estimated quantitative steroid profile versus the respective quantitative profile obtained with the accredited GC/QQQ are presented. For T and E, the LOQ in GC/QQQ was 1 ng/mL, while in GC/Q-TOF, it was 2 ng/mL. All correlation coefficients shown in Figure 3 were above 0.94 showing high agreement between the two screening platforms.

Figure 3. Correlation of SP measured with GC/QQQ and GC/Q-TOF characterized by correlation coefficients

4.3.5. Application of GC/Q-TOF to Real Samples and Verification of Reference Materials

Figure 4 shows the ion chromatogram of a real sample of the AAS nandrolone main metabolite 19-norandrosterone di-TMS estimated at 3.9 ng/mL is presented together with the respective full scan spectrum, to be compared with a Quality Control Positive sample spiked at 5 ng/mL. The mass accuracies of the most characteristics ions in the real sample and in the Quality Control Positive spiked samples are presented in Table 3. Similarly, Figure 5 shows the extracted ion chromatograms of newly discovered long-term metabolites of the AAS dehydrochloromethyltestosterone [6] M3 and epi-M3 in a sample obtained from an excretion study sample provided by WAADS. The mass accuracies of the main characteristic ions of M3 from this sample are presented in Table 4. This procedure is useful to verify the identity of non-commercially available reference materials, where the certificate of analysis does not exist.

Figure 4. Analysis of real sample of 19NA: (A) extracted ion chromatogram at 20 ppm mass window, of 19NA in an original urine sample estimated at 3.9 ng/mL. (B) full scan spectrum 19NA in an original sample. (C) extracted ion chromatogram at 20 ppm mass window, of 19NA in a positive control at 5 ng/mL. (D) full scan spectrum 19NA in a positive control.

Table 3 Mass accuracies for the main ions for 19NA in the original samples and positive control.

Theoretical mass	Real sample		QC sample	
	Experimental mass	Mass error(ppm)	Experimental mass	Mass error(ppm)
420.2874	420.2828	-10.9	420.2874	0
405.2640	405.2630	-2.5	405.2635	-1.2
315.2139	315.2122	-7.0	315.2114	-9.5

(All errors are negative, showing systematic negative bias).

Figure 5. Extracted ion chromatograms at 20 ppm mass window, of newly discovered long-term metabolites of the AAS dehydrochloromethyltestosterone M3 and epi-M3 in a real case and in a blank urine sample.

4.4. Conclusions

The development of a new GC/Q-TOF method for the screening and confirmatory GC/MS analysis of small molecules not subjected in LC/MS analysis for the WADA antidoping system is presented. The presented method was validated at LOD of 50% MRPL for the majority of the target analytes in FS acquisition mode. Our data shows that the SP profile obtained with high-resolution GC/Q-TOF meet the WADA specifications [24]. The quality of the FS fragment mass spectra obtained for representative AAS was proven by the achieved mass accuracy of the main

characteristic ions at the level of 15 ppm mass window. The specificity of the method for the studied AAS is similar to the routine method used at ADLQ based on tandem MS in a GC/QQQ instrument. This study demonstrated the robustness in terms of manufacturer and in-house ADLQ maintenance and instrument failures of the GC/Q-TOF instrument in a typical routine analysis workload where more than 700 samples were analyzed in a period of 3 months. The acquisition of FS data in the same MS cycle, together with possible tandem MS acquisitions, permits the retrospective analysis of the acquired GC/TOF data of sample analyzed for official antidoping purpose to detect other non-targeted substances. These non-targeted substances could be illegal designer drugs or AAS long-term metabolites that prolong AAS detection abuse in the human urine samples, and detection with our approach is possible even for analytes, which were not known at the time of the data acquisition.

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Comparison of Gas Chromatography Quadrupole Time-Of-Flight and Quadrupole Orbitrap Mass Spectrometry in Anti-doping Analysis: I. Detection of Anabolic-androgenic Steroids

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Abstract

World Anti-doping Agency (WADA) encourages drug testing laboratories to develop screening methods that can detect as many doping substances as possible in urine. The use of full scan high-resolution acquisition (FS/HR) with GC/MS for the detection of known and unknown TMS derivatives of AAS provides anti-doping testing bodies with a new analytical tool. The AAS extracted from urine samples by generic liquid-liquid extraction, after enzymatic hydrolysis, and trimethylsilyl (TMS) derivatization step. The extracted urine analyzed by GC/Q-TOF and GC/Q-Orbitrap to compare the performance of both instruments for the detection of 46 AAS in human urine. The quantitation of endogenous anabolic steroids and the ability of the two analytical platforms to comply with the requirements for testing as part of the WADA Athlete Biological Passport (ABP) was also assessed. Data presented shows that the analytical performance is in compliance with the WADA specifications for both instruments. The LOD(s) for both instruments are well below the 50% MRPL sensitivity level. The mass errors in the current study for the GC/Q-Orbitrap platform are lower compared to the respective of the GC/Q-TOF. The data presented herein proved that both molecular profiling platforms can be used for antidoping screening. The mass accuracies are excellent in both instruments, however the GC/Q-Orbitrap shows superior due to higher resolution compared to GC/Q-TOF.

5.1 Introduction

The World Anti-doping Agency (WADA) Prohibited List (WPL) [1] comprises prohibited classes of drugs with pharmacological activity. The small molecules included on the WPL are detected using mass spectrometry (MS) coupled with either liquid chromatography (LC) or gas chromatography (GC). The criteria used to select the method of analysis are determined mainly by the sensitivity, specificity and matrix effects that fulfil the specifications of the WADA International Standard for Laboratories (ISL) [2] and the WADA Minimum Required Performance Levels (MRPL) technical document TD MRPL [3]. The molecules analyzed by GC/MS are usually those with chemical structures that result in low ionization efficiency with LC/MS [4].

In the sports anti-doping field, GC/MS screening typically utilizes low-resolution GC triple quadrupole technology (GC/QQQ) [5]; however, a few methods have been published that utilize full-scan (FS) and high-resolution (HR) acquisition modes. In 2007, a time-of-flight (TOF) screening method was published [6] that could be used for the analysis of synthetic anabolic-androgenic steroids (AAS). However, the WADA specifications in 2007 were less demanding than the contemporary regulations. In another study, a limited screening method based on two-dimensional (2D) GC coupled to time-of-flight mass spectrometry was published that could be used for selected anabolic agents [7]. In another, more comprehensive study from the same research group, a screening method based on quadrupole-Orbitrap GC/MS was published for use in the detection of a large number of substances in urine, such as synthetic AAS, β -agonists, stimulants, narcotics, metabolic modulators and diuretics, that fulfilled the WADA sensitivity specifications [8]. Finally, in another recent study [9], a routine screening method that used GC/MS with a triple quadrupole instrument was adapted for use with a QTOF GC/MS instrument and subjected to full validation, in which the FS/HR MS acquisition mode was applied to detect and quantify exogenous and endogenous steroids [10].

However, FS/HR GC/MS technologies are more widely in other analytical fields than in anti-doping analysis. A recent review that focused on the use of GC/MS with TOF mass analyzers describes their use for the analysis of a large number of organic contaminants and residues present at trace levels for environmental, food safety and biological applications [11]. A recent example of the use of FS/HR GC/MS in the food analysis field combined atmospheric pressure chemical

ionization (APCI) with the use of a QTOF mass analyser for the analysis of the volatile components of olive oil [12].

The use of GC/MS coupled with TOF and QTOF mass analyser technologies and two-dimensional GC (GC×GC) has been recently reviewed [13]. The author observed that the popularity of GC×GC is increasing and the number of components that can be simultaneously detected is limited by characteristics of the MS system such as the dynamic range, resolution and scanning speed, and eluting GC×GC peaks. Moreover, several recent applications of this method to toxicology, clinical chemistry, food and environmental analysis has been recently reviewed [13]. In toxicology, the volatile organic compounds (VOCs) produced during the early stages of bodily decomposition can be detected using thermal desorption coupled to GC/GC/TOF MS, which could be used for a wide variety of forensic or epidemiologic purposes, such as during natural catastrophes with large numbers of collapsed buildings [14]. In clinical chemistry, the quantitative analysis of organic acids in urine has been performed using GC/GC/TOF MS to determine abnormal patterns that can indicate the presence of inherited disorders of organic acid metabolism [15]. In food analysis, GC/GC TOF MS has been applied to the investigation of dioxin-like micropollutants in animal-derived food matrices [16]. In environmental analysis, the profiling of short- and medium-chain chlorinated paraffins in sediment and fish samples using GC/GC/TOF MS with negative ionization has been demonstrated [17].

Along with molecular profiling using TOF mass analysers, the use of the GC/Q-Orbitrap has been recently developed. The use of FS/HR GC/Orbitrap MS with electron ionization was evaluated for use in pesticide residue analysis in fruit and vegetables [18]. In food analysis, GC and LC coupled to Orbitrap MS has been applied to the identification of substances that have migrated from nano-films in food packaging [19].

The advantages of the FS/HR-MS method for anti-doping screening analysis have been described by Friedmann *et al* [20]. The HR mass filter results in enhanced specificity and sensitivity and the reduction of background noise, and full scan acquisition allows for the detection of a virtually unlimited number of analytes, which can be identified by reprocessing of the acquired data files. Using FS/HR-GC/MS for the identification of designer drugs [21,22], and as well as novel metabolites that allow for the prolonged detection of substances with “zero tolerance,” such as AAS [23,24] has been demonstrated.

Synthetic AAS are prohibited [1] and present a challenge [3] for anti-doping screening laboratories, since their presence should still be detected at low concentrations for a long period of time after administration of banned substances. Moreover, endogenous AAS [10], such as testosterone (T) and prohormones, are administered exogenously to enhance endogenous AAS levels. Because there is no difference in the mass spectra produced by exogenously administered AAS and their endogenously produced counterparts, an indirect methodology based on the ratio of AAS metabolites is currently used to identify possible abuse of substances. To alleviate these challenges, the WADA introduced the steroid profile and WADA Steroidal Athlete Biological Passport (ABP), which contains the quantified results of endogenous AAS screening analyses conducted during the career of the professional athlete for the following parameters: Testosterone (T), Epitestosterone (E), Androsterone (A), Etiocholanolone (Etio), 5 α -androstane-3 α ,17 β -diol (5 α adiol), 5 β -androstane-3 α ,17 β -diol (5 β adiol) and the following ratios: T/E, A/Etio, 5 α adiol/5 β adiol, A/T, 5 α adiol /E, for each analyzed sample [10]. The concentrations of the markers, in addition to the ratios of AAS metabolites, are also used in the identification of possible abuse [25].

The goal of this study is the comparison of two FS/HR-GC/MS molecular screening technologies, which are based on the Agilent GC/Q-TOF and the Thermo GC/Q-Orbitrap, using the same sample aliquots and the same GC parameters and conditions for both instruments. Both the QTOF and Orbitrap mass analysers allow for high mass resolution, but the Orbitrap mass analyser generally produces higher-resolution spectra at a lower scanning speed and higher dynamic range, depending on the applied acquisition parameters, due its increased ion trapping ability. We present an analytical platform comparison study that uses these two MS analyzers for the detection of anabolic steroids and that includes the qualitative screening of synthetic AAS, the quantitative profiling of endogenous AAS and the reprocessing of the electronic data files for preventive analysis. In this article, the performance of the GC/Q-TOF and GC/Q-Orbitrap in the detection and quantitation of exogenous and endogenous AAS in the same urine samples is compared. The analysis comprises a limited number of analytes, including representative exogenous AAS and all of the endogenous AAS that are present in the steroidal ABP.

5.2 Materials and methods

5.2.1 Chemicals and Reagents

Sodium hydrogen carbonate and diethyl ether were supplied by Merck (Darmstadt, Germany). Methanol (HPLC grade), 2-propanethiol, di-potassium hydrogen phosphate, potassium dihydrogen phosphate, ammonium iodide and sodium bicarbonate were supplied by Sigma Aldrich (Darmstadt, Germany). β -Glucuronidase derived from *Escherichia coli* (*E. coli*) was supplied by Roche (Mannheim, Germany). MSTFA (N-methyl-N-(trimethylsilyl) trifluoroacetamide) was supplied by Carl Bucher (Waldstetten, Germany).

5.2.2 Reference Materials

The following internal standards (ISTD) were purchased from LGC (Wesel, Germany): D5-etiocholanolone (d5 Etio), D4-androsterone glucuronide (d4A Glu), D3-testosterone (d3T), D3-epitestosterone (d3E), and D5-5 β -androstane-3 α -17 β -diol (D5-5 β Diol). The remaining reference standard materials were purchased from LGC (Wesel, Germany), TRC (Toronto, Canada), Sigma Aldrich (Darmstadt, Germany), Steraloids (Newport, USA), and Cerilliant (Round Rock, USA). Standard stock solutions of the analytes were individually prepared in methanol. For validation purposes, standard working solutions containing the analytes were prepared in methanol by subsequent dilution of the stock solutions. The analytes from the endogenous steroid profile were diluted in a different working solution. All the solutions were stored at -20°C in amber vials.

5.2.3 Sample Preparation

The sample preparation was described in detail for a previous study [9]. In brief, it included a liquid-liquid extraction with diethyl ether at pH 9-10 and a desalting step, which is an approach that is commonly used to extract doping substances from the urine matrix. A clean extract was obtained after the separation of the organic layer from the aqueous phase following centrifugation and the cooling of the extraction tubes in an ethanol basin at -80°C. Prior to the extraction step, deconjugation of the Phase II steroid glucuronide conjugates was performed by enzymatic hydrolysis using the *E. coli*-derived β -glucuronidase as indicated in [10]. The final step of the sample preparation was the derivatization of the extracts with trimethyl silane (TMS), which was performed in a mixture of MSTFA, ammonium iodide and propanethiol; in these conditions, both the hydroxyl and the keto steroidal groups were derivatized [26] by the TMS group.

5.2.4 Instruments and Analytical Conditions

5.2.4.1 GC/EI/Quadrupole Time-Of-Flight Analysis Conditions

The GC/MS system used in the current study was an GC 7890 coupled with a 7200 Q-TOF mass spectrometer (G3850-64101; Agilent, Delaware, USA) equipped with a BPX5 5% phenyl polysilphenylene-siloxane capillary column (30 m length, 0.25 mm ID, 0.1 μ m film thickness; SGE, Victoria, Australia) and a back-flush system. The combination of the quadrupole device with the TOF MS analyzer provides the capability to conduct MS/MS experiments. Helium was used as a carrier gas at a constant flow of 1.1 mL/min. The injection port temperature was set to 280 °C, and the injection volume was set to 2 μ L with a split ratio of 20:1. The oven temperature was initially set at 160 °C, increased at 10 °C/min to 200 °C, then increased at 2 °C/min to 220 °C, followed by an increase at 6 °C/min to 292 °C and 50 °C/min to 310 °C, where it was held for 3 min. The analysis run time was 29.36 minutes. The interface temperature was set to 280 °C and the ion source was set to 250 °C. Electron ionization (EI) of the compound ionization was performed using 70 eV of electron energy. A 2 GHz extended dynamic range (EDR) acquisition mode was used for the TOF data acquisition. The acquisition rate was 5 spectra per sec (200 msec per spectrum), and the number of transients per spectrum was set to 2718. GC/Q-TOF has the capacity to acquire MS data in FS/HR mode with a mass accuracy of <5 ppm mass error in EI mode, depending on the concentration of the analytes. The applied MS range (m/z 80-670) allows for the measurement of small molecules typically analyzed with GC/Q-TOF. Automated mass calibration was performed after every three injections using perfluorotributylamine (PFTBA, Agilent).

5.2.4.2 GC-EI-Quadrupole Orbitrap Analysis Conditions

The second GC/MS system used in the current study was a GC/Q-Orbitrap (Q Exactive GC, Thermo Scientific, Bremen, Germany), equipped with an SGE BPX5 column (30 m length, 0.25 mm ID, 0.1 μ m film thickness). This system consisted of a TriPlus RSH autosampler, a TRACE 1310 GC with a hot split/splitless injector, an EI source, and a hybrid quadrupole Orbitrap (Q Exactive) mass spectrometer with HCD (higher energy collision-induced dissociation). The sample introduction was performed using the TriPlus RSH autosampler. Helium was used as carrier gas with a constant flow set at 1.1 mL/min. The same analysis conditions for the GC parameters described in the previous section (GC/EI/Quadrupole time-of-flight analysis

conditions) was used. EI at 70 eV was used for the compound ionization with a source temperature set at 250 °C. Full scan acquisition mode was applied with a mass range of m/z 80-670 with 1 μ sec scans, a resolving power of 60,000 at m/z 200 and an AGC target set at $3 \cdot 10^6$. The mass calibration procedure was performed before each acquisition batch using PFTBA. The internal mass calibration during the measurement was conducted using three different background ions that originated from the column bleed (m/z 207.03236/ $C_2H_{15}O_3Si_3^+$, 281.05115/ $C_7H_{21}O_4Si_4^+$ and 355.06994/ $C_9H_{27}O_5Si_5^+$) with a search window of ± 2 ppm.

5.2.5 Qualitative Analysis

The validation of the screening method on both instruments was performed according to ISL guidelines [2]. In this procedure the following parameters were evaluated and validated: the identification capability, the specificity and the limit of detection. To assess the retention time for each compound, a mixture of standards at a high concentration (10-fold of the MRPL) was injected into both instruments. The evaluation of the compound identification capability based on the retention time was performed using 10 different blank urine samples that were spiked with a standard mixture of 46 synthetic anabolic steroids at concentrations of 50% of the MRPL [3]. To evaluate the specificity of the developed methods, 10 blank urine samples were analyzed in order to demonstrate the absence of any interference. The limit of detection (LOD) was determined by spiking the urine samples with a mixture of standards at 10%, 25%, 50% and 100% of the MRPL. The LOD is the lowest concentration at which a compound can be detected with sufficient data points and the elimination of background interference. The validation results showed that the analytical methods that were used in this study to detect AAS in urine are in compliance with the criteria laid out in the WADA ISL guidelines.

5.2.6 Quantitative Analysis

The method used for quantitative compound profiling was validated on both instruments. The validation process was carried out over a period of three days. The linearity, precision, accuracy and combined measurement uncertainty were evaluated. The calibration curves for quantification purposes were generated from stripped urine samples, which were blank urine samples collected from female children and depleted of endogenous steroids using C18 SPE extraction and collection of the eluent to avoid any interference with the endogenous steroids in the urine matrix, that were spiked with the standards. The calibration curves were obtained by measuring the levels of

endogenous AAS included in the steroid profile of the ABP at seven different concentrations of standards; concentrations of 2-400 ng/mL were used for T and E, 100-8000 ng/mL for A and Etio, and 4-800 ng/mL for 5 α adiol and 5 β adiol. The ratio of the peak height (m/z) that was obtained for the analyte to the peak height of the internal standard was calculated and plotted against the concentration of the added analyte. Linearity was determined by using the weighed linear regression model ($W=1/X$, where X is the concentration of the analyte). The precision and accuracy of the method were determined using a level of spiked quality control (QC) samples that corresponded to level 5 of the calibration curve, which were prepared in four different aliquots. The analysis was performed using 4 replicates for each level for each day ($n=4$) over a period of three days ($n=12$). The intermediate precision was determined from the data obtained from the QC samples collected during the 3 days of the experiments. Both the intermediate precision and accuracy (% bias) that were calculated based on the QC samples were used to estimate the combined measurement uncertainty (MU) for each steroid according to the following equation:

$$\text{Combined } MU = \sqrt{\text{intermediate precision}^2 + \text{Bias}^2}$$

5.3 Results and Discussion

The current study was conducted using the same samples and chromatographic conditions in two different laboratories: Anti-Doping Lab Qatar (ADLQ) in Qatar (GC/Q-TOF) and RIKILT-Institute of Food Safety (GC/Q-Orbitrap) in the Netherlands. The same sample set was analyzed on both analytical platforms within two weeks period in order to reduce possible variability due to sample stability. The methods applied herein were based on that of a previous study [9], with the exception of the GC/Q-Orbitrap MS method, which is described in the Materials and Methods section. The entire sample preparation procedure was conducted at ADLQ, where the aliquots were subsequently analyzed using Q-TOF. Afterwards, the aliquots were safely sent to RIKILT at room temperature and reanalyzed using the GC/Q-Orbitrap instrument with the same chromatographic conditions, including identical injection port liner, chromatographic column and temperature parameters. The only difference in the two chromatographic systems was the inclusion of a back-flush device installed on the Q-TOF instrument. In this study, representative AAS were selected for analysis. AAS are considered to be drugs with anabolic pharmacological activity that is highly beneficial for the enhancement of athletic performance, even long after administration.

Consequently, AAS are required to be detected by anti-doping laboratories at the lowest possible concentrations, even below the values specified by WADA [3]. To this end, AAS were analyzed at concentrations close to their limit of detection (LOD). The measured AAS analytes included endogenous AAS that are part of the ABP SP, AAS analytes that are easily detected by GC analysis, as well as analytes that are problematic for GC analysis due to matrix interferences.

The MS tuning and calibration procedures were applied to both instruments according to the manufacturers' specifications. The full width at half maximum (FWHM) resolution was set to 60,000 (specified at m/z 200) during the tuning procedure for the Q-Orbitrap and to 12,000 (specified at m/z 272) for the Q-TOF. These conditions, in addition to the conditions described in the instruments and analytical conditions section, provided sufficient data points to identify and quantify the analyte peaks in the FS/HR-GC/MS data; there were 20-30 and 30-50 data points across the chromatographic peaks for the Q-Orbitrap and the Q-TOF data, respectively. For the GC/Q-Orbitrap, the mass accuracy reached 2 ppm error due to the use of lock mass recalibration with ions (m/z 207.03236, 281.05115, and 355.06994) that originated from the column bleed. The GC/Q-TOF method did not include a lock mass procedure during acquisition; however, the mass calibration procedure was repeated after three sample injections.

5.3.1 Qualitative Analysis Results

Table 1 summarizes the qualitative parameters used to validate the AAS screening conducted using both instruments. The GC/MS method provided sufficient data quality to discriminate between the MS signals derived from each of the investigated analytes using both instruments. Figure 1 shows the comparison of the mass accuracies for the molecular ions and other fragment ions produced by the analyzed AAS from Table 1, which were spiked at 2.5 ng/mL as a function of the m/z . Table 2 summarizes the reproducibility of the mass accuracy for the GC/Q-TOF and GC/Q-Orbitrap analyses of 10 different urine samples using the base peaks of the selected AAS. Overall, the mass errors for the GC/Q-Orbitrap platform in the current study are lower than those of the GC/Q-TOF system. Considering that the analyzed samples and chromatographic systems in both analytical platforms were identical, it can be concluded that the mass accuracy of GC/Q-Orbitrap is higher compared to GC/Q-TOF, which is attributed to higher MS resolution of the Orbitrap mass analyzer. Actually, the image current detection coupled with efficient fast Fourier transformation has been successfully implemented in mass analysers such as Orbitrap, allowing the highest mass

resolution achievable today. Orbitrap mass analyzers operate with good sensitivity due to its trapping capability, high mass resolving power up to 150,000 at 200 m/z with reasonable duty cycle (< 1 second) and mass accuracy below five part per million (ppm). On the other side, the current TOF technology can achieve mass resolving power up to 50,000 at 400 m/z and mass errors which are above 10 ppm.

The identification capability parameters [2], which are based on the detectability of the AAS within the different urine matrices at 50% of the MRPL concentration [3], are shown in Table 1. The LOD for each AAS is also shown in Table 1.

Table 1 : Summary of the results of the qualitative analysis.

	Analytes (TMS derivatives)	Chemical formula	RT (min)	Ions (<i>m/z</i>)			Spiked conc ^a (ng/mL)	LOD (ng/mL)		Detection in 10 urine samples	
1	18-Normetanol	C ₂₃ H ₃₈ O ₁ Si ₁	8.7	358.2686	253.1951	216.1873	2.5	GC/Q-TOF	1.25	GC/Q-TOF	10
2	1-Androstene 3 β ,17 β -diol	C ₂₅ H ₄₆ O ₂ Si ₂	14.49	405.2637			2.5	GC/Q-TOF	1.25	GC/Q-TOF	9
3	1-Testosterone	C ₂₅ H ₄₄ O ₂ Si ₂	15.45	206.1121	194.1121		2.5	GC/Q-TOF	1.25	GC/Q-TOF	9
4	3 α OH-Tibolone	C ₂₇ H ₄₆ O ₂ Si ₂	16.31	443.2796	353.2295		2.5	GC/Q-TOF	0.63	GC/Q-TOF	10
5	3 β OH-Tibolone	C ₂₇ H ₄₆ O ₂ Si ₂	15.19	443.2796	353.2295		2.5	GC/Q-TOF	0.63	GC/Q-TOF	10
6	3'OH Stanozolol ml	C ₃₀ H ₅₆ N ₂ O ₂ Si ₃	24.17	560.3644	545.3409		1	GC/Q-TOF	1.00	GC/Q-TOF	8
7	17 α -Methyl-5 α -androstane-3 α ,17 β -diol	C ₂₆ H ₅₀ O ₂ Si ₂	15.69	450.3344	270.2342	255.2107	1	GC/Q-TOF	1.00	GC/Q-TOF	9
8	17 α -Methyl-5 β -androstane-3 α ,17 β -diol	C ₂₆ H ₅₀ O ₂ Si ₂	15.87	450.3344	270.2342	255.2107	1	GC/Q-TOF	1.00	GC/Q-TOF	9
9	6-Oxo-androstenedione	C ₂₈ H ₄₈ O ₃ Si ₃	18.34	516.2906	501.2671		2.5	GC/Q-TOF	0.63	GC/Q-TOF	10
10	Bolasterone met (7 α ,17 α -dimethyl-5 β -androstane-3 α ,17 β -diol)	C ₂₇ H ₅₂ O ₂ Si ₂	17.11	374.2999	284.2499	269.2264	2.5	GC/Q-TOF	1.25	GC/Q-TOF	10
11	Boldenone met (5 β -androst-1-ene-17 β -ol-3-one)	C ₂₅ H ₄₄ O ₂ Si ₂	12.43	194.1121	206.1121		2.5	GC/Q-TOF	1.25	GC/Q-TOF	10
12	Calusterone met (7 β ,17 α -dimethyl-5 β -androstane-3 α ,17 β -diol)	C ₂₇ H ₅₂ O ₂ Si ₂	16.8	374.2999	284.2499	269.2264	2.5	GC/Q-TOF	2.50	GC/Q-TOF	10
13	Calusterone	C ₂₇ H ₄₈ O ₂ Si ₂	18.56	460.3187	445.2953		2.5	GC/Q-TOF	2.50	GC/Q-TOF	10
14	Clenbuterol	C ₁₈ H ₃₄ N ₂ Cl ₂ O ₁ Si ₂	6.15	300.1007	335.06890		0.1	GC/Q-TOF	0.10	GC/Q-TOF	7

	Analytes (TMS derivatives)	Chemical formula	RT (min)	Ions (<i>m/z</i>)			Spiked conc ^a (ng/mL)	LOD (ng/mL)		Detection in 10 urine samples	
15	Clostellol met (4-chloroandro-4-en-3 α -ol-17-one)	C ₂₅ H ₄₃ O ₂ ClSi ₂	17.49	466.2485	468.2587		2.5	0.63	0.63	10	10
16	Danazol met (Eflisterone)	C ₂₇ H ₄₄ O ₂ Si ₂	18.64	456.2874	441.2640		2.5	1.25	0.63	9	10
17	Drostanolone PC	C ₂₆ H ₄₈ O ₂ Si ₂	16.75	448.3187			2.5	0.63	0.63	10	10
18	Drostanolone met (Drostanolone 3 α 17one)	C ₂₆ H ₄₈ O ₂ Si ₂	14.26	448.3187			2.5	0.63	0.63	9	10
19	Epimetendiol	C ₂₆ H ₄₈ O ₂ Si ₂	12.55	358.2686	343.2452	448.3187	1	1.00	0.25	9	10
20	Fluxymesterone met(9 α -fluoro-18-nor-17,17-dimethyl-4,13-diene-11 β -ol-3-one)	C ₂₆ H ₄₃ O ₂ F ₁ Si ₂	15.35	462.2780	447.2545	357.2076	2.5	1.25	1.25	10	10
21	Formebolone met (Dealdehyde-formebolone)	C ₂₉ H ₅₄ O ₃ Si ₃	19.14	534.3375	339.2170		2.5	0.63	0.63	10	10
22	Formestane		19.25	518.3062	503.2828		20			10	10
23	Furazabol	C ₂₃ H ₃₈ N ₂ O ₂ Si ₁	21.54	402.2697	387.2462		2.5	2.50	0.63	9	10
24	Furazanol met(16 β -hydroxyfurazabol)	C ₂₆ H ₄₆ N ₂ O ₃ Si ₂	24.35	490.3042	218.1153		2.5	1.25	0.63	10	10
25	Mesterolone met(1 α -methyl-5 α -androstan-3 α -ol-17-one)	C ₂₆ H ₄₈ O ₂ Si ₂	15.47	448.3187	433.2953		2.5	0.63	0.63	10	10
26	Mesterolone PC	C ₂₆ H ₄₈ O ₂ Si ₂	16.3	141.0730	433.2953		2.5	1.25	1.25	10	10
27	Methasterone met(2 α ,17 α -dimethyl-5 α -androstan-3 α ,17 β -diol)	C ₂₇ H ₅₂ O ₂ Si ₂	16.24	449.3266	374.2999		2.5	2.50	2.50	10	10

	Analytes (TMS derivatives)	Chemical formula	RT (min)	Ions (<i>m/z</i>)			Spiked conc ^a (ng/mL)	LOD (ng/mL)		Detection in 10 urine samples	
28	Methasterone PC	C ₂₇ H ₅₀ O ₂ Si ₂	18.3	462.3343	419.2796	332.2530	2.5		GC/Q-TOF	GC/Q-TOF	9
29	Methenolone	C ₂₆ H ₄₆ O ₂ Si ₂	17.24	446.3031	431.2796	195.1199	2.5	1.25	0.63	5	5
30	Methenolone met (3 α -hydroxy-1-methylen-5 α -androstane-17-one)	C ₂₆ H ₄₆ O ₂ Si ₂	15.06	446.3031	431.2796	251.1826	2.5	1.25	0.63	8	9
31	Methyl-1-testosterone	C ₂₆ H ₄₆ O ₂ Si ₂	17.24	446.3031	431.2796	356.2536	2.5			7/inter	inter
32	Mibolerone	C ₂₆ H ₄₆ O ₂ Si ₂	17.78	446.3031	431.2796	301.1982	2.5	1.25	0.63	10	10
33	19-Norandrosterone	C ₂₄ H ₄₄ O ₂ Si ₂	11.73	405.2640	315.2139		1	0.25	0.25	9	10
34	19-Noretiocholanolone	C ₂₄ H ₄₄ O ₂ Si ₂	13.13	405.2640	315.2139		2.5	0.63	0.63	10	10
35	Norbolethone m1(13 β ,17 α -diethyl-5 α -gonane-3 α ,17 β -diol)	C ₂₇ H ₅₂ O ₂ Si ₂	18.28	144.0965	157.1034		2.5	1.25		8	10
36	Norbolethone m2(13 β ,17 α -diethyl-5 β -gonane-3 α ,17 β -diol)	C ₂₇ H ₅₂ O ₂ Si ₂	19	144.0965	157.1034		2.5	1.25		10	10
37	Norclostebol	C ₁₈ H ₂₅ ClO ₂	20.15	452.2328	417.2640		2.5	0.63	0.63	10	10
38	Norethandrolone m2(17 α -Ethyl-5 β -estrane-3 α ,17 β -diol)	C ₂₆ H ₅₀ O ₂ Si ₂	17.52	241.1951	331.2452		2.5		0.63	10	10
39	Oxabolone PC	C ₂₇ H ₄₈ O ₃ Si ₃	18.67	506.3062	507.31405		2.5	0.63	0.63	9	10
40	Oxymesterone	C ₂₉ H ₅₄ O ₃ Si ₃	20.81	534.3375	519.31405	389.2327	2.5	0.63	0.63	10	10
41	Stenbolone	C ₂₆ H ₄₆ O ₂ Si ₂	16.23	220.1278	208.1278		2.5	1.25	0.63	9	10

	Analytes (TMS derivatives)	Chemical formula	RT (min)	Ions (<i>m/z</i>)			Spiked conc ^a (ng/mL)	LOD (ng/mL)		Detection in 10 urine samples	
42	Testosterone	C ₂₅ H ₄₄ O ₂ Si ₂	16.83	432.2874	209.1357	417.2640	EAAS	GC/Q-TOF	GC/Q-Orbitrap		
43	5 α -Androstan-3 α ,17 β -diol	C ₂₅ H ₄₈ O ₂ Si ₂	14.21	241.1951	256.2186		EAAS				
44	5B-Androstan-3 α ,17 β -diol	C ₂₅ H ₄₈ O ₂ Si ₂	14.55	241.1951	256.2186		EAAS				
45	5B-Androstan-3 α ,17 β -diol d5, ISTD	C ₂₅ H ₄₃ D ₅ O ₂ Si ₂	14.15	246.2265	261.2499		ISTD				
46	Androsterone	C ₂₅ H ₄₆ O ₂ Si ₂	13.8	434.3031	329.2295	419.2796	EAAS				
47	Androsterone d4, ISTD	C ₂₅ H ₄₂ D ₄ O ₂ Si ₂	13.5	438.3282	333.25463	423.3047	ISTD				
48	Epitestosterone	C ₂₅ H ₄₄ O ₂ Si ₂	15.99	432.2874	209.1356	417.2640	EAAS				
49	Epitestosterone- D3, ISTD	C ₂₅ H ₄₁ D ₃ O ₂ Si ₂	15.86	435.3063	420.2828		ISTD				
50	Etiocholanolone	C ₂₅ H ₄₆ O ₂ Si ₂	14.11	434.3031	329.2295	419.2796	EAAS				
51	Etiocholanolone d5, ISTD	C ₂₅ H ₄₁ D ₅ O ₂ Si ₂	13.78	439.3345	334.2609	424.3109	ISTD				

- Spiked concentration at 50% of the MRPL.
EAAS: endogenous androgenic-anabolic steroids
ISTD: internal standard

Table 2: Reproducibility of mass accuracy in different urine matrix for both instruments.

inst.	Analytes	Theor. m/z	Average	STD	max	min
Q-TOF	5 β -androst-1-ene-17 β -ol-3-one	194.1121	3.15	2.96	9.27	0.00
	6-oxo- androstenedione	516.2906	7.62	3.73	13.17	2.32
	1 α -methyl-5 α -androstane-3 α -ol-17-one	433.2953	2.67	1.73	5.77	0.69
	17 α -Ethyl-5 β -estrane-3 α ,17 β -diol	331.2458	5.30	5.72	15.09	0.30
Q-orbitrap	5 β -androst-1-ene-17 β -ol-3-one	194.1121	0.40	0.23	0.52	0.00
	6-oxo- androstenedione	516.2906	1.01	0.09	1.16	0.97
	1 α -methyl-5 α -androstane-3 α -ol-17-one	433.2953	0.18	0.22	0.46	0.00
	17 α -Ethyl-5 β -estrane-3 α ,17 β -diol	331.2458	1.81	0.48	2.72	1.21

Theor. Theoretical

Figure 2 shows the extracted ion chromatograms for the main fragment ions derived from the following AAS compounds: 1-testosterone, 5 β -androst-1-ene-17 β -ol-3-one (boldenone metabolite), 3'OH stanozolol, epimetendiol, bolasterone, 9 α -fluoro-18-nor-17,17-dimethyl-4,13-diene-11 β -ol-3-one (flouxymesterone-18nor), 13 β ,17 α -diethyl-5 β -gonane-3 α ,17 β -diol (norbolethone metabolite), 19-norandrosterone (nandrolone metabolite), oxymesterone and clenbuterol. An improvement in the detection of 3'OH stanozolol using GC/Q-Orbitrap was noted, which was due to the lower level of matrix interference it had compared to that of GC/Q-TOF. Moreover, clenbuterol was detected unambiguously using GC/Q-Orbitrap with the FS/HR mode at 0.1 ng/ml; it was only possible to detect this compound in MS/MS mode using GC/Q-TOF [9].

The detection of other compounds, such as boldenone, bolasterone and $13\beta,17\alpha$ -diethyl- 5α -gonane- $3\alpha,17\beta$ -diol, was subject to interference using either instrument.

Figure 2 Extracted ion chromatograms of main fragment ions of some AAS screened by GC/Q-TOF and GC/Q-Orbitrap at concentration 50% of MRPL

Generally, the chromatographic data obtained from routine screening using GC and LC/MS is independently evaluated by two analysts in order to identify suspicious ions that may be the result of a prohibited substances abuse. These suspicious signals activate specific confirmatory procedures that include testing of a new aliquot of the suspicious urine sample. A routine batch of samples analyzed by GC/MS or LC/MS methods may generate tens of thousands of ion chromatograms that have to be reviewed one by one. Consequently, the quality and reproducibility of the ion chromatograms to be reviewed is important in reducing errors during the evaluation of doping tests. In an HR instrument, the mass accuracy of the ion chromatograms influences the quality of their evaluation. If the mass extraction window is too wide, then the matrix interference is enhanced; if it is too narrow, then there is an increased risk of not detecting a prohibited substance due to mass error. In the current study, the mass extraction windows were optimized and set to 20 ppm for GC/Q-TOF and 5 ppm for GC/Q-Orbitrap, and the same m/z values were used for the detection of the analytes. The Orbitrap method can be further optimized via the use of the chromatographic back-flush device and the inclusion of MS/MS acquisition, both of which were applied only to the GC/Q-TOF analysis in the current study. Both the GC/Q-TOF and the GC/Q-Orbitrap instruments were equipped with a quadrupole device that allowed for the acquisition of MS/MS.

5.3.2 Quantitative analysis results

The development of a new GC/MS anti-doping screening method that cannot measure endogenous AAS has no practical utility [10, 25]. Therefore, the current study included screening of the six endogenous AAS that are listed in Table 3 for both instruments. The data presented in Table 3 demonstrates that the analytical performance of both instruments is in compliance with the WADA specifications [10]. The MU is less than 20% for A and Etio, 25% for 5 α adiol and 5 β adiol and less than 20% for T and E. The endogenous concentrations of androsterone and etiocholanolone may be high relative to those of the other AAS (e.g., 8 $\mu\text{g/mL}$), so to avoid detector saturation, the full scan MS mode was not used for Q-TOF, but instead the transition of m/z from 434.3031 to 419.2796 was retraced at lower abundances using the MS/MS data. The GC/Q-Orbitrap method was conducted in FS mode during testing of all of the steroids. It should be noted, however, that the Orbitrap platform is less prone to detector saturation than Q-TOF because the Orbitrap is an ion trapping instrument that can adapt to higher analyte concentrations by reducing the ion

acquisition time via automatic gain control and, thereby, allow for the detection of a larger dynamic concentration range than Q-TOF. Overall, the data acquired from both instruments was in compliance with the specifications given in the technical document TD2016EAAS [10]. In another study, a comparison of steroid profile data obtained using GC/Q-TOF and GC/QQQ was previously made [9].

Table 3: Summary of quantitative data analysis results.

Compound name	Calibration range (ng/ml)	bias%		intermediate precision%		MU%	
		GC/Q-TOF	GC/Q-Orbitrap	GC/Q-TOF	GC/Q-Orbitrap	GC/Q-TOF	GC/Q-Orbitrap
Androsterone	100-8000	15.1	10.9	4.3	4.02	15.7	11.6
Etiocholanolone	100-8000	9.1	5.7	6.2	4.3	11	7.2
5a-androstandiol	4-800	5.8	6.5	0.8	4.7	5.9	7.7
5b-androstandiol	4-800	5.1	6.1	1.9	5.3	5.5	8.1
Testosterone	2-400	9.1	17.2	1.2	1.1	9.2	17.2
Epitestosterone	2-400	8.4	2.1	9	1.5	12.3	2.6

5.3.3 Analysis of a proficiency test sample

The analysis of a proficiency testing sample was also successfully conducted using both instruments. The sample was obtained from an individual to whom nandrolone was administered; the two main metabolites of nandrolone, 19-norandrosterone and 19-noretiocholanolone, were detected. Figure 3 presents the FS/HR spectrum of 19-norandrosterone obtained at a concentration of 6.2 ng/mL using both the GC/Q-TOF and GC/Q-Orbitrap platforms.

Figure 3 Full scan spectrum of proficiency test sample (19-norandrosterone) obtained by (A) GC/Q-TOF and (B) GC/Q-Orbitrap

5.4 Conclusion

The goal of the current study was the comparison of the performance of the GC/Q-TOF and GC/Q-Orbitrap molecular profiling technologies during routine anti-doping screening and the assessment of the qualitative and quantitative analyses resulting from the use of both platforms. The data presented herein demonstrate that both molecular profiling platforms can be used for anti-doping screening. It was also shown that it is possible to combine FS/HR acquisition with MS/MS to enhance specificity in order to facilitate screening for AAS. The LODs for both instruments were well below 50% of the MRPL sensitivity level. The identification criteria data shows that a limited number of compounds were not able to reach 100% detectability in the various urine matrices. The mass accuracies were excellent for both instruments; however, the GC/Q-Orbitrap had superior mass accuracy due to its higher resolution. The superiority of the GC/Q-Orbitrap was also demonstrated by the lower matrix effects found in the urine samples in relation to the mass

accuracies. Overall, both instruments proved to be sufficiently robust for routine anti-doping testing and analysis. The results of the reprocessing of the existing data files to identify substances that previously escaped detection in FS mode will be presented in a future report.

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Ultra-Fast Retroactive Processing of Liquid-Chromatography High-Resolution Full-Scan Orbitrap Mass Spectrometry Data in Anti-Doping Screening of Human Urine

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Abstract

Retroactive analysis of previous testing urine samples has become an important sports antidoping tool. Retroactive reprocessing of old data files acquired from a generic screening procedure can reveal detection of initially unknown substances, like illegal drugs and newly identified metabolites. To be able to efficiently search through hundreds to thousands of liquid-chromatography high-resolution full-scan Orbitrap mass spectrometry data files of anti-doping samples, a combination of MetAlign and HR_MS_search software has been developed. MetAlign reduced the data size ca. 100-fold making local storage of massive volume of data possible. The newly developed HR_MS_Search module can search through the reduced data files for new compounds (mass or isotope pattern) defined by mass windows and retention time windows. A search for 33 analytes in 940 reduced data files lasted 10 seconds. The output of the automatic search was compared to the standard manual routine evaluation. The results of searching were evaluated in terms of false negatives and false positives. The newly banned β 2-agonist Higenamine and its metabolite, Coclaurine, were successfully searched in reduced data files originated from a testing period that these substances were not banned, as an example of retroactive analysis. The freeware MetAlign software and its automatic searching module HR_MS_Search facilitated the retroactive reprocessing of reduced full scan high-resolution LC/MS screening data files and created a new tool in antidoping laboratories' network.

6.1 Introduction

The World Anti-Doping Agency (WADA) is the worldwide leading body in human sports anti-doping control. Doping in sports is defined by the List of prohibited substances with performance enhancement capability, published annually by WADA [1]. The WPL refers to prohibited pharmacological actions related to pharmacological classes, while the named therein prohibited drugs, are included as examples and it does not constitute an exhaustive list. The drugs of the WPL can be characterized as peptides or small molecules. The WADA Accredited Laboratories perform the Initial Testing Procedure (ITP) or screening of small molecules using Gas Chromatography/Mass Spectrometry (GC/MS) and Liquid Chromatography/Mass Spectrometry (LC/MS), in parallel for all urine samples, to comply with the analytical needs created by the WPL. For the LC/MS part of ITP, the analysis performed by Orbitrap high-resolution Full Scan Liquid Chromatography Mass Spectrometry (FS/HR-LC/MS) has particular advantages, because 1) of the sensitive qualitative detection of the analytes, 2) the full scan MS acquisition can be conducted in both positive and negative ionization polarity within a single analysis for all analytes, 3) it can be combined in future with quantitative intact Phase II metabolites analysis [2-4]. Because of its untargeted nature, this technology may not only help in ITP of a large list of known doping substances, but also acquire MS signals from doping substances that are not known yet but belong to the prohibited pharmacological classes of the WPL List.

Due to the technological advances and increasing knowledge of metabolism, new slowly excreted metabolites of known Anabolic Androgenic Steroids (AAS) were discovered; these long-term metabolites allow the possible detection of AAS abuse for longer time after the administration has been stopped, compared to the previously known metabolites [5-8]. Furthermore, new designer drugs – molecules outside the official pharmaceutical system- have been produced and marketed escaping national legislations [9-11]. It is having been proven that the re-analysis of old samples previously reported as negatives has resulted in reporting more positive cases for doping use than the initial testing [12-13].

Consequently, the possibility of re-testing stored negative samples, when new pharmaceutical and metabolic knowledge is available, is an important anti-doping tool that may act as a deterrent against doping. However, this creates a logistical problem, since the re-analysis (from sample

preparation and instrument analysis to data review and detection) of all the available stored urine samples from the past for each new substance is virtually impossible, because the sample urine volume is limited, and the needed human, material and instrumental resources are important [3, 14]. In practice, only specifically selected samples can be re-tested [12, 13]. If, however, initially the data have been acquired in full-scan high-resolution mode, it might suffice to only re-process the previously acquired FS/HR-LC/MS data files, since they contain all non-fragmented compound information that an MS can acquire.

The use of FS/HR-MS in LC and GC in anti-doping has been reported and applied recently [2, 3, 15 - 19]. However, re-processing of thousands of data files is also a logistically difficult task, because the size of high-resolution mass spectrometric data files per sample is in the range of 100-500 MB. This task requires a strong Information technology (IT) infrastructure, which is not usually available to the WADA Accredited Laboratories. In addition, conventional manual processing of thousands of data files requires substantial human resources for the creation of the ion chromatograms and the visual evaluation.

A solution to perform fast re-analysis of large numbers of data files is to use pre-processed size-reduced data files in which all essential analytical information of substances is still available. This size reduction can be obtained by eliminating noise and baselines from chemical background followed by peak-picking. The previously described MetAlign software, which has a long history to analyze metabolomics LC and GC MS/MS data, can perform this task as part of its use to process untargeted metabolomics data of several formats [20, 21, 22]. Herein, its use to obtain a 100-1000-fold data reduction of raw data files is presented. Obtaining such size-reduced data makes local storage of thousands of FS/HR-LC/MS files on a solid-state drive (SSD) feasible. Because the size-reduced files do not need any further pre-processing, retrieving information – such as mass, intensity, retention time – from hundreds to thousands of files is a very fast exercise [23]. A new module called HR_MS_Search has been developed in this study which allows to search for isotope pattern matches for multiple substances simultaneously in hundreds to thousands of reduced data files in the redms_acc format, which is the output format of MetAlign of the size reduced pre-processed LC/MS data. This HR_MS_Search software is now part of the MetAlign software suite.

The current study examines the performance of the MetAlign data reduction and subsequent searching by the HR_MS_search module for LC/MS ITP retroactive reprocessing. The goal was

the use of this software for searching of large numbers of reduced data files from ITP FS/HR-LC/MS, in order to prioritize samples for confirmation. The study has been applied on existing data files from routine ITP analysis at ADLQ. The searching performance was evaluated using the risk rate of false negatives and false positives as main criteria. In ITP, the main interest of the WADA Accredited Laboratories is to eliminate false negatives (i.e. evaluating positive samples as negative). On the other hand, too many ITP false positive samples (i.e. evaluating negative samples as positive) creates an additional unnecessary workload of additional investigation analyses and a waste of laboratory resources to prove that the sample was negative. Therefore, this study is focused not only on the speed and the easiness-of-use of the HR_MS search module, but also on how to minimize ITP false negatives and false positives when using automatic searching, in order to assess this retroactive analysis tool in ITP. The overall MetAlign evaluation was conducted as a new antidoping deterrent tool.

6.2 Materials and Methods

6.2.1 Samples, Materials, Instruments and data acquisition

ITP sample data files after FS/HR-LC/MS analysis were used in the current study. They were originated from the routine screening analytical data of ADLQ. The detailed sample preparation, reference materials, instrumental LC/MS analytical procedure were described elsewhere [2]. The sample preparation was conducted as following: 5 mL of urine aliquots, spiked with internal standard solution, were applied for enzymatic hydrolysis using 100 μ L of β -Glucuronidase from *E. coli* at pH 7, adjusted by addition of 1 mL phosphate buffer. The hydrolysis was conducted at 50° C for 1.5-hour duration conditions. After cooling the aliquots were extracted with 5mL of ethyl acetate at pH 9-10 adjusted by a solid mixture $\text{NaHCO}_3\text{:Na}_2\text{CO}_3$ (10:1) (w/w). After extraction, centrifugation and the separation of the organic from the aqueous phase, the organic layer was evaporated under stream of nitrogen at 40° C, reconstituted with 200 μ L of reconstitution solvent (mobile phase A/B 80:20; v/v) and mixed with 20 μ L of the non-processed original urine to the final extract. 5 μ L of the mixture were injected into the LC/MS system. The addition of the non-extracted urine part to the reconstituted extract was important for the detection small molecules that are not extracted with the applied extraction protocol, such as melidonium, ethylglycuronide,

FG4592 and other HIF stabilizers, AICAR, finasteride carboxylic acid metabolite, ritalinic acid, dextran and hydroxyethylstarch.

The LC/MS analysis [2] was performed by a Dionex UHPLC system (Thermo Scientific, Bremen, Germany) equipped with QExactive bench top Orbitrap based MS (Thermo Scientific, Bremen, Germany). The chromatographic separation was performed using a Zorbax Eclipse Plus C18 column (100 × 2.1 mm i.d., 1.8 µm particle size; Agilent Technologies, Santa Clara, CA, USA). Water containing 5 mM HCOONH₄ and 0.02% (v/v) HCOOH (solvent A) and mixture of acetonitrile/water (90:10 v/v) containing 5 mM HCOONH₄ and 0.02% formic acid (solvent B) were used as mobile phase solvents. The chromatographic program was modified from [2] as following: the total analysis run time per sample was 20 minutes with a constant flow rate set at 0.2 mL/min throughout the entire run. The initial conditions were set to 95% A and 5% B for the first minute of the run. Solvent B was then increased to 90% on the 9th minute, followed up to 100% on the 11th minute after which it remained constant for 3 minutes. After the 14th minute, Solvent B was reduced to 5% within 30 seconds to start the post-run equilibrium for the remaining duration of the 20 min run.

The mass spectrometer was equipped with a heated electrospray ionization source (HESI2) and operated with positive–negative polarity switching full scan MS acquisition mode [2]. Nitrogen was used as sheath gas, ion sweep gas, and auxiliary gas. The ion spray voltages were set to 4000 V in positive mode and 3800 V in negative mode. The settings of orbitrap for the FS acquisition were as followed: scan range m/z from 100-1000 at 17500 resolving power, automatic gain control (AGC) target was set at 10⁶ and duty cycle was 100 ms.

In Table 1, the 33 analytes, examined in the current study are shown together with their preferred detection polarity ionization mode. Those analytes were incorporated by spiking in the positive quality control (QC) samples prepared in each analytical batch at a concentration level corresponding to 50% of the Minimum Required Performance Limit (MRPL) level (see also Table 1) [4]. The analytes included herein were considered representative for the more than 300 analytes included in the LC/MS ITP at ADLQ and belong to various drug classes, such as AAS, b2-agonists, narcotics, diuretics, stimulants and others [2].

In this study a total of 940 data files were processed, which originated from 20 ITP analytical batches of ADLQ. The samples were analyzed over a period of several months using two instruments with identical LC/MS configuration and method settings [2 and paragraphs above]. Retention time differences between the two instruments were less than 0.1 min. Out of the total 940 data files, 860 were routine athletes 'urine samples, 20 blank negative QC urine samples and 60 were positive QC spiked urine samples. 53 out of the 860 urine samples were manually evaluated as containing of one or more of the substances of Table 1 (also present in the QC samples).

Table 1 List of substances used in this evaluation study

Analyte	QC sample Concentrations (ng/mL)	Ionisation Polarity
3'-Hydroxystanozolol	1	+
4'-Hydroxystanozolol	1	+
16-Hydroxystanozolol	1	+
4-Methylhexaneamine	50	+
Thiazides ACB Artifact ¹	100	-
Amphetamine	50	+
Benzoylcegonine	50	+
Boldenone	2.5	+
Cannabis (THCCOOH)	75	-
Canrenone	50	+
Chlorothiazide	50	-
4-Hydroxyclophene	10	+
Codeine	5	+
Ephedrine	50	+
Furosemide	50	-
Gestrinone	2.5	+
Hydrochlorothiazide	50	-
Indapamide	50	+
Letrozole metabolite ²	10	-
Methylphenidate	50	+
Methandienone long-term metabolite ³	1	+
Pemoline	50	+
Pentazocine	25	+
Raloxifene	10	+
Ritalinic Acid	50	+

Analyte	QC sample Concentrations (ng/mL)	Ionisation Polarity
Salbutamol	10	+
3-Hydroxy-4-methoxytamoxifen.	10	+
Testolactone	10	+
THG ⁴	2.5	+
Tramadol	25	+
Epitrenbolone	2.5	+
Trenbolone	2.5	+
Trimetazidine	10	+

QC=concentration in QC spiked sample; polarity=polarity used for substance detection.

¹4-Amino-6-chloro-1,3-benzenedisulfonamide. ²Bis(4-cyanophenyl) methanol. ³18-Nor-17B-hydroxymethyl,17A-methylandroster-1,4,13-trien-3-one. ⁴Tetrahydrogestrinone: 17-hydroxy-18A-homo-19-nor-17A-pregna-4,9,11-trien-3-one.

6.2.2 Standard ADLQ LC/MS Data Processing Setup for ITP

For each routine ITP analytical batch, the generated ion chromatograms were manually reviewed by two analysts, after grouping printouts per analyte [24] for all the samples of the batch (maximum 60 samples including QC). The printouts were generated by applying an extraction mass window of ± 5 parts per million (ppm). The reviewing was based on experience in evaluating criteria for analyte detection by direct comparison to QC signals. The applied criteria were based on: a) peak abundance sufficiently above the noise and background to facilitate a probable confirmation follow-up procedure, b) the retention time to match with the QC sample and c) applicable ion ratios within analyte to match. Data acquisition files were stored on instrument computers, until backed up on a weekly basis by the EMC Avamar server located in the ADLQ data center. Backups were kept on hard drives and off-loaded to tapes on an annual basis. The retrieval of old backed-up data files was accomplished through the same EMC Avamar software.

6.2.3 Data Analysis Protocol and Hardware for the MetAlign Software Suite

6.2.3.1 Hardware

Although MetAlign runs on any modern PC with a Windows 7 64-bit platform or better [21], here it was run on a hyper-threaded 16-core PC (32 virtual cores; 3 GHz; 64 GB RAM) equipped with

a solid-state disc under a Windows 7 64-bit operating system. HR_MS_Search was run on a PC (W7 64-bit) equipped with an SSD.

6.2.3.2 MetAlign Software and Settings

The MetAlign software used in this study is freeware. MetAlign settings specific for ADLQ data were used for the size reduction of the positive and negative mode data. Positive and negative mode data within the same original data file were processed separately. Currently up to 1000 data sets can be processed per batch; 940 ADLQ files in the herein configuration took ca. 4 hours per polarity mode to process for the size reduction. The size reduced data files were stored on an SSD in a folder structure in a way comparable to how the original raw data files were stored by date. A thorough description of MetAlign software data reduction algorithm can be found elsewhere [20].

6.2.3.3 HR_MS_Search

HR_MS_Search was used as freeware searching module together with typical Excel-compatible input and output sheets. An Excel-compatible search template contained information per substances to be searched, i.e. accurate masses, retention times, mass error window (7.5 ppm), retention window (± 0.1 minute). It was therefore possible to define a search for isotope patterns. The Excel-compatible output provided all the information present in all the searched size-reduced data files. The output for each data file, where the analyte was detected, comprised delta retention, ppm errors for each defined mass, the number of masses including the molecular ion, as well as a match factor for the isotope pattern, in case of an isotope pattern for a substance was found. Searching 940 size-reduced data files for multiple masses of one substance took approximately 10 seconds.

6.2.4 MetAlign/HR_MS_Search Software Evaluation Protocol

In order to evaluate the performance of the MetAlign approach (2.3.2 and 2.3.3), the searching output had to be compared to the manual evaluation outlined in 2.2 comprising the following parameters: a) number of QC and routine samples positives detected by MetAlign/HR_MS_search, where detection was true i.e. the analyte was detected in the data file and existed in the sample, b) number of false negatives reported by MetAlign compared to the ADLQ manual evaluation, i.e. the substances existed in the sample/data file but is not detected by MetAlign/HR_MS_search, c)

number of false positives compared to the ADLQ manual evaluation, i.e. the substances did not exist in the sample/data file and were included to the MetAlign/HR_MS_search output file.

Subsequently, the influence of additional filter criteria on false positive and false negative rates was examined. The following filters were individually applied to examine their influence: a) filter on mass accuracy error smaller than 3 ppm, b) filter on the existence of a 2nd mass signal where applicable, with the printout accuracy and mass error parameters of the same as of the base peak, c) filter on abundance of signals higher than the 10% of the average QC peak abundance of the same substance.

6.2.5 Search for a New Substance and a Metabolite (Higenamine and Coclaurine: added to the WPL in 2017)

The retroactive reprocessing capabilities of MetAlign were tested with Higenamine, which was newly identified as prohibited β 2-agonist substance introduced to the WPL in 2017. Higenamine and its metabolite Coclaurine (methylated Higenamine) were obtained as reference standards (TRC, Toronto, Canada) and their solutions were injected into the chromatographic system as described in 2.1 to obtain their retention times and detection ions. Both substances were detected in positive mode as protonated molecular ions (m/z 272.1281 for Higenamine and m/z 286.1438 for Coclaurine). All data files were searched for these substances (mass error window of ± 5 ppm; retention window of ± 0.1 minute); samples containing possible signals only for both substances were selected for follow-up. The selected samples underwent confirmatory analysis, which comprised repetition of the entire confirmatory analysis from the original urine sample, as described above in 2.1. The LC/MS confirmatory analysis was conducted in both full scan and targeted MS² acquisition mode: for Higenamine the precursor ion m/z was 272.1281 and the product ions m/z were 107.0493, 255.1010, 161.0594, 272.1281, 123.0441, 145.0646; for Coclaurine the precursor ion m/z was 286.1438 and the product ions m/z were 269.1166, 175.0751, 237.0906, 286.1438, 209.0958, 137.0595. The collision energy was 35eV. The WADA Technical Document for Identification Criteria [25] was applied to confirm the detection or the absence of both substances.

6.3 Results and discussions

6.3.1 In general

For the MetAlign/HR_MS_search approach, the evaluation comprised the following parameters: the required IT infrastructure for the application, the processing speed, the processing performance scores and the reliability of the automatic processing.

6.3.2 Comparison of Hardware and Processing Time

6.3.2.1 Analysis by The Normal Manual Routine

The normal manual ITP (see 2.2) for a batch of 40 data files and the usual 300 analytes resulted in the generation of 12000 ion chromatograms. Careful visual evaluation was needed to evaluate all suspicious signals to minimize false negatives prior deciding for confirmation by a subsequent independent analysis on a new sample aliquot. The evaluation of 940 data files manually for all 33 analytes using this method herein needed not only the retrieval of the data files from backup media, but also 12 working hours for two senior analysts to evaluate the generated ion chromatograms.

6.3.2.2 Analysis by MetAlign/HR_MS_Search (automatic approach)

6.3.2.2.1 Data Size Reduction for Local Storage On SSD

The size reduction process was a one-time event done in full automation (easily done overnight) and therefore required very minimal human resources. Once the size-reduced data files had been made, they could be stored in a ready-to-search folder structure. In the current study 940 data files (20 batches) needed separate size reduction processing for the positive and negative polarities, which lasted together ca. 8 hours on the hardware configuration described in 2.3.1. On average a typical original data file here was 115MB, while a positive polarity size-reduced file was ca. 900 KB and a negative one was ca. 600 KB. Once stored locally in a size-reduced format, the data files can be searched over and over without further processing needs.

6.3.2.2.2 Searching a Database of Size-Reduced Data Files

Performing a search with HR_MS_search for multiple masses from one substance as described in 2.3.2 on 940 size-reduced files took about 10 seconds. This search time scales with the number of

files and the number of substances. For example, a search on 10000 size-reduced files (together ca. 10 GB) lasted ca. 100 seconds.

6.3.3 Comparison of Automatic to The Manual Approach

The manual evaluation by two senior analysts was a visual demanding task comparing multiple ion traces simultaneously. The reviewing was based on experience in applying criteria (see 2.2). The analyst decided if a peak was enough above noise and background to facilitate a subsequent confirmation; furthermore, the retention was recorded and, if applicable, ion ratios were considered.

The routine ITP manual evaluation was used as reference method. Comparing automatic searching outcome to manual evaluation was therefore biased towards the manual approach. In Table 2, a comparison of results is shown between the manual and automatic approach. First, it was checked if the substances of Table 1 could be found in the QC samples. The MetAlign performance was evaluated by the number of spiked QCs detected in comparison to that found in the instrument's reports processed by analysts. In Table 3, a summary of the MetAlign software searching performance of Table 2 can be found in relation to the sensitivity and specificity validation parameters. A total of 714 out of 717 spiked analytes peaks present in the QCs across the batches were detected and only 3 peaks were missed. Investigation of the 3 false negatives in QCs showed that the missed signals were of very low intensities (just above noise levels) for ritalinic acid and clomiphen 4-OH metabolite (4-hydroxy metabolite of clomiphen); consequently, they were not considered as false negatives. In Figure 1A, the example of the missed signal of ritalinic acid is presented, which was due to the low peak intensity from material deterioration in dilution condition. Ritalinic acid is a relatively unstable substance in the solution conditions of ADLQ.

Table 2 List of results based on the manual and automatic (MetAlign\HR_MS_Search) evaluation approach

Substance	Manual	Auto	FN	FP	Filter 3ppm		Filter 2 nd ion		Filter 10% QC	
					FN	FP	FN	FP	FN	FP
3'-hydroxy-Stanozolol	3	23	0	20	0	8	1	0	0	11
4-hydroxy-Stanozolol	3	44	0	41	1	14	2	0	0	14
16-hydroxy-Stanozolol	1	22	0	21	0	6	0	2	0	2
4-Methylhexaneamine	1	36	0	35	0	20	0	0	0	0
Thiazides ACB Artifact	2	6	0	4	0	3	0	1	0	0
Amphetamine	3	50	0	47	0	8	0	10	0	1
Benzoyllecgonine	1	787	0	786	0	479	0	44	0	233
Boldenone	1	632	0	631	0	457	0	40	0	248
Cannabis (THCCOOH)	4	73	0	69	0	45	0	42	0	9
Canrenone	1	292	0	291	0	135	0	22	0	11
Chlorothiazide	1	31	0	30	0	22	0	12	0	2
4hydroxy Clomiphene	1	7	0	6	0	2	0	6	0	2
Codeine	19	235	0	216	0	106	0	46	0	13
Ephedrine	13	858	0	845	0	808	0	172	0	82
Furosemide	1	3	0	2	0	1	0	1	0	0
Gestrinone	0	1	0	1	0	1	0	0	0	1
Hydrochlorothiazide	1	44	0	43	0	40	0	40	0	12
Indapamide	1	3	0	2	0	2	0	2	0	0
Letrozole Metabolite	1	1	0	0	1	0	0	0	0	0
Methylphenidate	2	450	0	448	0	176	0	6	0	0
Methandienone long-term metabolite	1	585	0	584	0	266	1	17	0	7
Pemoline	0	101	0	101	0	38	0	3	0	0
Pentazocine	0	0	0	0	0	0	0	0	0	0
Raloxifene	0	12	0	12	0	4	0	1	0	1
Ritalinic Acid	11	548	0	537	0	250	2	10	0	395
Salbutamol	9	841	0	832	0	638	0	227	0	40
Tamoxifene metabolite	1	7	0	6	0	3	0	1	0	1

Substance	Manual	Auto	FN	FP	Filter 3ppm		Filter 2 nd ion		Filter 10% QC	
					FN	FP	FN	FP	FN	FP
Testolactone	16	157	0	141	11	48	0	38	0	45
THG	1	30	0	29	0	7	0	6	0	11
Tramadol	13	745	0	732	0	526	0	117	0	6
Epitenbolone	0	46	0	46	0	32	0	1	0	1
Trenbolone	0	46	0	46	0	28	0	0	0	2
Trimetazidine	1	88	0	87	0	42	0	1	0	7

Auto=automatic; FN=number of false negatives; FP=number of false positives; 3ppm = 3ppm criterion (see text), second ion=second ion criterion (see text); 10% QC=signals >10% QC average (see text).

Table 3 Numbers of table in percentages (from Table 2)

Substance	Without Filter		Filter 3ppm		Filter 2nd ion		Filter 10% QC	
	sensitivity	specificity	sensitivity	specificity	sensitivity	specificity	sensitivity	specificity
3'-hydroxy-Stanozolol	100	97.9	100	99.1	99.9	100	100	98.8
4-hydroxy-Stanozolol	100	95.6	99.9	98.5	99.8	100	100	98.5
16-hydroxy-Stanozolol	100	97.8	100	99.4	100	99.8	100	99.8
4-Methylhexaneamine	100	96.3	100	97.9	100	100	100	100
Thiazides ACB Artifact	100	99.6	100	99.7	100	99.9	100	100
Amphetamine	100	95	100	99.1	100	98.9	100	99.9
Benzoylgonine	100	16.4	100	49	100	95.3	100	75.2
Boldenone	100	32.9	100	51.4	100	95.7	100	73.6
Cannabis (THCCOOH)	100	92.7	100	95.2	100	95.5	100	99
Canrenone	100	69	100	85.6	100	97.7	100	98.8
Chlorothiazide	100	96.8	100	97.7	100	98.7	100	99.8
4hydroxy Clomiphene	100	99.4	100	99.8	100	99.4	100	99.8
Codeine	100	77	100	88.7	100	95.1	100	98.6
Ephedrine	100	10.1	100	14	100	81.7	100	91.3
Furosemide	100	99.8	100	99.9	100	99.9	100	100
Gestrinone	100	99.9	100	99.9	100	100	100	99.9

Substance	Without Filter		Filter 3ppm		Filter 2nd ion		Filter 10% QC	
	sensitivity	specificity	sensitivity	specificity	sensitivity	specificity	sensitivity	specificity
Hydrochlorothiazide	100	95.4	100	95.7	100	95.7	100	98.7
Indapamide	100	99.8	100	99.8	100	99.8	100	100
Letrozole Metabolite	100	100	99.9	100	100	100	100	100
Methylphenidate	100	52.3	100	81.3	100	99.4	100	100
Methandienone long-term metabolite	100	37.9	100	71.7	99.9	98.2	100	99.3
Penolone	100	89.3	100	96	100	99.7	100	100
Pentazocine	100	100	100	100	100	100	100	100
Raloxifene	100	98.7	100	99.6	100	99.9	100	99.9
Ritalinic Acid	100	42.9	100	73.4	99.8	98.9	100	58
Salbutamol	100	11.5	100	32.1	100	75.9	100	95.7
Tamoxifene metabolite	100	99.4	100	99.7	100	99.9	100	99.9
Testolactone	100	85	98.8	94.9	100	96	100	95.2
THG	100	96.9	100	99.3	100	99.4	100	98.8
Tramadol	100	22.1	100	44	100	87.6	100	99.4
Epitenbolone	100	95.1	100	96.6	100	99.9	100	99.9
Trenbolone	100	95.1	100	97	100	100	100	99.8
Trimetazidine	100	90.7	100	95.5	100	99.9	100	99.3

Figure 1. A. An example of Quality Control urine sample spiked with ritalinic acid at 50 ng/mL (compound with stability problems in solution) in the left ion chromatogram at time of 5.97 minutes to be compared with a blank urine sample in the right ion chromatogram. **B.** An example of Quality Control urine sample spiked with benzoylecgonine at 50 ng/ml in the left ion chromatogram at 6.10 minutes to be compared with a blank urine sample provided voluntarily by an ADLQ staff member in the right ion chromatogram, which also comprised a signal at 6.10 minutes originating from the matrix.

In all other non-QC data files, where substances were detected by the manual approach and analytically confirmed, the automatic approach found them too. Therefore, the false negative rate in this study was zero (FN in Tables 2 and 3). However, the automatic approach found many more signals that fit the search profile (mass error window of 7.5 ppm and retention window of ± 0.1 minutes). These were referred to as ITP false positives (FP in Tables 2 and 3) and were part of the low intensity background in the data files. In Figure 1B, the example of the benzoylecgonine signals, that generated a high rate of FP, is presented. The Quality Control blank urine sample in Figure 1B shown a signal in the retention time of benzoylecgonine. For this signal, the analyst was alerted by MetAlign to create a human driven decision, i.e. whereas this signal needed further investigation as suspicious, or could be neglected for reasons outside the automatic searching (see application of filters below).

To see if the number of false positives in the automatic approach could be reduced without increasing false negatives, some extra filters in the Excel-compatible results were applied. When decreasing the mass error window to 3 ppm, the number of false positives was decreased by 37%, but in turn caused 13 false negatives to appear (from 0% up to 12%). Most false negatives were for testolactone. Careful inspection of original data showed that a near isobaric compound co-eluted with testolactone, which evidently mixed their masses at the given resolution increasing mass error. A second option was to include signals only having a second isotope present. This approach decreased the number of false positives by 87%, but 6 false negatives then occurred (from 0% up to 5%). These false negatives were from smaller signals for which the second isotope was absent or too close to noise to have a detectable accurate mass signal. The third option was the introduction of an intensity limit based on a reference standard in the QC samples. The cut-off value at 10% of the average signal in the QC samples resulted in an 83% reduction of false positives without any new false negatives occurring (stayed at 0%). The output spreadsheets could be sorted on hits based on intensities. Therefore, after performing the search on a database of reduced data files, any intensity threshold could be applied. Although the best selection method was based on the relative intensity to QC, additional filtering of the output by taking into account the presence of a second isotope and a more precise mass could also help in narrowing down potential candidate samples for confirmation analysis.

An additional way to select signals was by changing the retention time window. In the automatic approach, the retention window can be defined separately for each compound. Care must be taken not to apply too small retention windows, because: 1) pH-dependent shifts in retention time might occur for some compounds that have a pKa close to the on column pH, 2) some compounds may be so high in concentration that saturation of the column may occur which alters peak shape and may result in peaks broader than the retention window and with apexes outside the window, 3) retention time may vary up to 0.1 minutes over time in applied batches. A retention window of ± 0.1 minute was a good value for those data files.

6.3.4 Real Retroactive Example: *Higenamine and Coclaurine* (see 2.5)

The HR_MS_Search software was used in order to examine the practicality of the approach to real antidoping conditions, where retroactive reprocessing was needed. The WADA specifications consider the protection of the clean athletes from the cheating athletes and from accusation of a

clean athlete for a false doping offense (FP). This aim has formulated the WADA analytical framework to become simultaneously sensitive in detection of doping substances and without any doubt when a doping substance has to be reported. In the retroactive reprocessing aiming to detect new substances, probably under lack of a complete human metabolic profile information, the antidoping laboratories maximize the strictness of the conditions of reporting a positive result. Such a strict approach was followed in the herein case, requiring the detection of both parent compound and metabolite in the same sample. Higenamine and Coclaurine standards were injected to obtain retention times. Then 940 data files were reprocessed both by HR_MS_Search software and manually via the LCQUAN Thermo software procedure aiming to identify the co-presence of Higenamine and Coclaurine. The HR_MS_Search reprocessing resulted in 25 findings. Four (of 25) samples were considered as real suspects, since both substances were also found by the manual routine search and for the reasons referred above in this paragraph. A second sample preparation of the above 4 suspect samples was performed together with a blank urine sample and a positive control urine sample spiked with the Higenamine and its metabolite at a concentration of 100 ng/mL. The re-analysis of the 4 suspicious samples, resulted in the confirmation of the presence of Higenamine and its metabolite in one urine in compliance with WADA identification and MRPL criteria [4, 25] of reporting. In Figures 2 and 3, the full description of the Higenamine and Coclaurine confirmatory MS data are found respectively.

Figure 2. Extracted ion chromatograms from the confirmatory procedure of the full scan and the productions used for the identification of Higenamine obtained for a blank urine specimen, the positive case urine sample and the spiked (100 ng/mL) positive control urine: A) full scan acquisition ion m/z 272.1272, B) ion 1 m/z 272.13 to 107.0493, C) ion 2 m/z 272.13 to 255.1010, D) ion 3 m/z 272.13 to 161.0594, E) ion 4 m/z 272.13 to 272.1272, F) ion 5 m/z 272.13 to 123.0441, G) ion 6 m/z 272.13 to 145.0646. Percentages of ion ratios at (B) – (G) are used in comparison for compliance to the identification and acceptance criteria of [24] between the suspicious sample (first percentage in parenthesis) and the positive control urine (second percentage in parenthesis): B) (100%, 100%), C) (44.4%, 44.8%), D) (33.3%, 33.0%), E) (15.4%, 16.7%), F) (11.4%, 11.8%), G) (9.8%, 10.6%).

Figure 3. Extracted ion chromatograms from the confirmatory procedure of the full scan and the product ions used for the identification of Coclaurine obtained for a blank urine specimen, the positive case urine sample and the spiked (100 ng/mL) positive control urine. The low intensity ion signal of coclaurine detected in the blank urine sample is due to the presence of traces of the compound in almost all urine, as coclaurine can be found in plenty of plant sources: A) full scan acquisition ion m/z 286.1438, B) ion 1 m/z 286.14 to 269.1166, C) ion 2 m/z 286.14 to 175.0751, D) ion 3 m/z 286.14 to 237.0906, E) ion 4 m/z 286.14 to 286.1438, F) ion5 m/z 286.14 to 209.0958, G) ion 6 m/z 286.14 to 137.0595. Percentages of ion ratios at (B) – (G) are used for compliance to the identification criteria of [24] between the suspicious sample (first percentage in parenthesis) and the positive control urine (second percentage in parenthesis): B) (100%, 100%), C) (51.9%, 58.3%), D) (46.6%, 39.2%), E) (20.1%, 24.0%), F) (17.5%, 22.3%), G) (22.1%, 22.6%).

6.3.5 MetAlign/HR_MS_Search Software as Quality Tool

The ability of HR_MS_Search software to produce summary from the reduced QC sample data files, is also of interest as part of a laboratory quality control scheme. Fast searching could output data from all spiked compounds in QC samples over an extended time period. From this output the extraction of the necessary data to create graphs of delta retention time vs. retention time range presented in Figure 4A, as well as mass error in ppm vs. mass range of the analytes (Figure 4B) for all the QC compounds was feasible.

Figure 4. Output data from spiked compounds in QC samples over an extended time period using MetAlign/HR_MS_Search. A. delta retention time vs. retention time in min, B. mass error in ppm vs. mass range in m/z.

6.4 Conclusion

The present study describes the use of MetAlign/HR_MS_Search software as a tool for retroactive reprocessing in anti-doping analysis for data files acquired in full scan MS mode by Thermo Orbitrap LC/MS. MetAlign processing consisting of search of 1000's of data files of urine samples can be performed in seconds. This capability, focused on WADA ITP procedures on samples from the past that could contain suspicious signals (of unknown illegal substances or new metabolites), created an important additional value of the old analytical data. Anti-doping laboratories could consider this as useful tool for selecting samples for a follow-up confirmation analysis. This automated approach performed satisfactorily with regard having no false negatives compared to the manual routine approach. The searching module created a number of FPs, a usual fact originating from urine background peaks. The rate of FPs could be reduced after application of specific per substance filter parameters like mass error ranges, abundance thresholds in case of signals corresponding to low initial concentration for the substance, existence of more than one m/z , or parameters outside the substance; like the existence of an additional relevant metabolite. Consequently, the searching parameters could be adjusted per analyte, in order to optimize case-by-case the searching results.

The HR_MS_Search software run on a standard IT infrastructure and could be used to generate laboratory quality control charts with regard to retention time and mass error precision over time. The wide application of the current approach could result in a substantial improvement of the deterrence in the antidoping system, in combination with the already applied WADA long-term sample storage policy.

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Chapter 7

Comparison of Gas Chromatography Quadrupole Time-Of-Flight to Quadrupole Orbitrap Mass Spectrometry in Human Urine Sports Antidoping Analysis: II. Retroactive Analysis of Anabolic Steroids

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Abstract

The retrospective analysis of samples to determine the presence of designer drugs or new metabolites is gaining importance in sports antidoping drug testing. The high-resolution full scan data acquisition enables the retrospective analysis of already acquired LC/MS and/or GC/MS data of samples that were measured in the past for the presence of unknown drugs or new metabolites, without the need to reanalyse the samples. The MetAlign software search module has been developed to facilitate the retroactive analysis of already acquired data files. In this study, the ability of MetAlign software to identify drugs and their metabolites traces was assessed with a two-electron impact ionisation full scan high-resolution GC/MS mass spectrometry platform equipped with time of flight and Orbitrap mass analyzers (GC/Q-TOF MS and GC/Q-Orbitrap MS), and the same sample aliquot, same sample preparation and the same GC parameters and conditions were applied for each analyser. The results show that the MetAlign search found more false positives in the GC/Q-TOF results than in the GC/Q-Orbitrap results. This can be explained by the extensive matrix interference and MS resolution: the m/z extraction widow of compounds is higher in GC/Q-TOF than in GC/Q-Orbitrap, and the mass errors are higher in GC/Q-TOF than in GC/Q-Orbitrap.

7.1 Introduction

Doping is the most unethical behaviour in sports, as it violates the spirit of sports and can pose a potential risk to athletes' health. The World Anti-Doping Agency (WADA) is the responsible international body that circulate the definition of doping and the specifications of the antidoping monitoring system through the WADA Code [1]. The WADA Code comprises the WADA Prohibited List (WPL) [3], which contains a wide range of prohibited pharmacological classes of substances and prohibited methods. WADA accredited anti-doping laboratories apply analytical procedures that should, as much as possible, cover the detection of the prohibited substances at the minimum required performance levels (MRPL) [4].

Designer anabolic androgenic steroids (AAS) are usually circulating without official approval from national pharmaceutical organizations and are easily available through the Internet and directed to athletes from illegal laboratory benches without being subjected to any clinical trials for toxicity, adverse health effects, etc. [5]. The antidoping system faces a challenging threat from the use of designer drugs by cheating athletes, because designer drugs are unknown to the authorities, including WADA laboratories, which results in the loss of their detection. The detection of unknown designer drugs requires analytical technology that provides sensitive and specific (especially for anabolic agents) data for as many prohibited known and unknown designer molecules as possible. The most powerful analytical platform for this purpose is high-resolution full scan acquisition mass spectrometry (FS/HR-MS), as described previously in Chapters 2, 3 and 4 [5-7]. The advantages of the use of FS/HR acquisition in the antidoping screening analysis has been demonstrated in previous publications [6-8]. The HR mass filter results in highly specificity, sensitivity data with an enhanced signal detection and reduced background noise. FS/HR acquisition allows the inclusion of a practically unlimited number of screening analytes in the method, without any prior hypothesis allowing for the detection of new compounds, such as designer steroids and their metabolites, which may prolong the detection of prohibited substances with "zero tolerance", such as anabolic-androgenic steroids (AAS) [10-12], in the acquired data [5], [9].

The storage of antidoping samples in safe conditions for a long period of time and the reanalysis of former negative samples, e.g. reanalysis after the reveal of the circulation of new designer drugs, is highly resource demanding, because extra urine volume is consumed for sample preparation and

laboratory resources. The creation of an antidoping framework, where the new information can be applied not only to current and future tests but also retrospectively on already analyzed data of negative reported samples, would considerably improve antidoping monitoring and would have an important role in preventing antidoping by publicly making the message known that all unknown drugs can be retrospectively detected in already analyzed antidoping samples. Consequently, cheating athletes will always have the fear that, even years after a negative reported antidoping test, they can be caught, despite the use of a designer drug unknown at the time when the sample was given or even after the calculation of the clearance times, since, especially for anabolic steroids for example, the detection of new long-term metabolites prolongs the detection of the use of prohibited compounds in urine [2]. Reanalysis is gaining importance in sports drug testing due to the impressive results that came out after the retesting of negative old samples from the Beijing 2008 and the London 2012 Olympic Games some weeks before the Rio 2016 Olympic Games took place [13,14]. Reprocessing of already acquired LC/MS or GC/MS data instead of retesting stored urine samples has many advantages, since data analysis has a low cost in human and computer resources and does not require additional urine sample or laboratory and sample storage resources, and therefore has a great potential for retrospective antidoping analysis. However, reprocessing thousands of datafiles is a difficult task due to the large volume of FS/HR-MS data files per sample, which is estimated at 600 MB, and the large number of analyzed samples. Moreover, extensive human resources from senior analysts are needed for the manual processing of thousands of datafiles. Using pre-processed size-reduced data is recommended to rapidly reanalyse large numbers of datafiles. The size reduction can be achieved by the elimination of noise and baselines from chemical background, followed by peak picking. MetAlign software is a tool that has been created by Arjen Lommen in 2009 [15] for pre-processing GC/MS and LC/MS data. MetAlign is a powerful tool, and it consists of a data reduction algorithm, searching and accurate mass detection of compounds, baseline corrections, and peak picking as well as the retention time alignment and *m/z* recalibration of up to 1000 data sets. The aim of data size reduction is to be able to reprocess many data files in a reasonable amount of time and to save storage space. Obtaining a 100-1000-fold data reduction makes the local storage of thousands of HR/FS data files on a fast-accessible solid-state drive (SSD) easily feasible [15, 16]. The important issue for data file size reduction is how much and what kind of analytical information is kept in the reduced data file and what kind and how much of the analytical information is lost compared to the original data. The entire

procedure should ensure that an analytical signal from prohibited substances in the reduced volume data file is retained.

The current chapter presents the application of the MetAlign software for the reprocessing of GC/MS data files for already acquired samples at the Antidoping Lab Qatar (ADLQ). However, in the future, further software development is needed to add additional features, such as the application of a filter based on the mass error and retention time. This chapter also presents the assessment of the MetAlign software performed on the data obtained with two different GC/MS electron ionisation (EI) FS/HR MS platforms, such as GC/Q-TOF MS and GC/Q-Orbitrap MS, and the same sample aliquot, same sample preparation and the same GC parameters and conditions were applied to each analyser. This work aims to be complementary to another study that has been completed for the FS/HR- LC/MS data files, as presented in Chapter 6 [7], to complete the data reprocessing screening framework of the WADA accredited laboratories [17].

7.2 Materials and methods

7.2.1 Chemicals, Reagents and Reference Materials

The description of the chemical, reagents and reference materials are previously described in detail in Chapter 5 [12].

7.2.2 Sample Preparation

The detailed sample preparation procedure is described previously in Chapter 5 [12]. In brief, the sample preparation procedure includes the deconjugation of the Phase II steroid glucuronide conjugates by enzymatic hydrolysis using the *E. coli*-derived β -glucuronidase, which is followed by liquid-liquid extraction with diethyl ether at a pH 9-10 and a desalting step. A clean extract was obtained after the separation of the organic layer from the aqueous phase. The final step of sample preparation was the trimethylsilyl (TMS) derivatization of the extracts. After that, the extract was injected and analyzed with two different FS/HR GC instruments.

7.2.3 Instruments and Analytical Conditions

The detailed analytical instrument procedure was described previously in Chapter 5 [12]. The first instrument consisted of a GC 7890 system coupled with a 7200 Q-TOF mass spectrometer (G3850-64101; Agilent, Delaware, USA) equipped with a BPX5 5% phenyl polysilphenylene-siloxane

capillary column (30 m length, 0.25 mm ID, 0.1 μm film thickness; SGE, Victoria, Australia). The instrument operated in FS in positive electron ionization (EI) acquisition mode. The acquisition rate was 5 spectra per sec (200 msec per spectrum), and the number of transients per spectrum was set to 2718. The applied MS range (m/z 80–670) allows for the measurement of small molecules analyzed with GC/Q-TOF with a mass resolution $>12,500$. The second GC/MS system used in the current study was a GC/Q-Orbitrap system (Q Exactive GC, Thermo Scientific, Bremen, Germany) equipped with the above-described SGE BPX5 column. Full scan in positive EI acquisition mode was applied with a mass range of 80–670 m/z and 1 μsec scans, a resolving power of 60,000 at m/z 200 and the automatic gain control (AGC) target set at $3 \cdot 10^6$.

The current study is a pilot study, which has been conducted to evaluate the developed search template. Only six AAS were selected from the list of compounds that has been examined in Chapter 5. The six AAS analytes examined in the current study are listed in Table 1. These analytes were spiked in 10 different urine samples at concentration levels corresponding to the 50% MRPL level, and a total of 40 data files, which were generated from the validation data in both the HR GC/MS platforms, as described in Chapter 5, were used for reprocessing and searching for the selected analytes. Since the same GC column and the same temperature program were used, the retention times of compounds were the same in the two HR/FS GC/MS systems.

Table 1. List of the AAS examined in the study.

Analytes (TMS derivatives)	Chemical formula	RT (min)	Ions (m/z)			Spiked conc* (ng/mL)
Drostanolone met (Drostanolone 3ol17one)	$\text{C}_{26}\text{H}_{48}\text{O}_2\text{Si}_2$	14.26	448.3187	433.2953		2.5
Mesterolone met(1 α -methyl-5 α -androstane-3 α -ol-17-one)	$\text{C}_{26}\text{H}_{48}\text{O}_2\text{Si}_2$	15.47	448.3187	253.1951	433.2953	2.5
Stenbolone	$\text{C}_{26}\text{H}_{46}\text{O}_2\text{Si}_2$	16.23	220.1278	208.1278		2.5
Norethandrolone m2(17 α -Ethyl-5 β -estrane-3 α ,17 β -diol)	$\text{C}_{26}\text{H}_{50}\text{O}_2\text{Si}_2$	17.52	241.1951	331.2452		2.5
Formebolone met (Dealdehyde-formebolone)	$\text{C}_{29}\text{H}_{54}\text{O}_3\text{Si}_3$	19.14	534.3375			2.5
Oxymesterone	$\text{C}_{29}\text{H}_{54}\text{O}_3\text{Si}_3$	20.81	534.3375	519.3140	389.2327	2.5

* concentrations = 50% MRP

7.2.4 Data Analysis Protocol and the MetAlign Software Application

7.2.4.1 Manual Assessment of GC/MS Chromatograms

All 40 datafiles were evaluated manually for the presence or absence of the 6 AAS. The printouts were generated after reprocessing the datafile by the instrument software with a mass error range of 20 part per million (ppm) for the GC/Q-TOF data and 5 ppm for the GC/Q-Orbitrap data. The datafiles of GC/Q-TOF were processed by MassHunter software, and the datafiles of GC/Q-Orbitrap were processed by TraceFinder. The printouts of the generated ion chromatograms after grouping per compound were manually evaluated by two senior scientists experienced in antidoping analysis for each sample to determine the presence or absence of the searched analytes. The printouts were evaluated, and the signal of the analytes were compared to the corresponding signals in the QC samples. The analyst assessed if the peaks were sufficiently above the noise and background levels, if the retention time matched that of the QC samples and if applicable the EI fragment ion ratios within the analytes were correct visually [18]. The current workflow of manual processing and automatic reprocessing method with MetAlign suggested to replace one analyst is presented in Figure 1.

Figure 1. Schematic workflow of manual processing and automatic processing with MetAlign used in this study

2.4.2 MetAlign Software Search Templates and Criteria Setup

Based on the information presented in Table 1, a search template was created for both sets of platform data. The masses in this list together with the retention times were used to identify the 6 AAS targeted in this analysis. The criteria used for automatic data processing with MetAlign are summarized in Table 2 for the GC/Q-TOF instrument and GC/Q-Orbitrap analyser. A csv (comma separated value) text format served as a search template file and was created to contain information on the substances to be searched with the MetAlign input module. The template contained the following information: accurate masses, retention time, mass error window and retention window. The intensity values are added to the template to monitor EI fragment ratios of the same compound. The ion with the highest intensity for each compound in the template was used as a quantifier. In this setup, the compound is only considered identified when hits with at least two ions and a match factor of at least 900 are achieved. The match factor of MetAlign has been created by following the concept of the standard matching formula of the MS library searching algorithm described in [19]. The searches have been done on the data files using the version of MetAlign software especially adopted for retrospective antidoping screening.

Table 2. Matching criteria used by MetAlign for 6 compounds analyzed by GC/Q-TOF and GC/Q-Orbitrap.

Compound name	ppm error		RT	Delta RT	Min. nr of ions	ion 1 (m/z)	ion2 (m/z)	Ion3 (m/z)
	TOF	Orbitrap						
Stenbolone (-methyl-5 α -androst-1-en-17 β -ol-3-one)	30	5	16.1	0.15	2	446.3031	208.1278	
Norethandrolone metabolite (17A-Ethyl-5B-estrane-3A,17B-diol)	30	5	17.4	0.15	2	331.2452	241.1951	
Drostanolone metabolite (Drostanolone- 3ol-17one)	30	5	14.1	0.15	2	448.3187	433.2952	
Formebolone metabolite (Dealdehyde-formebolone)	30	5	19.0	0.15	1	534.3375		
Oxymesterone (17 α -methylandrost-4-en-4,17 β -diol-3-one)	30	5	20.7	0.15	3	534.3375	519.3141	389.2327
Mesterolone metabolite (1 α -methyl-5 α -androstane-3 α -ol-17-one)	30	5	15.3	0.15	2	448.3187	253.1951	433.2953

7.3 Result and Discussion

To assess the quality of automatically reprocessed HR- GC/MS data acquired on the GC/Q-TOF and GC/Q-Orbitrap platforms, we have first created a search template for the searched AAS compounds, which included the intensity values of at least 2 fragments of compounds to be searched to be able to perform matching based on EI fragment ratios. The search gave output files in the Excel compatible csv format. The quality of the performance of MetAlign was evaluated by counting the number of false positive in the output files of the blank urine samples (non-spiked samples) and false negatives identified in the output files of the 6 AAS spiked QCS samples. To evaluate the MetAlign search results for GC/Q-TOF, the following parameters have considered: 1- the number of false negatives in the spiked QCS samples reported by MetAlign compared to the manual evaluation, i.e., when the analyte is present in the spiked sample but MetAlign failed to detect it. 2- the number of false positives reported as AAS by MetAlign compared to the manual evaluation in the blank urine samples.

7.3.1 MetAlign Software Search Templates and Criteria Setup for GC/Q-TOF

Based on the compound matching parameters presented in Table 2 for the GC/Q-TOF data search, the performance of the MetAlign software was evaluated by comparing the number of the analytes corresponding to the 6 spiked AAS in the QCs samples detected by MetAlign with the number of the analytes corresponding to the 6 spiked AAS found in the report evaluated manually by two expert analysts using the report generated by the instrument' software (Agilent Mass Hunter Quantitative Analysis for QTOF). The results obtained with the MetAlign software and manual screening are summarized in Table 3.

Table 3. List of results based on the manual and automatic (MetAlign Search) evaluation approaches for GC/Q-TOF

Analyte	Manual	Automatic	False negatives	False positives
Stenbolone (-methyl-5 α -androst-1-en-17 β -ol-3-one)	10	10	0	0
Norethandrolone m2(17A-Ethyl-5B-estrane-3A,17B-diol)	10	10	0	0
Drostanolone met (Drostanolone- 3ol-17one)	10	10	0	0
Formebolone met (Dealdehyde-formebolone)	10	16	0	6
Oxymesterone (17 α -methylandrost-4-en-4,17 β -diol-3-one)	10	6	4	0
Mestrelone met (1 α -methyl-5 α -androstane-3 α -ol-17-one)	10	5	5	0

Stenbolone, drostanolone metabolite, norethandrolone metabolite and formebolone metabolite were identified by MetAlign searching as well as by the manual approach in all the 10 spiked samples by using the following search profile: a mass error window of 30 ppm and retention window of ± 0.15 minutes, as described in Table 2. Moreover, the false positive rate for the abovementioned analytes was zero.

For stenbolone, ions m/z 446.3031 and m/z 208.1278 were used for the identification of the analytes. Both ions were well detected in the spiked samples by the manual approach and automatic approach within the optimized criteria presented in Table 2. Furthermore, the automatic approach did not detect any signals that fit within the search profile in the blank samples. Accordingly, the false positive rate and false negative rate are zero for stenbolone.

In all the data files of the spiked samples, drostanolone metabolites were detected by the manual approach, while the automatic approach identified them as well with m/z 448.3187 (molecular ion M^+) and m/z 433.2952 (M^+-CH_3). Therefore, the false negative rate was zero for this analyte. Additionally, no signals from the background of the blank samples fit the search profile, which led to a zero false positive rate.

Similarly, a comparison between the manual evaluation and the MetAlign search resulted in a zero false positive and false negative rate for the norethandrolone metabolite. The identification of the norethandrolone metabolite was based on the detection of peaks with m/z 331.2452 and m/z

241.1951. The norethandrolone metabolite was identified by the manual approach as well as by the automatic approach in all the spiked samples. Moreover, neither approach identified any signals that met the norethandrolone metabolite identification criteria in the blank samples.

The formebolone metabolite was detected in all the spiked samples by the automatic and manual approaches, and therefore, the false negative rate is zero for this analyte. However, the results of the automatic approach showed 6 false positives cases for formebolone metabolite identification in the blank samples. The investigations show that the high number of misidentifications of the formebolone metabolite is most likely due to the MetAlign software wrongly selecting a closely eluted background peaks (within the delta retention time of 0.15 minutes) as shown in Figure 2, for the extracted ion chromatogram (EIC) of the molecular ion of 534.3375 m/z . The output file of the peaks identified by the MetAlign search software was checked manually to confirm that the correct peaks were detected in all the spiked samples. The false positive rate for the formebolone metabolite analyte can be minimized by reducing the error tolerance for a retention time to 0.1 minutes and a mass error to 20 ppm in the search template. After applying the new approach, reducing the error tolerance for retention time and mass error in the automated search template, the results show a zero false positive rate for the formebolone metabolite.

Figure 2. EIC of QC urine spiked with the formebolone metabolite at a 2.5 ng/mL concentration in the bottom compared with a blank urine sample in the top EIC, which shows a signal within the retention time window at 0.15 minutes.

The analysis of the GC/Q-TOF data files by MetAlign shows false negative results for oxymesterone and the mesterolone metabolite. Investigations of the false negatives showed that oxymesterone was not observed by the automated search due to the low intensity of the peak, which was similar to the noise background level, as shown by the EIC of 519.3141 ($M^+ - CH_3$) m/z in Figure 3. By removing the ion of 519.3141 m/z from the search template and exclusively using the most abundant fragment ions of 534.33753 (M^+) and 389.2327 m/z for the identification, the identification of oxymesterone by automated search template improved and the number of false positive reduced to zero.

Figure3. EIC of spiked QC urine with oxymesterone at 2.5 ng/mL in the bottom to be compared with a blank urine in the top EIC.

Moreover, the results in Table 3 shows 50% false negative rate for the detection of mesterolone metabolite and the peaks were not identified by the automated search due to the low intensity of peak with 253.1951 m/z , which were around the noise background level. The proposed solution for the search improvement is to remove the ion of 253.1951 m/z from the search template and to search only for the most abundant fragment ions such as the one with 448.3197 and 433.2953 m/z .

7.3.2 Metalign Software Search Templates and Criteria Setup for GC/Q-Orbitrap

Based on the criteria presented in Table 2 for the GC/Q-Orbitrap data search, a comparison of the results of the manual approach and automatic approach using MetAlign for the samples spiked with the 6 AAS analytes was performed and is presented in Table 4. The MetAlign performance was evaluated by comparing the number of the detected analytes in the spiked QC and blank

samples to that found in the report provided by the vendor software and processed by the expert analysts.

Table 4: List of results based on the manual and automatic (MetAlign Search) evaluation approaches for GC/Q-Orbitrap

Analyte	Manual	Automatic	False negatives	False positives
Stenbolone (-methyl-5 α -androst-1-en-17 β -ol-3-one)	10	10	0	0
Norethandrolone m2(17A-Ethyl-5B-estrane-3A,17B-diol)	10	10	0	0
Drostanolone met (Drostanolone- 3ol-17one)	10	10	0	0
Formebolone met (Dealdehyde-formebolone)	10	10	0	0
Oxymesterone (17 α -methylandro-4-en-4,17 β -diol-3-one)	10	6	4	0
Mesterolone met (1 α -methyl-5 α -androstane-3 α -ol-17-one)	10	4	6	0

As presented in Table 4, stenbolone, norethandrolone and the drostanolone metabolite that were spiked in the QC samples were identified by the MetAlign search as well as by the manual approach. Stenbolone analytes were detected with a molecular ion of 446.3031 and 208.1278 m/z . The automated search results showed that stenbolone was identified in all the spiked QC samples and no signals fit the search profile of stenbolone in the blank samples. Therefore, the false negative and false positive rate is zero for stenbolone.

The comparison between the manual and automatic approach evaluation results also showed a zero false negative and zero false positive rate in the case of the norethandrolone metabolite when the criteria shown in Table 2 was used. Both ions with 331.2452 and 241.1951 m/z were used for identification and were detected by the automatic search as well as by the manual search in the spiked QCs samples. In addition, no misidentification of background peaks or interferences that could lead to false positive results was observed in blank urine samples by automatic search.

Similar to stenbolone and the norethandrolone metabolite, the false negative and false positive rate are zero for the drostanolone metabolite. The identification of the drostanolone metabolite was based on the molecular ion of 448.3187 and 433.2953 ($M^+ - CH_3$) m/z . The drostanolone metabolite

was identified in the spiked QC samples by both approaches. Moreover, the automatic approach did not identify any background peak or interference that could lead to false positive results in the blank urine samples.

The formebolone metabolite was detected in all the spiked QC samples by the automatic and manual approaches, therefore the false negative rate is also zero for this analyte. The results also showed a zero false positive rate in blank samples detected by the automatic approach due to the high mass accuracy, allowing for the use a mass window of 5 ppm to generate the extracted ion chromatograms. The previous setup led to the generation of clear EICs for the formebolone molecular ion of 534.3375 m/z in the blank samples without a closely eluted background peak, as shown in Figure 4.

Figure 4. EIC of m/z 534.3375 in the QC urine spiked with the formebolone metabolite at a 2.5 ng/mL concentration level (right plot) compared with a blank urine sample (left plot).

The results of automatically searching of the GC/Q-Orbitrap data showed false negatives in the case of oxymesterone and the mesterolone metabolite. The analysis of the data files by MetAlign shows more false negative results for the oxymesterone metabolite than the manual approach shows. Observations showed that the oxymesterone metabolite was not identified by the automated search due to the low intensity of the ion with 519.3141 ($M^+ - CH_3$) m/z , which was within the noise background level. The identification of the oxymesterone metabolite by the automated search can be improved by removing the low intensity ion of 519.3141 m/z from the search template and to use the most abundant ions of 534.33753 (M^+) and 389.2327 m/z . The results of the proposed new automated search setting showed a zero false negative rate for oxymesterone metabolite identification.

Moreover, the results in Table 4 show that 60% of the mesterolone metabolite peaks were not identified by the automated search due to the low intensity of the ion with 253.1951 m/z , which was within the noise level of the background, as shown in Figure 5. To reduce the number of false negatives in the automatic approach, ion 253.1951 m/z was removed from the search template, and the search was applied only for the most abundant ions at 448.3197 and 433.2953 m/z . The result with the new search setting showed a zero false negative rate for mesterolone metabolite identification.

Figure 5. EIC of the ion with m/z 253.1951 m/z from the QC urine spiked with the mesterolone metabolite at a 2.5 ng/mL (right plot) and a blank urine (left plot).

In general, the automatic search of the GC/Q-TOF data found more false positive signals than that of the GC/Q-Orbitrap results, showing the benefit of the higher mass resolution and higher mass accuracy of the Orbitrap mass analyzer by allowing a smaller error tolerance for the peak m/z of the targeted analytes, as explained in the discussion of chapter 5 [12].

7.4 Conclusion

The current chapter shows how MetAlign software can be used for the identification of AAS in FS/HR GC/Q-TOF and GC/Q-Orbitrap data. An extensive re-processing of acquired GC/Q-TOF and GC/Q-Orbitrap data with MetAlign software is ongoing to include more compounds to allow the comprehensive assessment of MetAlign to automatically identify compounds in already acquired data. The presented search template should be applied to other AASs and prohibited substances to conclude and determined the applicability of MetAlign search templated on FS/HR-GC/MS data under the WADA ISL. Additional efforts should be made to improve the MetAlign

search results, such as increasing the number of studied analytes and adding more filter criteria. The proposed additional filter criteria are as follows: a smaller mass accuracy error filter and signal intensity filter and to examine their influence on false positive and false negative rates. The use of retention time alignment to reduce the variability of the compound retention time in raw data and implementing an automatic algorithm to identify the best mass and retention time tolerance search parameter [20-23] are also proposed to improve the MetAlign search. The optimized parameter should be thoroughly validated before reduced data files are considered and before retrospective identification of new designer drugs is implemented to eventually replace human analyst manual assessment of FS/HR-GC/MS data.

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Chapter 8

Summary and Future Perspective

The overall aim of this PhD Thesis is to study the use of high-resolution full scan (FS/HR) LC/MS and GC/MS small molecule platforms for comprehensive antidoping screening. FS/HR allows to accurately identify and quantify the endogenous anabolic steroids included in the Athlete Biological Passport (ABP) measured with GC/MS and to consider new steroidal ABP biomarkers such as intact phase II sulfate metabolites and to apply new mass spectrometry data processing approach for the detection of new unknown designer doping agents.

The main conclusion of this PhD Thesis is that the new FS/HR GC/MS and LC/MS methods presented in this PhD research are validated for high-throughput antidoping screening and are ready to be applied as routine procedures in any WADA Accredited Laboratory equipped with the respective analytical technologies.

This PhD Thesis includes chapters to address these topics using various analytical platforms. **Chapter 1** provides a general introduction into antidoping screening, the framework defined by WADA specifications for athletes testing and the central role of anabolic androgenic steroids (AAS) and designer drugs used by cheating athletes to enhance their sport performance. The outline of this Thesis is presented in Chapter 1.

Chemically modified steroids, also known as designer AAS that are manufactured with the purpose of evading anti-doping tests and legislations of prohibited substances, remain a major concern in the fight against doping. **Chapter 2** focuses on the main challenges related to designer AAS use by athletes and to anti-doping screening endeavours. These include major efforts made by anti-doping screening laboratories and drug-testing authorities to control the illegal spread of designer AAS through rigorous doping controls and policy by applying strict analytical and regulatory guidelines. The review presented in **Chapter 2** presents the recent improvements of analytical protocols as well as used sample preparation and instrumental analytical techniques currently used for the successful detection of designer AAS. The performance of novel high-resolution fast scanning mass spectrometers and high-throughput bioassays for the successful detection of AAS is discussed together with the use of the *in-silico* prediction of AAS metabolites and availability of their synthetic reference materials for anti-doping screening purposes. The importance of reprocessing the LC-MS(/MS) data of already analyzed anti-doping control samples and the importance of urine sample stabilization and long-term storage are also discussed.

Chapter 3 presents how LC/MS screening methods are developed and validated to detect the endogenous and exogenous prohibited substances. This chapter also focuses on the expansion of the analytical detection range of small molecules to the detection of sulfate Phase II metabolites and the quantitative determination of endogenous sulfate steroids in their intact form. In this study, 304 prohibited substances are validated within the estimated parameters, which are in compliance with International Standard for Laboratories (ISL) specifications [1]. A robust screening procedure was developed, and the obtained validation parameters showed an excellent identification performance at 50% of the WADA minimum required performance limits (MRPL) [2] and the lack of interference for the target analyte's peak. All the validation data was collected within a 3-month period by performing sample preparation and the analysis with two FS/HR-LC/MS systems. The quantitative validation results of the sulfo-conjugated steroid profile show an excellent analytical performance with respect to linearity, accuracy (2.4–17%), intermediate precision (1.2–4.1%), and combined measurement uncertainties (3.2–18%) over the calibration range. The sulfo-conjugated steroid profile can be studied in all samples without any additional analysis and without creating any interferences with the gluco-conjugated steroid profile, as required by the WADA specification [3]. Polarity switching allowed for the detection of basic and acidic drugs within the same chromatographic run. Positive and negative extracted ion chromatograms were simultaneously used as detection criterion to eliminate false identifications. The high-resolution full scan LC/MS data obtained with the polarity switching MS acquisition mode allowed the collected data to be reprocessed retrospectively, new metabolites and unknown prohibited designer drugs could be searched.

The objective of **Chapter 4** was to develop and validate a new screening method capable of detecting small molecules by using GC/Q-TOF as a complementary analytical platform to the currently used Orbitrap LC/MS screening method described in **Chapter 3**. The method was validated for the detection of 73 analytes with an LOD that was 50% of the MRPL [2] in full scan (FS) acquisition mode. Moreover, the method was validated for the quantification of six endogenous steroids, including testosterone (T), epitestosterone (E), androsterone (A), etiocholanolone (Etio), 5A-androstan-3A,17B-diol (5AA diol), and 5B-androstan-3A,17B-diol (5B diol), which was considered as steroid profile (SP) included in the ABP. The results indicate that the SP profile obtained with high-resolution GC/Q-TOF meets the specifications of WADA [3]. The combined uncertainty for the determination of the endogenous steroids, as estimated

during method validation, meets the requirements of WADA. The acquisition of the FS data allows the retrospective analysis of the already acquired GC/Q-TOF datafile for samples analyzed for official anti-doping purposes to detect unknown designer drugs, that were not known at the time of data acquisition or new AAS long-term metabolites that prolong AAS detection abuse in human urine samples.

The study presented in **Chapter 5** compared the performances of the GC/Q-TOF and GC/Q-Orbitrap for qualitative and quantitative screening analyses using the same quality control urine samples. The method had been validated for a limited number of analytes, including representative exogenous AAS and all the endogenous AAS present in the steroidal ABP. The acquired data indicated that both platforms are suitable for anti-doping screening. Regarding qualitative analysis, the validated LODs of both instruments were well below 50% of the MRPL [2]. The identification parameters which are based on the detectability of the AAS within the different urine matrices at 50% of the MRPL, showed that a limited number of compounds were unable to reach 100% detectability in the different urine matrices. Additionally, the results indicate that the quantitative analysis of the endogenous AAS obtained with high-resolution GC/Q-TOF and GC/Q-Orbitrap met the specifications of WADA [4]. The combined uncertainty for the determination of the endogenous steroids, as estimated during method validation, meets the requirements of WADA and is less than 20% for A and Etio, 25% for 5AA diol and 5B diol and less than 20% for T and E in both platforms. Overall, the data showed that both instruments are functional and suitable for routine anti-doping testing and analysis. **Chapter 7** presents the results of a pilot study used for the reprocessing of the existing high-resolution GC/MS data files generated from the study presented in **Chapter 5**.

In **Chapter 6**, the performance of the MetAlign search software is assessed for use as a retroactive re-processing tool for data files acquired in FS mode by Thermo Orbitrap LC/MS to identify doping cases during the International Testing Procedure (ITP). MetAlign processing has the capability of searching thousands of reduced datafiles in seconds. This functionality produces an essential value for the already acquired analytical data especially for the earlier collected samples physically not available any longer that could contain unknown substances or new metabolites. In this study, a total of 940 datafiles were reprocessed with the MetAlign search software, and the results show that no false negatives were determined when this automated approach was used, and

these results were compared to the manual routine assessment results. However, the searching module generated several false positives, as expected, due to the presence of urine background peaks. The application of specific parameter filters, such as accurately setting mass error ranges, implementing abundance thresholds below which detection of a low concentration substance is uncertain and using multiple m/z values such as natural stable isotopes, for example, could assist in reducing the false positive rate generated by the automatic approach. The searching parameters could be adjusted for each analyte to optimize case-by-case compound identification. The current approach has the potential to act as a massive deterrent to doping when combined with the already applied WADA long-term sample storage policy.

Chapter 7 presents a pilot comparative study, as a continuation of **Chapter 5**. **Chapter 7** focuses on how the MetAlign software can be used for the identification of six exogenous AAS in FS/HR GC/Q-TOF and GC/Q-Orbitrap data that had been generated using the data obtained in the validation study presented in **chapter 5**. The result shows the successful identification of the six exogenous AAS with a zero rate of false positive and false negative rate. The study should be extended to include more compounds to obtain more reliable statistics to conclude and determine the applicability of MetAlign automatic search templated on FS/HR-GC/MS data under the WADA ISL [1]. This can be achieved by using the MetAlign software to extensively re-process the GC/Q-TOF and GC/Q-Orbitrap data acquired during previous sport events. Moreover, a thorough validation of the optimized parameters should be conducted to consider the applicability of using the MetAlign search software on the reduced data files and the potential of retrospectively identifying new designer drugs and the eventually replacement of human analysts (manual assessment) with an automatic assessment of the FS/HR-GC/MS data for antidoping screening.

Future perspectives

Anti-doping agencies are playing a “cat-and-mouse game” against illegal private and governmental institutions to fight the use of doping agents by athletes to maintain the fairness of sports and protect the health of athletes. The advances in analytical technologies and bioinformatics to process and interpret the acquired data to find designer AAS and their long-term metabolites is certainly not the maximum of what can be achieved to detect doping activities. In my host institution at the Antidoping Lab Qatar (ADLQ) and in other WADA antidoping laboratories world-wide, there are numerous efforts that aim to further improve anti-doping

screening efficiency by improving various aspects, such as the accuracy of the detection by lowering false positive and negative doping detections, by decreasing the analysis time and cost of the screening and laboratory infrastructure required for sample storage, preparation and analysis and by determining how to more efficiently process the collected data obtained with various analytical platforms by not necessarily using mass spectrometry. For example, in ADLQ, we will further study sulfo-conjugate endogenous anabolic androgenic steroids (EAAS-S) related to testosterone (T) metabolism and quantified from routine urine anti-doping samples. It is also important to determine the reference values of EAAS-S in female and male elite athlete populations to set statistical reference limits, distributions of nominal concentrations and compound ratios. Such work is currently being conducted by my colleagues with my involvement by combining a GC/MS/MS analysis for the gluco-conjugate EAAS and LC/MS analysis for the sulfo-conjugate EAAS-S. The gluco-conjugate EAAS and sulfo-conjugate EAAS-S concentrations and the ratios between them are considered potential biomarker candidates for enhancing the specificity and sensitivity of the urinary steroid profile, which could enhance the anti-doping accuracy of WADA-implemented ABP system.

In my opinion, the most important aspect to improve anti-doping screening is to implement further actions to reach the full implementation of FS/HR-GC/MS and FS/HR-LC/MS screening procedures. This should be combined with routine use of accurate automatic data processing and machine learning algorithms that combine multiple information from the collected data to indicate possible doping events.

In regard to the steroid profile biomarkers originated from both the GC WADA EAAS systems and the proposed additional biomarkers in the LC sulfate conjugated EAAS-S [4], the following future studies should be conducted, following ethical approval:

- Data should be collected from at least 10 000 routine anti-doping athletes' samples for both gluco-conjugate EAAS and sulfo-conjugate EAAS-S without confounding factors [4] to provide a substantial athlete population reference data set for both genders from various ethnicities.
- Data should be collected from T administration studies with the same subject receiving placebo, transdermal and intramuscular injections.
- Data should be collected from clinical studies related to multiple micro-dosing administrations of oral and transdermal T and oral or transdermal administrations of prohormones DHEA, DHT and androstenedione. The micro-dosing should be defined in

each study as 5-10 times less than the suggested dose. It is crucial that the same group of volunteers are used for all studies to avoid individual biological variance. At least two males/females with low T/E phenotypes should be included in the group of volunteers planned in such future clinical studies at the ADLQ.

- A clinical study should be performed to determine the stability of the sulfate steroids in urine samples obtained from healthy females throughout two menstrual cycles, as menstrual cycles significantly influence steroid hormone levels. From the baseline samples obtained over the entire period of this future clinical study, the stability of the sulfate steroid profile in healthy male/female subjects should be examined.
- The entire set of antidoping FS/HR-LC/MS data will be subjected to further metabolomic identification. It is important to develop a metabolomics identification pipeline to identify as much metabolites present in routine urine samples measured during routine antidoping screening.

More data must be collected using the FS/HR-GC/MS platform to assess the detection accuracy of AAS at concentrations close or below the MRPL. This Thesis included only AAS and their metabolites. Therefore, the use of FS/HR-GC/MS combined with well-advanced data processing tools such as MetAlign should be extended to other classes of prohibited substances, such as stimulants, narcotics, and β 2-agonists, to enhance the generic nature of the method. The detection of sulfate exogenous AAS metabolites in urine should be developed and validated using FS/HR-LC/MS screening testing, since sulfate metabolites possibly have lower excretion rates and longer detection times than other metabolites. This would improve the detection of AAS abuse after the doping event. Finally, bioinformatics tools such as MetAlign should be implemented and used routinely during antidoping screening in an automatic way to analyse both FS/HR-GC/MS and FS/HR-LC/MS data files for the automatically identification of known and unknown doping substances in urine samples of athletes.

I personally think that the development of these new and accurate anti-doping screening approaches will improve deter athletes from doping abuse, will contribute to maintain the fairness of sports during training and competition events, and will maintain the health of competing athletes.

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Samenvatting en toekomstperspectief

Het algemene doel van dit proefschrift is het bestuderen van het gebruik van analytische moleculaire profileringplatforms op basis van High-Resolution Full Scan (FS/HR) LC en GC voor uitgebreide antidoping-screening met als uiteindelijk doel het nauwkeurig kwantificeren van endogene anabole steroïden in het Athlete Biological Passport (ABP) alsmede het toepassen van nieuwe methodes voor verwerking van massaspectrometrie-data voor de detectie van nieuwe, onbekende designer-dopingmiddelen. Dit proefschrift bevat verschillende hoofdstukken waarin deze onderwerpen met behulp van verschillende analytische platforms worden uitgewerkt.

Hoofdstuk 1 geeft een algemene introductie over antidoping-screening, het kader van de WADA specificaties voor het testen van atleten en de centrale rol van anabole androgene steroïden (AAS) en designer-dopingmiddelen die door valsspelende atleten worden gebruikt om hun sportprestaties te verbeteren. Ook wordt een overzicht van dit proefschrift gepresenteerd in **hoofdstuk 1**.

Chemisch gemodificeerde steroïden, ook wel bekend als designer AAS, worden gefabriceerd met als doel het ontwijken van antidopingtests en wetgeving inzake verboden stoffen en blijven een groot probleem in de strijd tegen doping. **Hoofdstuk 2** richt zich op de belangrijkste uitdagingen die verband houden met het gebruik van designer AAS door atleten en met antidoping-screening. Deze omvatten de grote inspanningen van antidoping-screeningslaboratoria en dopingtest-autoriteiten om de illegale verspreiding van designer AAS te beheersen door strenge dopingcontroles en -beleid door strikte analytische en regelgevende richtlijnen toe te passen. Het overzicht in **hoofdstuk 2** presenteert de recente verbetering van analytische protocollen, alsmede de momenteel gebruikte monstervoorbereidings- en instrumentele analytische technieken voor het succesvol detecteren van designer AAS. De prestaties van nieuwe snelle en hoge resolutie massaspectrometers en high-throughput bioassays voor de succesvolle detectie van AAS worden besproken samen met het gebruik van *in silico* voorspelling van AAS metabolieten en de beschikbaarheid van hun synthetische referentiematerialen voor antidoping-screeningsdoeleinden. Het belang van het opnieuw verwerken van de LC-MS(/MS) data van reeds geanalyseerde antidoping-controlemonsters en het belang van stabilisatie van urinemonsters en hun langdurige opslag worden ook besproken.

Hoofdstuk 3 laat zien hoe LC/MS-screeningsmethoden worden ontwikkeld en gevalideerd om endogene en exogene verboden stoffen te detecteren. Het hoofdstuk richt zich ook op de uitbreiding van het analytische detectiebereik van kleine moleculen naar de detectie van Fase II-

metabolieten met sulfaat en de kwantitatieve bepaling van endogene sulfaatsteroïden in hun intacte vorm. In deze studie worden 304 verboden stoffen gevalideerd binnen de geschatte parameters in overeenstemming met de specificaties van de International Standard for Laboratories (ISL) [1]. Een robuuste screeningprocedure is ontwikkeld en de verkregen validatieparameters toonden een uitstekende identificatieprestatie bij 50% van de minimaal vereiste WADA-prestatielimieten (MRPL) [2] zonder interferentie voor de piek van het doelanalyt. Alle validatiegegevens werd binnen een periode van 3 maanden verzameld door monstervoorbereiding en analyse uit te voeren met twee FS/HR-LC/MS-systemen. De kwantitatieve validatieresultaten van het profiel van sulfo-geconjugeerde steroïden laten uitstekende analytische prestaties zien met betrekking tot lineariteit, nauwkeurigheid (2,4–17%), gemiddelde precisie (1,2–4,1%) en gecombineerde meetonzekerheden (3,2–18%) over het kalibratiebereik. Het sulfo-geconjugeerde steroïde profiel kan in alle monsters worden bestudeerd zonder enige aanvullende analyse en zonder enige interferentie met het met gluco-geconjugeerde steroïde profiel zoals vereist door de WADA-specificatie [3]. Wisseling van polariteit maakte de detectie mogelijk van basische en zure drugs binnen dezelfde chromatografische run. Positieve en negatieve extracted-ion chromatogrammen werden gelijktijdig gebruikt als detectie criterium om verkeerde identificaties te elimineren. De hoge-resolutie full scan LC/MS data opgenomen met polariteitsschakeling maakten het mogelijk om de verzamelde data retrospectief te verwerken en te zoeken naar nieuwe metabolieten en onbekende, verboden designerdrugs.

Het doel van **hoofdstuk 4** was de ontwikkeling en validatie van een nieuwe screeningmethode die kleine moleculen kan detecteren door GC/Q-TOF te gebruiken als een complementair analytisch platform naast de momenteel gebruikte Orbitrap LC/MS-screeningmethode beschreven in **hoofdstuk 3**. De methode is gevalideerd voor de detectie van 73 analyten met een LOD van 50% MRPL [2] in full scan (FS) acquisitiemodus. De methode is ook gevalideerd voor de kwantificering van zes endogene steroïden die als steroïdeprofiel (SP) in het ABP opgenomen zijn, zoals testosteron (T), epitestosteron (E), androsteron (A), etiocholanolon (Etio), 5A-androstan-3A,17B-diol (5AAdiol), en 5B-androstan-3A,17B-diol (5Badiol). De resultaten laten zien dat het SP-profiel verkregen met hoge-resolutie GC/Q-TOF voldoet aan de specificaties van de WADA [3]. De gecombineerde onzekerheid voor de bepaling van de endogene steroïden, zoals ingeschat tijdens de validering van de methode, voldoet aan de vereisten van de WADA. Het opnemen van FS-data maakt het mogelijk om retrospectieve analyse te doen van de GC/Q-TOF-datafile van een

monster dat is geanalyseerd voor officiële antidopingdoeleinden om onbekende designerdrugs op te sporen, die niet bekend waren ten tijde van de dataverzameling of nieuwe AAS lange-termijn metaboliëten die de detectieperiode van AAS-misbruik in menselijke urinemonsters verlengen.

De studie die in **hoofdstuk 5** besproken wordt vergelijkt de prestaties van de GC/Q-TOF en GC/Q-Orbitrap voor kwalitatieve en kwantitatieve screeninganalyses met dezelfde kwaliteitscontrole-urinemonsters. De methode is gevalideerd voor een beperkt aantal analyten, waaronder representatieve exogene AAS en alle endogene AAS die aanwezig zijn in het steroïde ABP. De data lieten zien dat beide platforms geschikt zijn voor antidopingonderzoek. Wat betreft kwalitatieve analyse waren de gevalideerde LOD's in beide instrumenten ruim onder 50% van het MRPL-gevoeligheidsniveau [2]. De identificatieparameters die zijn gebaseerd op de detecteerbaarheid van de AAS in de verschillende urinematrices bij 50% van de MRPL-concentratie, toonden aan dat voor een beperkt aantal verbindingen 100% detecteerbaarheid niet bereikt werd in de verschillende urinematrices. Bovendien geven de resultaten aan dat de kwantitatieve analyse voor endogene AAS verkregen met hoge-resolutie GC/Q-TOF en GC/Q-Orbitrap voldeed aan de specificaties van WADA [4]. De gecombineerde onzekerheid voor de bepaling van de endogene steroïden zoals geschat tijdens de methodevalidatie voldoet aan de vereisten van de WADA en is minder dan 20% voor A en Etio, 25% voor 5AAdiol, en 5Badiol en minder dan 20% voor T en E op beide platforms. Over het geheel genomen toonden de data aan dat beide instrumenten geschikt zijn voor routinematige antidopingcontroles en -analyses. **Hoofdstuk 7** beschrijft de resultaten van een teststudie voor het heranalyseren van de bestaande hoge-resolutie GC/MS-databestanden die zijn gegenereerd voor de studie die beschreven is in **hoofdstuk 5**.

In **hoofdstuk 6** wordt de prestatie van de MetAlign software beoordeeld voor het gebruik als een retroactieve herverwerkingsapplicatie voor databestanden die in FS-modus zijn opgenomen op een Thermo Orbitrap LC/MS om dopinggevallen te identificeren tijdens de Internationale Testprocedure (ITP). Verwerking met MetAlign geeft de mogelijkheid om binnen enkele seconden duizenden kleinere databestanden te doorzoeken. Deze functionaliteit verschaft een essentiële meerwaarde voor de reeds opgenomen analytische data, vooral voor de eerder verzamelde monsters die niet meer fysiek beschikbaar zijn en die onbekende stoffen of nieuwe metaboliëten zouden kunnen bevatten. In deze studie werden in totaal 940 databestanden opnieuw verwerkt met

MetAlign en deze liet geen vals-negatieve resultaten zien als een geautomatiseerde aanpak werd gebruikt in plaats van een handmatige routinebepaling. De zoekmodule genereert echter een aantal vals-positieve resultaten, zoals verwacht vanwege de aanwezigheid van achtergrondpieken in de urine. De toepassing van specifieke parameterfilters, zoals nauwkeurig ingestelde bereiken voor de fout in de bepaalde massa, drempelwaarden voor de intensiteit waaronder de detectie van een stof met een lage concentratie onzeker is, en het gebruik van meerdere m/z waarden zoals natuurlijke stabiele isotopen, zou kunnen helpen bij het verminderen van het percentage van vals-positieve resultaten dat wordt gegenereerd door de automatische aanpak. De zoekparameters kunnen voor elke analyt worden aangepast om de identificatie van de verbinding per geval te optimaliseren. De huidige aanpak heeft het potentieel om een enorm afschrikmiddel te vormen, in combinatie met het reeds toegepaste WADA-beleid voor lange-termijn monsteropslag.

Hoofdstuk 7 beschrijft een vergelijkende pilotstudie, als vervolg op het werk in **hoofdstuk 5**. Deze richt zich op hoe de MetAlign-software kan worden gebruikt voor identificatie van zes exogene AAS in FS/HR GC/Q-TOF data en GC/Q-Orbitrap data die zijn gegenereerd in de validatiestudie die beschreven is in **hoofdstuk 5**. Het resultaat toont de succesvolle identificatie aan van de zes exogene AAS met een percentage van nul voor zowel vals-positieve als vals-negatieve resultaten. De studie zou moeten worden uitgebreid met meer verbindingen om een hogere statistische betrouwbaarheid te verkrijgen voordat een conclusie kan worden getrokken over de toepasbaarheid van het automatisch zoeken met MetAlign van FS/HR-GC/MS-gegevens onder de WADA ISL [1]. Dit kan worden bereikt door een uitgebreide herverwerking met MetAlign-software van GC/Q-TOF en GC/Q-Orbitrap data die tijdens eerdere sportevenementen zijn verkregen. Bovendien moet een grondige validatie van de geoptimaliseerde parameters worden uitgevoerd om de toepassing van MetAlign op de gereduceerde databestanden te evalueren en voor retrospectieve identificatie van nieuwe designerdrugs en uiteindelijk om de menselijke data analist (gebruik makend van handmatige beoordeling) te vervangen door automatische beoordeling van FS/HR-GC/MS-data voor antidoping-screening.

Toekomstperspectief

Antidopingagentschappen spelen een 'kat-en-muisspel' met illegale particuliere en overheidsinstellingen om het gebruik van dopingmiddelen door atleten te bestrijden om zo de

eerlijkheid van sport te behouden en de gezondheid van atleten te beschermen. De geavanceerde analytische technologieën en bioinformatica om de verkregen data te verwerken en te interpreteren om zo designer AAS en hun lange-termijn metabolieten te vinden vormen zeker niet het maximale dat kan worden bereikt om dopingactiviteiten te detecteren. In mijn gastinstelling in het Antidoping Lab Qatar (ADLQ) en in andere WADA-antidopinglaboratoria over de hele wereld zijn er tal van inspanningen gericht op het verder verbeteren van de efficiëntie van de antidoping-screening op verschillende onderdelen, zoals de nauwkeurigheid van de detectie door het verlagen van vals-positieve en negatieve dopingdetecties, verkorten van de analysetijd en de kosten van de screening en de laboratoriuminfrastructuur die nodig is voor opslag, voorbereiding en analyse van monsters, evenals het efficiënter verwerken van de verzamelde data die zijn verkregen met verschillende analytische platforms die niet noodzakelijkerwijs massaspectrometrie gebruiken. Zo zullen we in het ADLQ verder werken aan sulfo-geconjugeerde endogene anabole androgene steroïden (EAAS-S) gerelateerd aan het testosteron (T)-metabolisme, gekwantificeerd in routinematige urine-antidopingmonsters. Het is ook belangrijk om de referentiewaarden van EAAS-S in de vrouwelijke en mannelijke populaties van elite-atleten te bepalen om statistische referentielimieten, verdelingen van nominale concentraties en verhoudingen van stoffen vast te stellen. Dit werk wordt momenteel uitgevoerd door mijn collega's in samenwerking met mij door GC/MS/MS-analyse voor de gluco-geconjugeerde EAAS en LC/MS-analyse voor de sulfo-geconjugeerde EAAS-S te combineren. De gluco-geconjugeerde EAAS en sulfo-geconjugeerde EAAS-S concentraties en de verhoudingen daartussen worden beschouwd als potentiële biomarkerkandidaten om de specificiteit en gevoeligheid van het urine-steroïdeprofiel te verbeteren, wat de antidoping-nauwkeurigheid van het door de WADA geïmplementeerde ABP-systeem zou kunnen verbeteren.

Naar mijn mening is het belangrijkste aspect om het antidopingonderzoek te verbeteren verdere actie om volledige implementatie van FS/HR-GC/MS en FS/HR-LC/MS-screeningprocedures te bereiken. Dit zou moeten worden gecombineerd met routinematig gebruik van nauwkeurige automatische dataverwerking en een machine learning-algoritme dat informatie uit de verzamelde data combineert om mogelijke dopinggevallen eruit te lichten. Wat betreft de biomarkers in het steroïdeprofiel die afkomstig zijn van zowel GC WADA EAAS-systemen als de voorgestelde aanvullende biomarkers in sulfo-geconjugeerde EAAS-S gemeten met LC [4], moeten de volgende toekomstige studies worden uitgevoerd na ethische goedkeuring:

- Gegevens verzamelen van ten minste 10 000 routinematige antidoping-monsters van atleten voor zowel gluco-geconjugeerde EAAS als sulfo-geconjugeerde EAAS-S zonder versturende factoren [4] om een substantiële hoeveelheid referentiedata van de atletenpopulatie te verkrijgen voor beide geslachten en van verschillende etniciteiten.
- Gegevens verzamelen uit T-toedieningsstudies waarbij dezelfde proefpersoon placebo, transdermale en intramusculaire injecties kreeg.
- Gegevens verzamelen uit klinische onderzoeken gerelateerd aan meervoudige microdoseringstoediening van orale en transdermale T en orale of transdermale toediening van de prohormonen DHEA, DHT en androsteendion. De microdosering moet in elk onderzoek worden gedefinieerd als 5-10 keer lager dan de aanbevolen dosis. Het is van cruciaal belang dat dezelfde groep vrijwilligers wordt gebruikt voor alle studies om individuele biologische variatie te vermijden. Ten minste twee mannen en vrouwen met een laag T/E-fenotype moeten worden opgenomen in de groep vrijwilligers die in dergelijke toekomstige klinische onderzoeken bij het ADLQ worden gepland.
- Klinisch onderzoek moet worden uitgevoerd om de stabiliteit van de sulfaatsteroïden in urinemonsters van gezonde vrouwen gedurende twee menstruatiecycli te bepalen, aangezien de menstruatiecyclus de steroïdhormoonspiegels aanzienlijk beïnvloedt. Uit de basislijn-monsters over de gehele periode van dit toekomstige klinische onderzoek moet de stabiliteit van het sulfaatsteroïdenprofiel bij gezonde mannelijke en vrouwelijke proefpersonen worden onderzocht.
- De volledige set van antidoping FS/HR-LC/MS data zal worden gebruikt voor verdere metabolomics-identificatie. Het is belangrijk om een metabolomics-identificatiepijplijn te ontwikkelen om zoveel mogelijk metabolieten te identificeren die aanwezig zijn in een routinematig urinemonster gemeten tijdens routine antidoping-screening.

Meer data moeten worden verzameld met het FS/HR-GC/MS-platform om de detectienauwkeurigheid van AAS te beoordelen bij concentraties dichtbij of onder de MRPL. Dit proefschrift behandelt alleen AAS en hun metabolieten. Daarom moet het gebruik van FS/HR-GC/MS in combinatie met geavanceerde dataverwerkingstools zoals MetAlign worden uitgebreid naar andere klassen van verboden stoffen zoals stimulantia, verdovende middelen, en β 2-agonisten om de algemene toepassing van de methode te verbeteren. De detectie van exogene sulfo-geconjugeerde AAS-metabolieten in urine moet worden ontwikkeld en gevalideerd met FS/HR-

LC/MS screeningstests, aangezien de sulfaatmetabolieten mogelijk lagere excretiesnelheden en langere detectietijden hebben. Dit zou de detectie van AAS-misbruik in de latere fase van het dopinggebruik verbeteren. Ten slotte moeten bioinformatica-tools zoals MetAlign worden geïmplementeerd en routinematig op automatische wijze worden gebruikt tijdens antidoping-screening om zowel FS/HR-GC/MS als FS/HR-LC/MS-databestanden te analyseren voor automatische identificatie van bekende en onbekende dopingstoffen in urinemonsters van atleten.

Persoonlijk denk ik dat de ontwikkeling van deze nieuwe en nauwkeurige methodes voor dopingonderzoek de afschrikking zal verhogen van het misbruiken van doping door atleten, zal bijdragen aan het behoud van eerlijkheid in sport, in zowel training als in wedstrijden, en de gezondheid van de deelnemende atleten zal waarborgen.

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Publications list

Part of this thesis

1. **Abushareeda W.**, Fragkaki A., Vonaparti A., Angelis Y., Tsivou M., Saad K., Kraiem S., Lyris E., Alsayrafi M., Georgakopoulos C., Advances in the detection of designer steroids in anti-doping, *Bioanalysis* 2014; 6 (6): 881-896.
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