Division of Pharmaceutical Chemistry and Technology
Faculty of Pharmacy
University of Helsinki
Finland

Development of Mass Spectrometric Methods for Analysis of Anabolic Androgenic Steroids

Laura Hintikka

ACADEMIC DISSERTATION

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Supervisors: Professor Risto Kostiainen
Division of Pharmaceutical Chemistry and Technology
Faculty of Pharmacy
University of Helsinki
Finland

Docent Tiia Kuuranne
Swiss Laboratory for Doping Analysis
Lausanne University Hospital
Switzerland

Reviewers: Docent Kati Hanhineva
Institute of Public Health and Clinical Nutrition
Faculty of Health Sciences
University of Eastern Finland
Finland

Professor Janne Jänis
Department of Chemistry
Faculty on Science and Forestry
University of Eastern Finland
Finland

Opponent: Professor Ilkka Ojanperä
Department of Forensic Medicine
Faculty of Medicine
University of Helsinki
Finland

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ABSTRACT

Anabolic-androgenic steroids (AAS) are synthetic compounds that are structurally related to testosterone. AAS are an important class of drugs that are commonly abused in sport and are classified as prohibited substances according to the World Anti-Doping Agency (WADA). AAS are metabolized extensively in the human body and excreted to urine mostly as glucuronides or sulphates. These conjugated compounds are present in human specimens in low concentrations and for a limited period. Sensitive and specific analytical methods are thus highly important to be able to detect these compounds for an extended time period in athletes. These compounds are usually analyzed from biological matrixes after cleavage of the glucuronide or sulfonate moiety. Direct analysis of intact conjugated steroid metabolites is an attractive option that involves more straightforward sample preparation. Nonetheless, the commercial availability of the conjugated reference material is limited.

The first aim of this study was to produce glucuronide-conjugated metabolites of AAS to be used in the development of methods for doping control. Glucuronide-conjugated metabolites of anabolic androgenic steroids were produced by *in vitro* enzyme-assisted synthesis. The products were characterized by nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS). The method offers a simple and stereospecific procedure of producing small amounts of reference material, which are needed in the development and application of analytical methods for AAS metabolites.

Secondly, these enzymatically synthetized AAS glucuronides were utilized in the development of a liquid chromatography – mass spectrometry (LC–MS) method for simultaneous detection of 12 glucuronide-conjugated AAS metabolites in human urine. Solid-phase extraction (SPE) of AAS glucuronides from urine, as well as LC separation and mass spectrometric detection, were optimized. The detection was performed by tandem mass spectrometer (MS/MS) combined via electrospray ionization (ESI). Novel LC–ESI-MS/MS method was validated, and the robustness and transferability of the developed method was studied in an interlaboratory comparison among seven laboratories. The recovery, detection limits, and repeatability of the developed method reached expectations. The interlaboratory comparison also revealed that the developed method is transferable to other laboratories equipped with triple quadrupole mass spectrometers. The developed method offers simpler and a more straightforward as well as sensitive approach to the analysis of exogenous steroids, without a hydrolysis process and time-consuming sample preparation.

The third part of this study involved gas chromatographic – mass spectrometric (GC–MS) methods, which with electron ionization (EI) are robust, sensitive, and well suited for quantitative measurements. High energy incorporated to the analytes, however, results in fragmentation and often to a loss of sensitivity. Another approach for a more sensitive and

specific method for analysis of anabolic steroids from urine, was to combine GC featuring good resolving power to softer ionization in atmospheric pressure (API). A microchip-based miniaturized heated nebulizer provides easy interfacing for GC–API-MS with high ionization efficiency. Two sensitive and selective gas chromatography – microchip atmospheric pressure photoionization - tandem mass spectrometry (GC–μAPPI-MS/MS) methods were developed, validated and successfully applied to the analysis of anabolic steroids in authentic excretion urine samples. The anabolic steroids were analyzed from urine samples without derivatization and as their trimethylsilyl (TMS) derivatives. The feasibility of GC–μAPPI-MS/MS in analysis of AAS in urine was studied and compared with conventional GC–EI-MS. Both GC–μAPPI-MS/MS methods showed good sensitivity and quantitative performance, demonstrating their potential to the analysis of biological samples. The advantage of GC–μAPPI-MS/MS is to provide soft and efficient ionization that produces abundant molecular ion or protonated molecule. The analysis of anabolic steroids without derivatization resulted in protonated molecules with fragmentation, but TMS derivatives produced intensive radical cations with only slight fragmentation, ensuring good selectivity and sensitivity. The TMS derivatives are also more volatile compounds providing better chromatographic performance. In addition, the fragmentation of the molecular ions of the TMS derivatized steroids in μAPPI-MS/MS is similar to EI, which allows for the use EI spectral libraries to support with the identification of TMS derivatized steroids. These methods offer valuable tools for anti-doping laboratories where new analytical strategies are needed to be able to detect an increasing number of prohibited compounds with various physico-chemical properties.

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Most importantly, I want to thank my family. Much has happened during these years, three children, self-build summer cottage and home, and several smaller projects. My mother and dad were always ready to help and support me. This would not have been possible without your assistance. Finally, I extend my warmest thank to my dear husband and our children for your patience and support. You gave me tons of other things to think and enjoy during this long-lasting project. I'm extremely happy to have you all beside me.

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications:

I Hintikka, L., Kuuranne, T., Aitio, O., Thevis, M., Schänzer, W., and Kostiainen, R. Enzyme-assisted synthesis and structure characterization of glucuronide conjugates of eleven anabolic steroid metabolites. Steroids 73 (2008) 257-265.

II Hintikka, L., Kuuranne, T., Leinonen, A., Thevis, M., Schänzer, W., Halket, J., Cowan, D., Grosse, J., Hemmersbach, P., Nielen, M.W.F., and Kostiainen, R. Liquid chromatographic-mass spectrometric analysis of glucuronide-conjugated anabolic steroid metabolites: method validation and interlaboratory comparison. J. Mass Spectrom. 43 (2008) 965-973.

III Hintikka, L., Haapala, M., Franssila, S., Kuuranne, T., Leinonen, A., and Kostiainen, R. Feasibility of gas chromatography - microchip atmospheric pressure photoionization - mass spectrometry in analysis of anabolic steroids. J. Chromatogr. A. 1217 (2010) 8290-8297.

IV Hintikka, L. Haapala, M., Kuuranne, T., Leinonen, A., and Kostiainen, R. Analysis of anabolic steroids in urine by gas chromatography–microchip atmospheric pressure photoionization-mass spectrometry with chlorobenzene as dopant. J. Chromatogr. A. 1312 (2013) 111-117.

The publications are hereafter referred to in the text by their roman numerals.

Author's contribution to the publications included in this thesis:

I	The experimental work was carried by the author. The optimization of enzyme-assisted synthesis was done by Tiia Kuuranne and the nuclear magnetic resonance related work was done by Olli Aitio. The manuscript was written by the author with contributions from the co-authors.
II	The experimental work was carried by the author. The manuscript was written by the author with contributions from the co-authors.
III	The experimental work, excluding the microfabrication, was carried by the author. The manuscript was written by the author with contributions from the co-authors.
IV	The experimental work, excluding the microfabrication, was carried by the author. The manuscript was written by the author with contributions from the co-authors.

ABBREVIATIONS

AAS	anabolic-androgenic steroid
ABP	athlete biological passport
ACN	acetonitrile
APCI	atmospheric pressure chemical ionization
API	atmospheric pressure ionization
APLI	atmospheric pressure laser ionization
APPI	atmospheric pressure photo ionization
AR	androgen receptor
cAPPI	capillary APPI
CI	chemical ionization
DA	dopant-assisted
EAAS	endogenous AAS
EI	electron impact
ELISA	enzyme-linked immunosorbent assay
ESI	electrospray ionization
eV	electron volt
D	dopant
DHT	dihydrotestosterone
GC	gas chromatography
Glu	glucuronic acid or glucuronide
HN	heated nebulizer
HPLC	high-performance liquid chromatography
HRMS	high resolution mass spectrometry
IA	immunoassay
i.d.	internal diameter
IE	ionization energy
ISTD	internal standard
LC	liquid chromatography
LLE	liquid-liquid extraction
LOD	limit of detection
LOQ	limit of quantification
LPME	liquid-phase microextraction
LTP	low temperature plasma
m/z	mass-to-charge ratio
MEPS	microextraction by packed sorbent
MRPL	minimum required performance limit
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MSTFA	*N*-Methyl-*N*-(trimethylsilyl) trifluoroacetamide
MTBE	methyl *tert*-butyl ether
NMR	nuclear magnetic resonance
PA	proton affinity
qTOF	quadrupole time-of-flight
QQQ	triple quadrupole
RIA	radioimmunoassay
RP	reversed phase
RSD	relative standard deviation
S/N	signal-to-noise ratio
S_N2	bimolecular nucleophilic substitution
SPE	solid phase extraction
SPME	solid phase micro extraction
SRM	selected reaction monitoring
SULT	sulfotransferase
TFC	turbulent flow chromatography
TMS	trimethylsilyl
TMSI	trimethylsilyl iodide
TOF	time-of-flight
UDPGA	uridine-5'-diphosphoglucuronic acid
UGT	uridine diphospho-glucuronosyltransferase
UHPLC	ultra-high performance liquid chromatography
UV	ultraviolet
VOC	volatile organic compound
WADA	World Anti-Doping Agency

STEROID GLUCURONIDES

5α-NG	5α-estran-17-one-3α-O-glucuronide
5β-NG	5β-estran-17-one-3α-O-glucuronide
d_4-5α-NG	[2,2,34,4-$^2H_4$]5α-estran-17-one-3α-O-glucuronide
5β-EPIMG	17β-methyl-5β-androst-1-ene-17-one-3α-O-glucuronide
5α-MTG	17α-methyl-5α-androstane-17β-ol-3α-O-glucuronide
5β-MTEG	17α-methyl-5β-androstane-17β-ol-3α-O-glucuronide
5α-MEG	1-methylen-5α-androstan-17-one-3α-O-glucuronide
AG	5α-androstane-3α-ol-17β-O-glucuronide; androsterone glucuronide
d_3-TG	[16,16,17-$^2H_3$]4-androsten-3-one-17β-O-glucuronide; testosterone glucuronide
NG	estr-4-en-3-one-17β-O-glucuronide; nandrolone glucuronide
d_3-NG	[16,16,17-$^2H_3$]estr-4-en-3-one-17β-O-glucuronide
5α-1-MEG	1-methyl-5α-androst-1-en-3-one-17β-O-glucuronide
MTG	17α-methyl-5β-androstane-3α-ol-17β-O-glucuronide; methyltestosterone glucuronide

STEROIDS

TES	testosterone
NAN	nandrolone
NANm	5α-estran-3α-ol-17-one
17MDN	17-methandienone
MDNm	17β-methyl-1-ene-5β-androstane-3α,17α-diol
MTm	17α-methyl-5β-androstane-3α,17β-diol
DNZm	ethisterone
MTS	methyltestosterone

1 REVIEW OF THE LITERATURE

1.1 Anabolic-androgenic steroids (AAS)

1.1.1 Origin and effects

Cholesterol is the most prevalent steroid in all animals and is essential for life. It is the precursor to all steroid hormones (Figure 1). In mammals, steroids are divided into six groups based on their individual structure and biology. Androgens, the male hormones, are one group of steroid hormones, of which testosterone is predominant in humans. Testosterone (Figure 2) is excreted mainly by testis in males, and by ovaries and placenta in females [1]. Average plasma levels of testosterone are 0.6 ng/ml in post pubertal males and 0.03 ng/ml in females. Testosterone and its active metabolites, e.g. dihydrotestosterone (DHT), are responsible for many changes that occur in puberty (growth, changes in skin, lowered voice) and they play an important role in stimulating and maintaining sexual function in man, increase lean body mass, stimulate body hair growth, and sebum secretion. The metabolic effects of androgens include reduction of hormone binding and other carrier proteins. Renal erythropoietin secretion is stimulated, and high-density lipoprotein levels are decreased [2]. Actions of testosterone are divided into two types: anabolic (promotion of muscular growth) or androgenic (development and maintenance of secondary male sexual characteristics). Anabolic-androgenic steroids are synthetic derivatives of testosterone. More than 600 testosterone derivatives have been synthesized in order to enhance the anabolic effect relative to the androgenic effect [3].

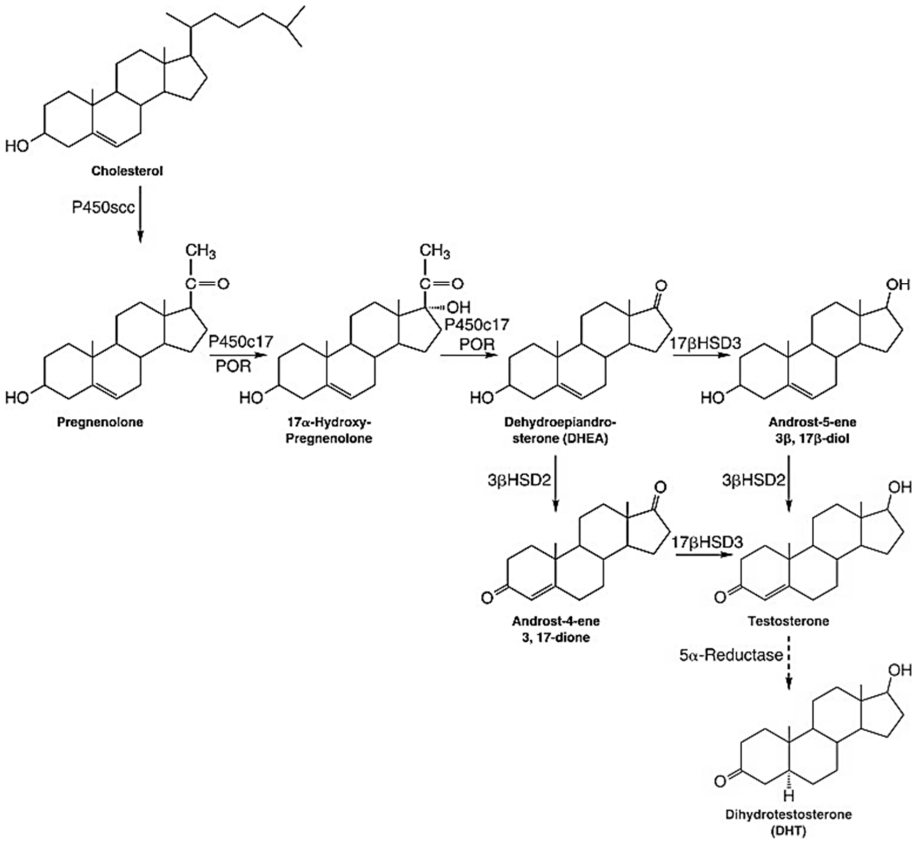


Figure 1. *Biosynthesis of testosterone [1]. Reprinted with the permission of Elsevier.*

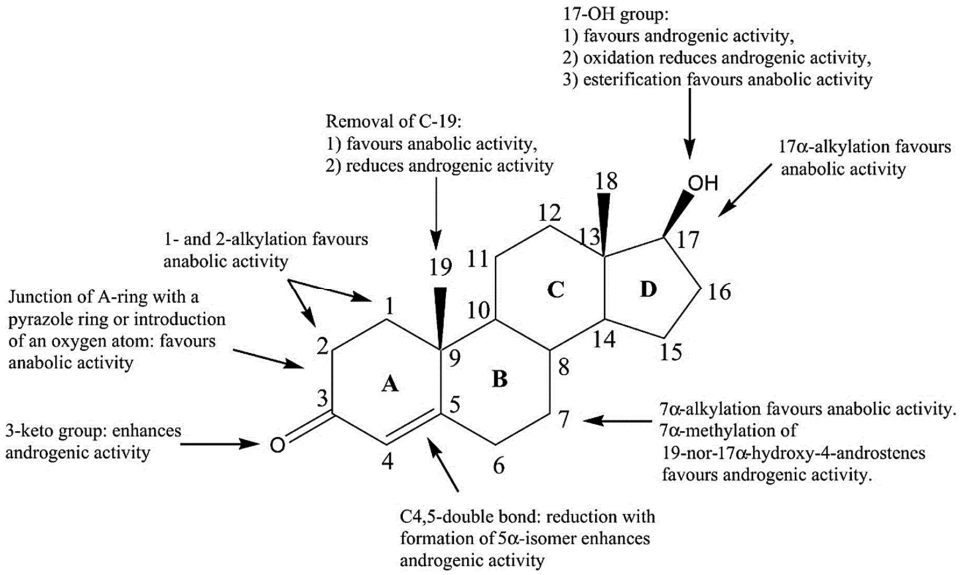


Figure 2. *Significant sites of testosterone for the expression of androgenic and anabolic activities [4]. Reprinted with the permission of Elsevier.*

1.1.2 Clinical use and abuse in sports

AAS are beneficial in therapeutic doses in treatments of several diseases and disorders. Hypogonadal or ageing men can be treated with androgen replacement therapy [5,6]. Androgen therapy offers support in the treatment of patients experiencing decreased testosterone plasma levels in HIV [7,8], chronic obstructive pulmonary disease [9], burns [10,11], renal failure, cancer, liver failure, and postoperative recovery. Anabolic steroids have the ability to stimulate erythropoiesis and can thus be used for treatment of anemic patients [12]. Androgens alone or together with estrogens have been used in the treatment of osteoporosis [13]. According to a report by Tomoda et al. [14], short-term administration of oxymetholone may have a beneficial effect on damaged myocardium in heart failure.

AAS are also misused in supratherapeutic doses to improve performance in sports and are classified as prohibited substances according to WADA [15]. The main desired effects of these testosterone-derived compounds are their potential ability to improve the physical performance of skeletal muscle and to balance catabolic conditions in the body after stress [3,16]. In addition, supratherapeutic doses of AAS increase muscle size [17-20]. These are desired effects also for non-athletes; recreational AAS abuse among adolescents has been increasing rapidly over the past years [21,22]. In addition to the desired effects on performance, androgens can also increase aggression and motivation for competition [23,24]. However, mediated mainly by their androgenic activity, the AAS have the potency also to cause serious health problems as side effects e.g. cardiovascular or liver diseases [16].

1.1.3 Structure and nomenclature

The molecular skeleton of androgens is formed by three six-member and one five-member carbon rings, which are labelled A-D (Figure 2). Usually there is at least one methyl group ($-CH_3$) at angular positions and many steroids also possess one or more double bonds. Most steroids have an oxygen atom at C-3. The tetracyclic structure of androgens has up to six chiral centers (C-5, -8, -9, -10, -13, and -14) allowing 2^6 isomers. Additional chiral centers occur if substituents are present (usually in C-17), but possible double bonds reduce the number of stereoisomers. The configuration in the chiral centers has to be stated in order to distinguish the three-dimensional shape of the molecule. The A/B ring junction can have a cis or trans configuration and it has to be specified if the C-5 is saturated. The 5α-orientation gives the molecule a planar shape, whereas the 5β-configuration causes a sharp bend in the structure resulting in different chromatographic, spectroscopic and chemical properties. The B/C ring junction has the 8β/9α-configuration (trans) in all common steroids and requires no illustration. Similarly, the C/D ring junction has trans configuration (13β/14α) in most of the steroids. Angular methyl groups in C-18 and C-19 have β-configuration [25].

Functional groups or conjugation in steroid structure, together with the shape of the molecule, determine the chemical and physical properties as well as interactions with hormone receptors (androgen receptor) or metabolizing enzymes. Modifications to structure intend to increase activity, sustain release into the circulation, or to permit oral delivery by reducing first-pass metabolism in the liver. An example of the latter is alkylation at the C17 position (Figure 2). On the other hand, modifications can increase the toxicity, for example 17α-alkylated orally active steroids are hepatotoxic [26]. Structure-activity relationship is determined by the binding affinity of steroid to the androgen receptor (AR). The 5α-configuration, for example, favors binding to AR making it more active compared to 5β structure [27]. The oxygen atom at C-3 can be in form of 3α- or 3β-hydroxyl group or as carbonyl (3-keto-group), which binds more strongly to AR than 3-hydroxy and makes 3-keto structures generally more active than compounds with the 3-hydroxy moiety. C-17 is also a common site for substituents. Estrogens and various androgens have a 17-oxo structure. Estradiol and testosterone possess 17β-hydroxy and some pregnanes have 17α-hydroxy groups: 17β-hydroxy favors binding to AR and makes compounds with such a structure highly more active. Any modification or elimination to 17β-hydroxy reduces the activity [25], whereas the presence of a 4-ene-one structure makes the molecule planar and highly active.

1.1.4 Human metabolism

Within the human body, the nonpolar AAS are transformed extensively by phase-I and phase-II metabolic reactions. Enzymatically catalyzed phase-I reactions (oxidation, reduction, hydroxylation, or epimerization) introduce more functionalities into steroid structure, which increase the polarity and serve as sites for phase-II reactions. Reduction of C4-C5 double-bonds to yield 5α- and 5β-saturated structures, 3-keto or 17-keto reduction, 1,2-hydrogenation, 6-, 12-, or 16-hydroxylations, 6,7-dehydrogenation, and 17-hydroxy oxidation are the main phase-I metabolic reactions for AAS [28]. Phase-I metabolism of nandrolone is presented as an example in Figure 3.

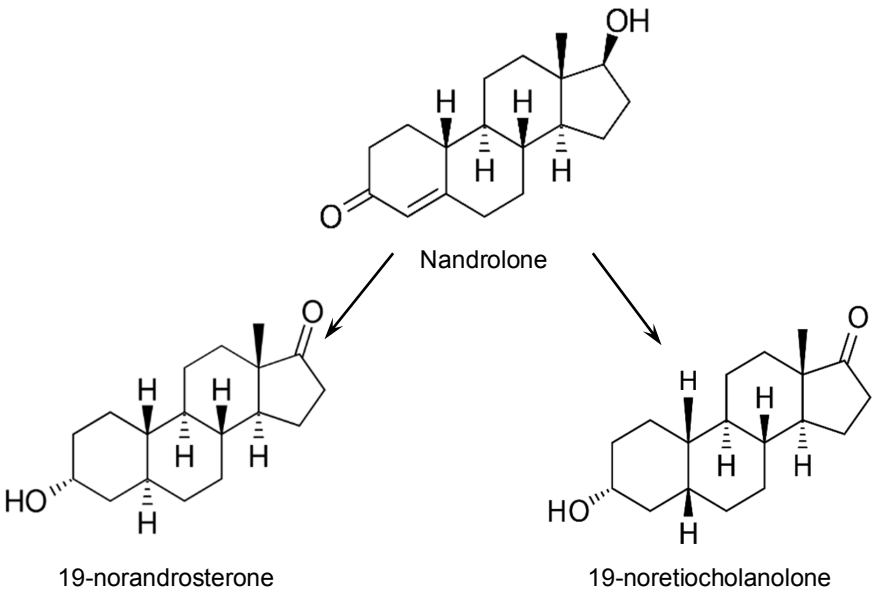


Figure 3. *Phase-I metabolism of nandrolone. Reduction in A-ring and 17-keto metabolism in D-ring yielding 5α/β-isomers, 19-norandrosterone (5α-estran-3α-ol-17-one), and 19-noretiocholanolone (5β-estran-3α-ol-17-one).*

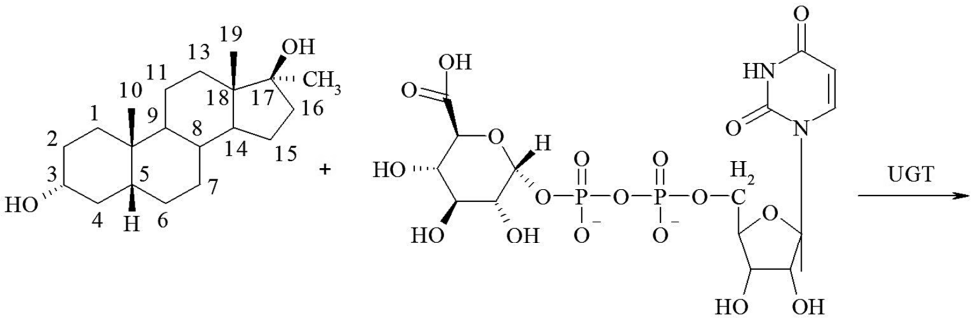
Phase-II reactions (glucuronidation, sulfation, methylation, acetylation, and conjugation with amino acids or glutathione) are conjugation reactions, which typically terminate the activity of the compound. In general, the conjugates are less toxic, less active, and more hydrophilic than parent compounds enhancing the excretion to urine. Less than 3% of androgens are excreted in urine in free form [29]. The most common pathways of AAS conjugation in human body are glucuronidation and sulfation. Both of these conjugation reactions are enzymatically controlled. Glucuronidation is catalyzed by uridine diphosphoglucuronosyltransferases (UGTs) and sulfation by sulfotransferase (SULT) enzymes. In AAS, the most common cites for conjugation reactions are 3- or 17-hydroxyl groups. Steroids possessing 3α- or 17β-hydroxyl groups are mostly excreted as glucuronides, whereas steroids with 3β-hydroxyl structure favor sulfation [30]. The spectrum and activity of phase-I and phase-II enzymes can differ dramatically both between individuals as well as within an individual over time. Both external (smoking, medication or nutrition) [31-34] and internal (age, gender, diseases and genetics) [31,35,36] factors may have an influence on phase-II enzyme activity. These differences in steroid metabolism should be taken into account when developing targeted screening methods for doping control.

1.1.5 Anabolic steroid glucuronides

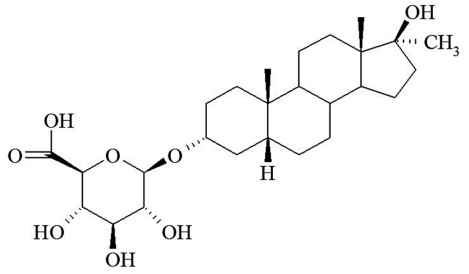
Glucuronidation, a major conjugation reaction for steroids, is a bimolecular nucleophilic substitution (S_N2) reaction. Several different functional groups can react with glucuronic acid to form O-, S-, N-, and C-glucuronides, which means that a wide variety of compounds are metabolized by glucuronidation [37]. UGTs, membrane-bound conjugating enzymes, catalyze the reaction where uridine-5'-diphosphoglucuronic acid (UDPGA) acts as a co-substrate. The polar glucuronide moiety is attached to the steroid structure with reversion of the configuration yielding a β-glycosidic bond. UGTs can be found in several tissues (e.g. liver, kidney, intestine, lung, and prostate), but the liver is considered to be the major site of glucuronidation [38-40]. According to recent knowledge, the UGT family contains 117 individual UGTs [41] and at least 25 are found in human [42]. UGT isoenzymes are categorized into four families—UGT1, UGT2, UGT3, and UGT8—on the basis of protein sequence similarity [40,42]. The enzyme families are further divided into subfamilies according to the sequence homology [43,44]. The most important enzymes involved in human steroid glucuronidation belong to the subfamilies UGT1A and UGT2B, but several isoenzymes belonging to UGT1 and UGT2 families are reported to catalyze steroid glucuronidation [39]. UGT2B family members 2B4, 2B7, 2B15, and 2B17 play an important role in androgen metabolism [45,46]. Glucuronide conjugation of a single steroid is often catalyzed by multiple UGT isoenzymes, due to their broad substrate specificity [47].

To overcome the risks and limitations of sample preparation, analysis methods targeting intact glucuronide-conjugated steroids are valuable for the pharmaceutical industry, as well as doping-control, clinical, and forensic laboratories. In such a case, steroid glucuronides are needed as reference material for method development and identification purposes. In vivo production of human AAS glucuronides often meets with both ethical and practical problems, due to the required isolation of pure metabolites from urine and inter-individual variation in metabolism. Chemical in vitro synthesis methods are most commonly applied for production of phase-I [48-50] and phase-II [50-55] metabolites of AAS. Some issues related to chemical synthesis, e.g. formation of racemic reaction mixtures and production of ortho ester or acyl transfer side-products [56,57], have been reported earlier. An alternative approach to chemical in vitro glucuronide synthesis is enzyme-assisted synthesis catalyzed with microsomal UGT enzymes (Figure 4). Enzyme-assisted synthesis allows production of stereo-specifically pure conjugates which can be considered as the main advantage over the chemical synthesis. Another advantage compared to chemical synthesis is the simplicity of the enzyme-assisted synthesis. Chemical synthesis is relatively laborious with several steps, but enzyme-assisted synthesis can be performed in one volume without protecting or deprotecting steps. Owing to the stereospecificity and simplicity, enzyme assisted synthesis offers a practical way to produce small amounts (milligrams) of glucuronides to be used, for example, in the development of an analytical method. Rat liver preparations as a source of conjugating enzymes have been used for glucuronidation of e.g., p-nitrophenol [58], nitrogen containing antidepressants [59], catechols [60,61] nitrocatechols [62,63],

androsterone, androstanediol, dihydrotestosterone [64], epitestosterone [65], testosterone [66,67], methyltestosterone [67], nandrolone [67], and neurosteroids [68]. Porcine and bovine microsomes have also been used as a source of conjugating enzymes [68]. Subcellular fractions, such as pooled human liver microsomes [69] or whole cell systems, i.e. human hepatocytes [70] and liver slices [71], have also been used as enzyme sources. Another enzymatic method, where E. coli β-glucuronidase is used as a source of glucuronylsynthase, has been reported [72,73] and utilized to produce a library of steroid glucuronides [74]. E. coli β-glucuronidase is usually applied to catalyze the deconjugation of glucuronide metabolites [75], where, conversely, the hydrolytic pathway is disabled by mutation and the resulting glucuronylsynthase enzyme can catalyze the formation of glucuronide product. Alternative assays have been used for glucuronide syntheses as well, e.g. fungus Cunninghamella elegans [76], recombinant human enzymes [47,69,77,78], and in vivo chimeric mouse model, a hybrid capable of producing human metabolites [79].



17α-Methyl-5β-androstane-3α,17β-diol Uridine-5'-diphosphoglucuronic acid (UDPGA)



17α-Methyl-5β-androstane-17β-ol-3α-*O*-glucuronide

+ UDP + H_2O

Figure 4. *Formation of 5β-MTG in glucuronidation reaction.*

1.2 Analysis of AAS

The World Anti-Doping Code specifies anabolic androgenic steroids as substances prohibited at all times, i.e. in and out of competition [15]. The testing methods for AAS in doping contexts begin with initial testing methods (screening analysis) to reveal suspicious samples, which are forwarded to confirmation procedures. Fast and robust methods are mandatory in screening assays while specificity is prioritized in confirmation analysis to achieve indisputable identification of the target compounds. One crucial parameter to consider is the time period (window of opportunity) in which these compounds can be detected in the athletes' samples. Exogenously administered AAS are present in human specimens, often in low concentration and within a limited time period. Sensitive detection and selection of a representative sample matrix are thus highly important in all anti-doping analyses, as well as in the experiments studying the new long-term metabolites for the extension of detection time.

1.2.1 Requirements

The majority of the anabolic steroids in the WADA prohibited list are of exogenous origin and, as such, analyzed only qualitatively. WADA has established sensitivity requirements for these qualitative methods. The minimum required performance levels (MRPL) for the exogenous non-threshold AAS are 2 ng/ml for dehydrochlormethyltestosterone, methandienone, methyltestosterone, and stanozolol, and 5 ng/ml for other exogenous non-threshold AAS in urine samples [80]. The laboratories have the flexibility to select the method for non-threshold compounds as long as it meets sensitivity requirements (limit of detection (LOD) at least 50% from the MRPL). Commonly used analytical methods are based on the detection of hydrolyzed phase-II metabolites with LC–MS or GC–MS after derivatization [81].

Doping analysis of anabolic steroids that are naturally present in the human body (endogenous anabolic steroids) requires diverse methods that are based on individual steroid profiling. The misuse of endogenous AAS (EAAS) is tracked with the steroidal module of the athlete biological passport (ABP) and "steroid profile" [82]. The ABP is a valuable tool that was recently implemented in anti-doping work [83,84]. It is based on the individual and longitudinal monitoring of hematological markers and was recently completed with the steroid profile module. Steroid profiles include concentration levels of endogenous steroids in urine and their respective ratios measured from urine samples [85]. Administration of anabolic steroids may significantly alter these normally robust ratios. The "steroid profile" is composed of markers (androsterone, etiocholanolone, 5α-androstane-3α,17β-diol, 5β-androstane-3α,17β-diol, testosterone, and epitestosterone) and their selected ratios. EAAS concentrations and their ratios may be altered following the administration of synthetic forms of endogenous AAS, such as testosterone precursors. The two-step procedure of EAAS testing begins with a screening procedure, the measurement of a "steroid profile" in

GC–MS or GC–MS/MS. In case of atypical findings, quantitative analysis is repeated for confirmation, together with an analysis based on gas chromatography–combustion-isotope ratio mass spectrometry (GC–C-IRMS) of the relevant markers. The latter is needed for the assessment of endogenous/exogenous origins of testosterone metabolites.

1.2.2 Sample matrixes

The sample matrixes currently used in routine doping control analysis are urine, serum, and whole blood; however, plasma [86], oral fluid [87], sweat [88] and dried blood spots [89] are proposed to offer additional information [90]. Urine is currently the most commonly used matrix in AAS analysis, as sufficient sample volume is easily obtained. In addition, concentrations of AAS metabolites in urine are relatively high for an extended period compared to blood [91,92]. Urine is a complex matrix containing thousands of different compounds and the steroids of interest are usually present at fairly low concentrations. Sample pre-treatment is needed in order to reduce matrix effects and to concentrate the sample, i.e. to improve the selectivity and sensitivity of the analysis.

1.2.3 Sample preparation

Sample preparation is necessary when analyzing anabolic steroids from urine samples due to the complexity of the matrix. The main goals of sample preparation are to remove interfering matrix elements and pre-concentrate the sample, in order to increase selectivity and sensitivity of the analysis. The commonly used sample preparation methods in doping analysis include liquid–liquid extraction (LLE), solid-phase extraction (SPE), and "dilute-and-shoot" methods. The applicability of different techniques for extraction of AAS from urine has been compared [93-95] in a doping analytical context.

Even though the majority of the AAS are excreted to urine as conjugates, they are frequently analyzed as their hydrolyzed counterparts rather than as intact conjugates. The direct analysis of AAS conjugates is possible by LC–MS [55,96,97], but these non-volatile and thermally unstable conjugates are not typically amenable to GC–MS. Therefore, the glucuronide-conjugated AAS are hydrolyzed enzymatically with β-glucuronidase from digestive juice of *Helix pomatia* (*H. pomatia*) or from *Escherichia coli* (*E. coli*) prior to GC–MS analysis [98]. Sulphate-conjugated AAS are not routinely included in human anti-doping screens, but they can be hydrolyzed with arylsulfatase [99] or via solvolysis [100,101]. The cleavage of both glucuronide and sulphate conjugates can be performed using *H. pomatia* which contains both β-glucuronidase and arylsulfatase activity [102]. However, problems associated with the production of artefacts [103], conversion between steroids [104] and incomplete hydrolysis [105,106] have been described when using *H. pomatia* in hydrolysis of AAS. Additionally, detection can be hindered by interfering peaks or elevated background noise [107]. The WADA technical document states that *E. coli* should be used as a source of β-glucuronidase in order to prevent these problems associated

with *H. Pomatia*, and to guarantee a harmonized approach within the anti-doping laboratories to ABP steroid profiling [82].

For LC–MS-screening, sample preparation can be as simple as a dilution with an appropriate solvent in order to reduce the matrix effect [108]. The dilution can be as minimal as a 10% addition of acetonitrile (ACN) [109], but the factor is usually higher being 1:1 [110-112] or 1:10 [113,114], or even 1:25 [108,115]. Dilute-and-shoot sample preparation methods are fast and cheap, but they have some drawbacks, such as increased limits of detection [97], higher matrix effects [97], and variation in retention times [108]. Dilute-and-shoot methods are suited to easily ionized compounds with higher detection limit requirements [108]. Usually more efficient and selective cleaning procedures are necessary to achieve lower detection limits.

Traditionally, LLE has been widely used for the analysis of AAS in urine, with different extraction solvents for example *tert*-butyl methyl ether [116-119], diethyl ether [81,120], ethyl acetate [121], or *n*-pentane [116]. Shortly after the introduction of the first prepacked and disposable SPE cartridge in the late 1970s, it was used for urinary steroid extraction [122] and nowadays it has become the method of choice for AAS extraction. A wide variety of different types of SPE cartridges have been used, for example XAD-2 [49] or C18 [119,123,124], alone or in combination with two different cartridges [125,126]. The performance of different SPE cartridges has been compared in the analysis of non-conjugated AAS [93,127-129]. Oasis HLB seems to be an efficient sorbent and in combination with LLE with methyl *tert*-butyl ether (MTBE) it has provided high recoveries and selectivity especially towards more polar steroids [93]. The Oasis HLB cartridge contains both hydrophilic and lipophilic groups and thus allows good recoveries for many anabolic steroids [130]. In an animal-sample context, five SPE cartridges were compared for extraction of steroid glucuronides and sulphates from urine samples [128]. A strong anion exchanger on a functionalized silica (SAX) cartridge gave the best purification for a large number of glucuronides and sulphates. In a study by Pu et al. [129], several different SPE cartridges were compared for AAS glucuronides in a complex matrix of equine urine. According to their results, Certify II with mixed mode sorbent combining non-polar C8 and strong anion exchange (SAX) functionalities resulted in the best recoveries for AAS glucuronides.

Alternatively, microtechniques such as solid phase microextraction (SPME) [131-133], liquid-phase microextraction (LPME) [94,134] or microextraction by packed sorbent (MEPS) [133] might be applied to minimize the sample throughput time or solvent amounts used. Other approaches to clean-up the urine samples have also been reported, such as highly selective immunoaffinity chromatography [135] and lately online turbulent flow chromatography (TFC) [136] and two-dimensional (2D) online trapping [137]. The TFC

and 2D online trapping enables direct injection of native urine samples providing clean-up and pre-concentration.

1.2.4 Analytical methods

Radioimmunoassays (RIA) were among the early methods used to analyze AAS and are still widely used in clinical laboratories owing to their ease of use, rapid throughput, and sensitivity [138,139]. Other detection methods such as direct immunoassay (IA) [140] or enzyme-linked immunosorbent assay (ELISA) [141] have also been used, but they have limitations such as non-specificity [142,143]. The mass spectrometric detection of AAS metabolites has replaced almost all other methods in anti-doping laboratories due to its selectivity and sensitivity [144]. It can be used in combination with gas or liquid chromatography to simultaneously separate and determinate multi component steroid mixtures. Owing to the simplicity and sensitivity accomplished by LC–MS methods, they are increasingly used in AAS analysis. Despite the continuing advance of LC–MS in AAS analysis, the necessity for GC–MS remains undeniable.

1.2.4.1 GC–MS

GC–MS with electron ionization has traditionally been the method of choice for AAS analysis in anti-doping routine. The method provides excellent chromatographic resolution, high sensitivity, and good quantitative performance. However, AAS are most often derivatized for GC–MS analysis in order to improve their volatility and thus the chromatographic performance. Trimethylsilylation using *N*-methyl-*N*(trimethylsilyl) trifluoroacetamide (MSTFA) [49,98,145,146] as a reagent is the most commonly used derivatization method with AAS. *Tert*-butylsilyl derivatives could be used in order to achieve higher mass increase, more intensive molecular ion and diagnostic ions above *m/z* 300 with EI [116,147]. Other derivatives used are, for example, acyl derivatives with steroids containing nitrogen, such as stanozolol or stable tri-, penta- or heptafluoro amines produced in conjunction with silylation of hydroxyl groups [148]. One disadvantage of the GC–EI-MS is that the derivatized AAS are strongly fragmented in EI process, which results in decreased sensitivity and/or specificity in selected ion monitoring or in selected reaction monitoring, since ion current is divided over several fragment ions and the structure-specificity of the fragments may be compromised as well. Chemical ionization (CI) provides an ionization method softer than EI. An intense protonated molecule with less fragmentation is typically observed in the analysis of AAS with CI [149].

Classical CI by using methane, isobutene, or ammonia as reagent gases has been commonly applied to the analysis of labile compounds in GC–MS. In this method, the ionization occurs in a vacuum. Atmospheric pressure chemical ionization (APCI) methods have been increasingly used in GC–MS (Table 1). Dzinic et al. [150] presented the interfacing of GC to MS by using APCI for the first time about four decades ago. Ten years later Revelsky et

al. [151,152] presented APPI interface for GC–MS. Although the potential of GC coupled with atmospheric pressure ionization to MS (GC–API-MS) has been demonstrated during the last decades, the technique has not made a breakthrough to routine analyses. There are now commercial interfaces for GC–API-MS with APCI and APPI, although several other techniques such as ESI [153], low temperature plasma (LTP) [154] and atmospheric pressure laser ionization (APLI) [155,156] have also been presented during the last years [157]. Recently, novel high sensitivity capillary APPI (cAPPI) interfaces have been introduced for GC–MS [158,159]. The cAPPI utilizes an extended heated transfer capillary (between the atmosphere and the vacuum of the MS) with an MgF_2 window, through which vacuum UV light (10 eV) from an external source enters the capillary. Since the sample is introduced, vaporized, and photoionized inside the extended capillary, ion transmission into MS is maximized resulting in excellent overall sensitivity. In the recent study by Haapala et al. [158], the feasibility of the GC–cAPPI-MS method was tested in the analysis of steroids in artificial urine. The method showed good quantitative performance and limits of detection down to pg/ml range.

Moreover, there is growing interest in the miniaturization of analytical devices by using microchip technology. The advantages of miniaturization include higher sensitivity, faster analysis, reduced sample and solvent consumption, and lower manufacturing cost. For these reasons, a miniaturized heated nebulizer [160] was developed for microchip-based APPI [161] and APCI [162] for LC–MS and GC–MS analysis. The performance of the heated nebulizer microchip as a GC–MS interface has been demonstrated in APPI and APCI modes, for example in the analysis of volatile organic compounds [162], drugs [163,164] polyaromatic hydrocarbons [163], and polychlorinated biphenyls [165].

Table 1. *GC–API-MS*

Analytes	Matrix	Ionization	Analyzer	Type	Pub. year	Ref.
VOCs and testosterone	-	µAPCI	QQQ	S	2006	162
		APPI	qTOF	S	2007	166
PAHs	-	µAPPI	QQQ	S	2007	163
PAHs	-	APCI APLI	TOF	S	2008	167
PCBs	soil	µAPPI µAPCI	ion trap	S	2008	165
VOCs	-	ESI APCI	QQQ	S	2008	153
SARMs	urine	µAPCI	Orbitrap	S	2010	164
impurities	pharmaceuticals	APCI	TOF	C	2010	168
metabolic fingerprinting	bacterial culture	APCI-I	TOF	C	2011	169
phenolic compounds	olive oil	APCI	TOF	C	2011	170
acrylic adhesives	food packing	APCI	QTOF	C	2012	171
PAHs and phthalates	-	APPI	QQQ	S	2012	172
volatile compounds	perfume	APPI (toluene)	LIT-orbitrap	S	2012	173
VOCs	-	LTP	qTOF	S	2013	154
metabolic profile (e.g. amino acids)	Human CSF	APCI	TOF	C	2013	174
flame retardants and plasticizers	electronic waste and car interiors	APCI	TOF	C	2013	175
neurosteroids	urine	APPI		S	2013	176
steroids	urine	cAPPI	ion trap	S	2013	158
methyl phosphonic acid esters	-	APPI	QQQ		2014	177
fatty acids	bacterial culture	APCI-II	TOF	C	2015	178
food contaminants	food packing	APCI	QTOF	C	2015	179
environmental pollutants	-	DA-APPI (toluene) and APLI	Orbitrap	S	2015	156
anabolic steroids	urine	APCI	QQQ	C	2017	180
anabolic steroids	urine	ESI	LTG orbitrap and QQQ	S	2017	181

LTP -low temperature plasma; VOC-volatile organic compound; S-self-made C-commercial; DA-dopant-assisted

1.2.4.2 LC–MS

Liquid chromatography connected to mass spectrometry (LC–MS) has become a more important technique over the last decade and is nowadays widely used with GC–MS to cover the wide range of target compounds in anti-doping analyses [90]. The advantage of LC–MS is that AAS can be analyzed without the time-consuming derivatization step that is necessary in GC–MS. The majority of the published LC–MS methods for AAS are focused on hydrolyzed steroids from glucuronide-conjugated fractions [107,120,182,183], although LC–MS could allow for direct analysis of intact conjugated steroid metabolites without time-consuming hydrolysis (Table 2).

The chromatographic separation of AAS is of great importance as many isobaric AAS cannot be separated by MS alone. A wide variety of different reversed phase columns have been used in AAS analysis (e.g. C18, C8, phenyl, and cyano) and their applicability has been compared [130]. Nowadays, ultra-high performance liquid chromatography (UHPLC) has become the technique of choice for steroid analysis as it provides improved resolution resulting in faster analysis, as well as improved specificity and sensitivity compared to high performance LC.

Electrospray ionization (ESI) [55,107,184], atmospheric pressure chemical ionization (APCI) [185-188], and atmospheric pressure photoionization (APPI) [189-193] have all been used in LC–MS analysis of AAS in biological samples in a human and animal testing context. LC–ESI-MS was applied for the first time in the analysis of steroids and their conjugates in 1992 [194] and after that ESI has been the most widely used ionization technique in the analysis of AAS by LC–MS (Table 2). ESI provides very soft and efficient ionization method for medium polar, polar and ionic compounds producing typically abundant protonated or deprotonated molecules or adduct ions (e.g. $[M+NH_4]^+$ or $[M+Na]^+$) with minimal fragmentation. ESI, as a soft ionization method, is especially suitable for labile compounds such as AAS conjugates, which are dissociated when more energetic ionization methods such as APCI and APPI are used. However, the ionization efficiency for neutral or non-polar compounds, such as AAS without a keto function at position 3 or conjugated double bond (4-ene-3-one), is limited with ESI [107]. Moreover, ESI is prone to matrix effects, which may have a significant contribution to the quality of the analysis [195]. For these reasons APCI and APPI have been used as alternative ionization methods in the analysis of AAS by LC–MS.

APCI and APPI provide high ionization efficiency for both non-polar and polar compounds and are thus well applicable to the analysis of non-conjugated AAS [185,186,188,196]. In APCI and APPI, the sample solution is vaporized by a heated nebulizer, forming gas-phase molecules of the analyte and solvent. Ionization, in the gas phase via proton transfer or

charge exchange reaction, is initiated by a corona discharge needle (APCI) [150] or by vacuum ultraviolet lamp emitting 10 eV photons (APPI) [197,198]. APCI and APPI provide improved ionization efficiency for less polar AAS, they are less susceptible to ion suppression and tolerate higher salt buffer concentrations than ESI. As a disadvantage in steroid analytics, the higher energy associated with APCI and APPI usually results in fragmentation of conjugated AAS hindering their direct analysis, especially regarding the information on intact structure and molecular weight. Methods have been published for steroid analysis by APPI with different dopants for example with toluene [189,191-193], acetone [189,191], chlorobenzene [176], anisole [191], THF [191], and also without dopant [199]. A profound review of APPI mass spectrometry and combinations with different separation techniques have been published recently [200].

For mass spectrometric measurements, the preferred analyzer type depends strongly on the purpose of the application and can be performed with several different types of analyzers. Generally, low-resolution instruments (e.g. triple quadrupole) are preferred for quantitative analysis, while high-resolution instruments are selected for untargeted approaches, identification, or for the analysis of large molecules. In AAS analyses, triple quadrupoles are versatile and commonly used as they offer good selectivity, sensitivity, quantitative performance, and several scan modes (SRM, full scan, product or precursor ion scan, and neutral loss scan). Selected-reaction monitoring (SRM) mode with triple quadrupole is the method-of-choice in routine and high-throughput AAS analysis owing to the high sensitivity and robust quantitative performance (Table 2). Despite structural modifications, the core structure of AAS often remains the same and produces general fragments which could be targeted as group-specific ions.

Similar to the drugs-of-abuse domain, the recent years have demonstrated so called “designer steroids” as a threat to anti-doping analyses [48,201]. Untargeted profiling has been found to be an efficient screening tool, offering complementary information to common testing methods. Precursor ion scanning offers a general screening method for anabolic steroids with common fragments [107,183,202]. With untargeted methods, and efficient data processing systems it is possible to monitor a large number of analytes in a single experiment with high sensitivity and specificity [203]. In addition to modern analytical equipment, comprehensive knowledge of the metabolism as well as mass spectrometric behavior of AAS are also key elements when establishing new methods.

Table 2. *Examples of published LC–MS/MS methods for the analysis of intact AAS conjugates. S=sulphates, G=glucuronides, ref.=reference*

Analytes	Matrix	Preparation	Separation	Ionization	Detection	LOD ng/ml	Ref.
S	-	-	HPLC (C18)	ESI+	QQQ		55
G, S	urine	SPE	HPLC (C18)	ESI+	QQQ	-	124
G, S	urine	SPE (C18 bond elute LRC)	HPLC (C18)	ESI+	QQQ	~3	195
							204
G, S	urine	SPE (Strata X)	HPLC (C18)	ESI-	QQQ	0.8-1	205
G	-	SPE (C18)	HPLC (C18)	ESI+	QQQ	-	47
G	urine	LPME SPE (C18) LLE (DEE, ethyl acetate)	HPLC (C18)	ESI+	QQQ	2-15	94
G, S	urine	SPE (C18 and NH_4^+)	HPLC (octadecyl)	ESI-	QQQ	-	206
mesterolone S, G	urine	LLE (MTBE and ethyl acetate) and direct inj.	HPLC (C18)	ESI+/-	qTOF	-	207
	urine	SPE (Oasis HLB)	UHPLC (C18)		qTOF		130
G, S	urine	SPE (C18+DEA) (S) SPE (Certify II) (G)	HPLC (C18)	ESI – (S) ESI+ (G)	Ion trap	-	129
S	urine	SPE (OasisWAX)	HPLC (C18)	ESI-	Ion trap	1 (LOQ)	208
G	urine	SPE C18	UHPLC	ESI-	QQQ		54
G, S	urine	LLE (ethyl acetate) SPE (SAX)	UHPSFC BEH, BEH 2-EP	ESI-	QQQ	0.1-0.5	209
G, S	urine	LLE (ethyl acetate) SPE (SAX)	UHPLC (C18)	ESI-	QQQ	-	128
G	urine	SPE (Oasis MCX)	HPLC (C18)	ESI+/-	QQQ	0.1	210
methenolone S	urine	LLE (ethyl acetate)	HPLC (C18)	ESI+/-	qTOF	-	211
androsterone G	urine	No sample preparation	No chromatogr.	DESI	QQQ	10	212
methandienone G	urine	LLE (MTBE) and SPE (C18)	UHPLC (C18)	ESI+/-	QQQ	-	213
stanozolol S	urine	SPE (C18)	UHPLC (C18)	ESI+/-	QQQ	-	214
4-chloro-metandienone S	urine	LLE (ethyl acetate)	UHPLC (C18)	ESI-	QQQ	-	215

2 AIMS OF THE STUDY

The overall aim of this research was to develop sensitive and specific mass spectrometric methods for the analysis of anabolic steroids in order to extend the detection time of target compounds in human urine samples collected for anti-doping purposes.

The more detailed aims of the studies were:

- Optimization of a rapid production pathway of stereochemically pure AAS glucuronides which are needed in the development of analytical methods in forensic or pharmaceutical sciences, as well as in doping control. (I)

- Production of steroid glucuronides via an enzyme-assisted synthesis route in order to fulfil the significant drawback of the conjugate analysis and to overcome the lack of reference material. (I)

- To achieve higher sensitivity and specificity by direct analysis of steroid metabolites in urine samples with a simplified sample preparation procedure and LC–MS-based method which can be transferred to a routine anti-doping laboratory. (II)

- To combine gas chromatography with an atmospheric pressure mass spectrometer using microchip technology and to demonstrate the performance of microchip APPI in the analysis of anabolic steroids in biological matrixes. (III, IV)

3 MATERIALS AND METHODS

3.1 Reagents and solvents

All solvents were of HPLC grade and all other reagents were of analytical grade. Liver microsomes were prepared from Aroclor 1254 induced (a single dose of 500 mg/4.5 mL olive oil/body weight) male Wistar rats (n = 5) at the department of Industrial Hygiene and Toxicology (Finnish Institute of Occupational Health, Helsinki, Finland) according to previously described procedure [216]. The treatment of the animals was approved by the local ethical committee for animal studies. BCA protein assay kit (Pierce, IL, USA) was used for the determination of protein concentration (18.5 mg/mL), which was used to standardize the amount of microsomal protein in the synthesis.

3.2 Steroids and steroid glucuronides

The selection of the compounds in this work represent the parent compounds and metabolites which can be detected in human urine after dosing with anabolic steroids (e.g. methandienone, methenolone, methyltestosterone, nandrolone, and testosterone), which are all reported to be misused by sportsmen [217]. The selection also represents compounds with small differences in the substitution of carbons 1, 3, 5, 10, and 17. Nomenclature, precursors, and structures of the AAS glucuronides, as well as sources of the steroid aglycones of this study, are presented in Table 3. AAS glucuronides were prepared via chemical synthesis at the German Sport University or via enzyme-assisted synthesis at the University of Helsinki, Finland (Table 3). Nomenclature, structures, and molecular weights of the studied anabolic androgenic steroids are presented in Table 4. Nandrolone (NAN) was purchased from Diosynth (Oss, The Netherlands) and all other anabolic steroids were from the National Measurement Institute (Sydney, Australia).

Table 3. *Nomenclature, precursors, structures, and molecular weights (M_w) of the AAS glucuronides, as well as sources of the steroid aglycones used as starting material. (DSHS = Institute of Biochemistry, Cologne, Germany; S = Steraloids, Wilmington, USA)*

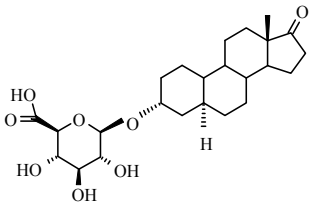
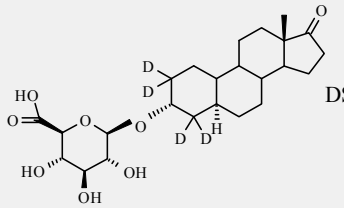
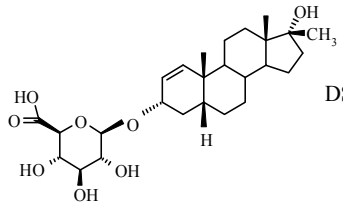
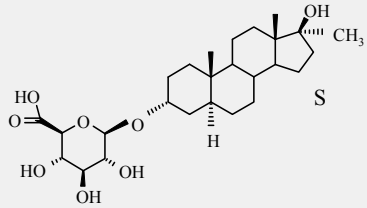
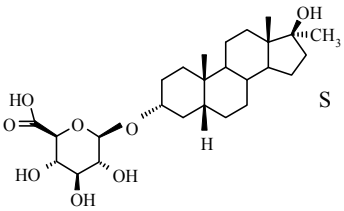
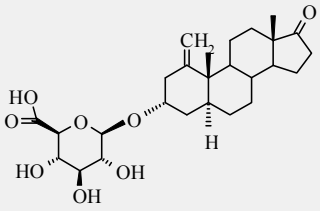
Abbrev.	Compound	Precursor	Structure	Source	Mw
5α-NG	5α-estran-17-one-3α-*O*-glucuronide	nandrolone		S	452
d_4-5α-NG	[2,2,4,4-$^2H_4$]5α-estran-17-one-3α-*O*-glucuronide			DSHS	456
5β-EPIMG	17β-methyl-5β-androst-1-ene-17α-ol-3α-*O*-glucuronide	methandienone		DSHS	480
5α-MTG	17α-methyl-5α-androstane-17β-ol-3α-*O*-glucuronide	mestanolone methyltestosterone oxymetholone		S	482
5β-MTG	17α-methyl-5β-androstane-17β-ol-3α-*O*-glucuronide	metandienone metandriol methyltestosterone		S	482
5α-MEG	1-methylen-5α-androstan-17-one-3α-*O*-glucuronide	methenolone		DSHS	478

***Table* 3.** *See previous page for the title of the table.*

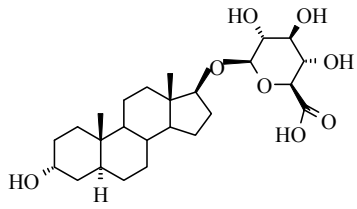
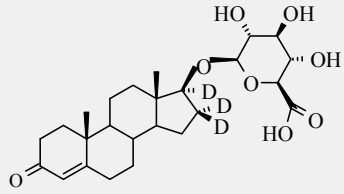
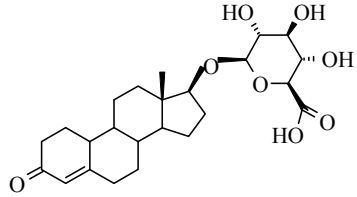
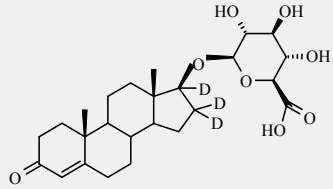
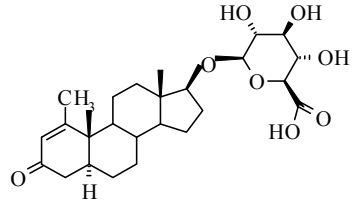
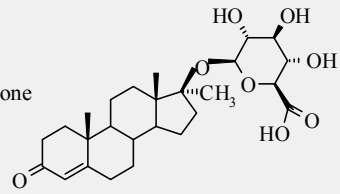
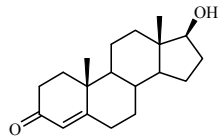
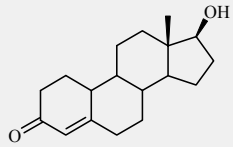
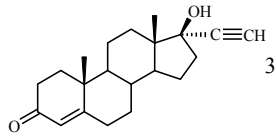
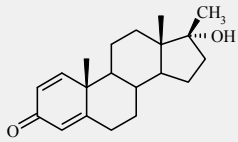
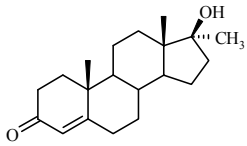
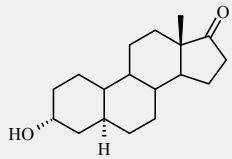
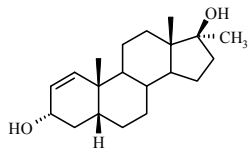
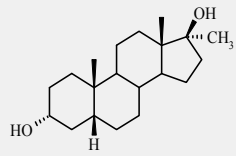
Abbrev.	Compound	Precursor	Structure	Source	Mw
AG	5α-androstane-3α-ol-17β-O-glucuronide	testosterone		DSHS	468
d_3-TG	[16,16,17-$^2H_3$]4-androsten-3-one-17β-*O*-glucuronide			DSHS	467
NG	estr-4-en-3-one-17β-*O*-glucuronide	nandrolone		Diosynth	450
d_3-NG	[16,16,17-$^2H_3$]estr-4-en-3-one-17β-*O*-glucuronide			DSHS	453
5α-1-MEG	1-methyl-5α-androst-1-en-3-one-17β-*O*-glucuronide	methenolone		DSHS	478
MTG	17α-methyl-5β-androstane-3α-ol-17β-*O*-glucuronide	methyltestosterone		DSHS	478

Table 4. *Nomenclature, structures, and molecular weights (M_w) of the studied anabolic androgenic steroids.*

Abbrev.	Trivial name	IUPAC name	Structure	M_w	M_w *bis*-O-TMS
TES	Testosterone	17β-Hydroxy-4-androsten-3-one		288	432
NAN	Nandrolone	17β-Hydroxy-19-nor-4-androsten-3-one		274	418
DNZm	Ethisterone Metabolite of danazol	17β-Hydroxy-17α-ethyl-4-androsten-3-one		312	456
17MDN	Metabolite of methandienone	17β-Methyl-17α-hydroxyandrosta-1,4-dien-3-one		300	445
MTS	Methyltestosterone (ISTD)	17α-Methyl-17β-hydroxyandrost-4-ene-3-one		302	446
NANm	Metabolite of nandrolone	5α-Estran-3α-ol-17-one		276	420
MDNm	Metabolite of methandienone	17α-Methyl-1-ene-5β-androstane-3α,17β-diol		304	448
MTm	Metabolite of methyltestosterone	17α-Methyl-5β-androstane-3α,17β-diol		306	450

3.3 Production and characterization of steroid glucuronides

In this study, enzymatic synthesis method was selected for the preparation of glucuronide-conjugated AAS-metabolites. Synthesis conditions were optimized in small-scale studies in a total volume of 100 µL with respect to concentration of the substrate 1–1000 µM (AAS or its phase-I metabolite), UDPGA 0.5–10 mM, and enzymatic protein 0.1–1.85 mg/mL. Optimization of each parameter was carried out in turn while keeping the other parameters constant in an overnight incubation. The concentrations of constant components were 50 µM (substrate), 5 mM (co-substrate), 0.5 mg/mL (protein) and incubation time of 2 h. β-glucuronidase inhibitor, *d*-saccharic acid 1,4-lactone (5 mM), was added to the reaction mixture to minimize the risk of hydrolysis by β-glucuronidases of the tissue preparation. Incubation reaction was carried out in 50 mM phosphate buffer (pH 7.4) with 5 mM $MgCl_2$. After centrifugation, the internal standard (ISTD, d_3-5β-MTG) was added to the supernatant and purified in SPE prior to LC–MS/MS analysis.

For the upscaled synthesis of AAS glucuronides, the steroid aglycone was diluted in 2 mL of methanol and filled up to the total volume of 20 mL of the earlier-described phosphate buffer. The reaction was initiated with the addition of UDPGA, carried out in a water bath of 37 °C for 12–15 h with continuous magnet stirring, and terminated by transferring the incubation mixture to an ice bath, and precipitating the enzymatic protein with dichloromethane, which also extracted the excess of starting material. The aqueous phase of the synthesis, containing the AAS glucuronide, was removed and further purified by solid-phase extraction (SPE) using a C_{18} cartridge (Isolute, 100 mg, International Sorbent Technology, UK) according to the method described earlier by Borts and Bowers [195], except for an additional SPE step, which was added to the procedure to ensure the purity of steroid glucuronide fraction with the final elution of 60% methanol in water. The AAS glucuronide fraction was then evaporated to dryness at 60 °C with nitrogen and reconstituted in LC eluent.

The synthesized anabolic steroid glucuronides were characterized by MS and NMR. The instrument used in MS and MS/MS measurements was Applied Biosystems API3000 (Perkin-Elmer SCIEX, Concord, ON, Canada) triple quadrupole instrument with a turbo ionspray source. Both positive and negative ion modes of ESI were used. The MS and MS/MS spectra were measured by using direct infusion of the purified compounds dissolved in 7.5 mM ammonium acetate in water–methanol (50–50, v/v). In negative ion mode, the pH of the solvent was neutral, whereas in positive ion mode the pH was adjusted to 4.2 with formic acid. The recorded mass range in MS and MS/MS experiment were *m*/*z* 100–600 and *m*/*z* 50–550, respectively. The ^{1}H NMR experiments were carried out on a Varian Unity 500 spectrometer at 23 °C using a gHX nano-NMR probe. Acetone-d_6 was used as solvent for MTG, 5β-EPIM, and 5β-EPIMG, whereas all the other compounds were dissolved in

methanol-d_4. The ^1H chemical shifts were referenced to residual acetone or methanol, 2.05 and 3.31 ppm, respectively.

3.4 Urine samples and sample preparation

The collected urine samples were stabilized with sodium azide (NaN_3) and stored in a refrigerator at +5 °C (I, II) or frozen (III, IV). Spiked urine samples were prepared by adding all the glucuronides or steroids as a mixture into a blank urine, which was a pool of six male and six female urine samples stored frozen until use. Excretion studies, approved by the local ethical committee, were performed with nandrolone, methenolone, methyltestosterone (II), and methandienone (III, IV). In the inter-laboratory comparison (II), each excretion study sample was provided at approximately 20 ng/mL ("low") and 200 ng/mL ("high") concentration.

The urine samples were prepared with solid phase extraction (SPE) (I, II) or LLE (III, IV). In direct LC–MS/MS analysis, the urine sample was buffered and extracted in SPE procedure with polymeric sorbent cartridge (Waters, Oasis® HBL, 30 mg/1 cc). The eluted methanolic fraction was further evaporated to dryness and the sample was reconstituted to LC–MS eluent. In the indirect GC–μAPPI-MS/MS analysis of AAS the urine sample was first hydrolyzed and then extracted with *n*-pentane. The buffered urine sample was hydrolyzed with β-glucuronidase of *Helix pomatia*, (55 °C for 2 h). After cooling to room temperature, the pH was adjusted to 9.2 and LLE was performed with n-pentane. The pentane layer was evaporated to dryness and the dry residue was either diluted to ethyl acetate (III) or derivatized with MSTFA (IV) prior to GC–μAPPI-MS/MS analysis.

3.5 LC–MS analysis of conjugated steroid glucuronides

The LC set-up consisted of a Hewlett-Packard model 1100 binary pump and autosampler. An endcapped cyano (CN) column (Phenomenex® Luna CN, 150 x 2 mm i.d., particle size 3 μm) was used. Eluent A was 5 mM ammonium acetate/acetic acid buffer (pH 3.5) in water and eluent B was 5 mM ammonium acetate/acetic acid buffer (pH 3.5) in acetonitrile:water (9:1, v/v). LC flow rate was 0.2 mL/min. The analytical run was initiated with a 1 min isocratic step of 5.6% B, then increased in linear ramp to 16.6% B in 1 min, and finally to 44.4% in 18 minutes. A 5 min clean-up step (90% B) was utilized prior to a final equilibration step of 2 min (5.6% B). The injection volume was 10 μL. The LC flow rate (0.2 mL/min) was post-column split 1:10 (Accurate; LC-Packings, The Netherlands) before introduction to the MS.

The mass spectra were recorded by an Applied Biosystems API3000 (Perkin-Elmer SCIEX, Concord, ON, Canada) triple quadrupole instrument with a turbo ionspray source. Analyses

were carried out in positive ion mode ESI. The ionspray voltage and orifice (declustering) voltage were 5500 V and 20 V, respectively. Purified air (custom-made; Atlas Copco, Belgium) was used as the nebulizing gas and the curtain gas. Nitrogen (Generator Systems 75-72; Whatman, USA) was applied as the collision gas and turbo gas. The turbo gas flow-rate was adjusted to 5 L/min at 350 ˚C. In direct injection studies with MS, the solvent flow rate was 0.6 mL/h and measured mass range was m/z 100-600, whereas in MS/MS the collision gas was nitrogen and collision energy (CE) was optimized with an offset range of 5-70 V (positive ESI) and 5-130 V (negative ESI) within full scan range of m/z 50-550.

3.5.1 Validation and inter-laboratory comparison

Anabolic steroid glucuronides were spiked as a mixture into blank urine, which was a pool of six male and six female urine samples stored frozen until use. Detection limits (signal-to-noise ratio (S/N) ≥ 3, determined by using standard deviation of the analyte peak height versus background noise), recovery (n=5), repeatability of injection (n=5), within-day repeatability (n=5), and between-day repeatability (n=5) were investigated. Linear and quadratic regression models of concentrations of AAS glucuronides in urine (1-60 ng/mL) were examined with respect to peak area.

Each laboratory used the distributed reference material for the tuning and adjusting of the LC and MS instrument parameters for an in-house method and were then requested to analyze individual and authentic urine samples resulting from excretion studies with nandrolone (Sample 1), methenolone (Sample 2), and methyltestosterone (Sample 3), comprising four selected steroid glucuronides as target compounds, namely 5α-NG (in sample 1), 5α-MEG (in sample 2), 5α-MTG, and 5β-MTG (both in sample 3). Each excretion study sample was provided at approximately 20 ng/mL (“low”) and 200 ng/mL (“high”), and the laboratories performed the analysis of both levels as triplicates, together with three replicates of drug-free urine samples fortified with each steroid glucuronide reference material (positive control, 0.4 nmol/mL, corresponding to 181-193 ng/mL). Maximum amount of identification (ID) points (18) was assigned to a specific target analyte which fulfilled the prevailing WADA-criteria [218] for relative ion ratios for each SRM-transition (n=3) in every sample replicate (n=3) at both concentration levels (n=2). Reference material and blank samples were provided by the German Sport University, distributed together with the authentic urine samples to the participating laboratories by courier mail, and stored in a refrigerator prior to analysis. Moreover, a recommended sample preparation protocol, suggested LC conditions, as well as the study protocol, were delivered to all participants.

Six out of seven participating laboratories utilized triple quadrupole mass spectrometers and one laboratory employed an ion trap analyzer. For the ionization, six laboratories used ESI with probe temperatures varying from 240 °C to 350 °C and one laboratory employed APCI

with 400 °C probe temperature. Either argon or nitrogen was applied as collision gas in triple quadrupole mass spectrometers and helium as damping gas in ion trap mass spectrometer. Specific instrumental information and modifications to the suggested protocol from each laboratory are described in Table 12.

3.6 GC–MS analysis of anabolic steroids

GC setup consisted of a Hewlett-Packard 5890 series II gas chromatography equipped with a CTC-A200S autosampler, a split/splitless injector, and a column transfer line. Helium was used as a carrier gas. The analytical column was a nonpolar forte BXP 5 from SGE (15 m x 0.25 mm. x 0.25 µm) with a 1.5 m long methyl deactivated fused-silica precolumn. The analytical column was connected to the transfer capillary of HN microchip with a press fit junction. The transfer capillary was threaded through a heated transfer line comprising a heated MSD transfer line (300˚C) connected to a self-made transfer line which was heated (290˚C) with a resistance wire. The HN microchip was placed in a custom-made clamp equipped with electrical connections for the heating and with tubing for the auxiliary gas.

The HN microchip (Figure 5) consists of a silicon plate and a Pyrex glass cover and features a sample introduction channel, an auxiliary gas inlet hole, a vaporizer channel, a thin film metal heater, and a nozzle. The channels and gas inlet are centrally located and DRIE etched (deep reactive ion etching) to the silicon wafer. The integrated platinum heater was sputtered on the Pyrex glass cover, which was then anodically bonded to the silicon wafer. The deactivated silica capillary (SGE, Victoria, Australia) for sample introduction and connector for auxiliary gas tubing (Nanoport, Upchurch Scientific Inc., Oak Harbor, WA) were glued to silicon wafer with high-temperature-resistant epoxy. A detailed description of the fabrication process has been described previously [160].

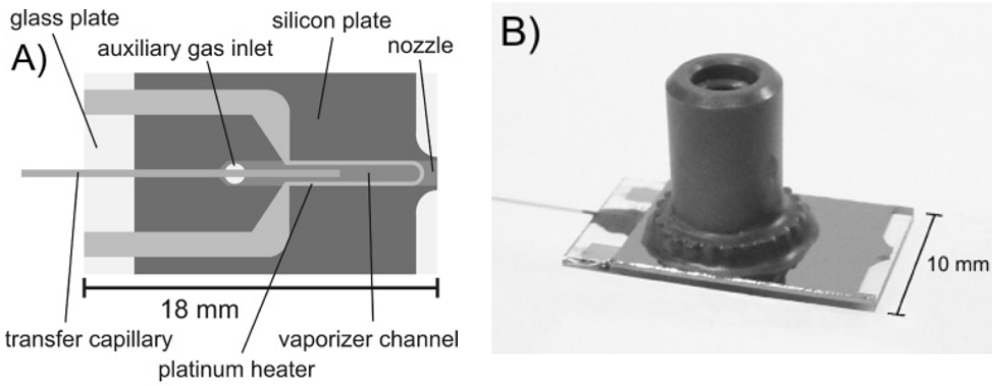


Figure 5. *(A) Schematic view of the microchip from the heater side. (B) A photograph of a microchip with Nanoport connector and transfer capillary. Reprinted with the permission of the American Chemical Society.*

The mass spectra were measured by a PE Sciex API 3000 triple quadrupole mass spectrometer, but the conventional ion source was replaced with a nanospray stand to enable positioning of the HN microchip at about 1 cm distance from the MS orifice. Analyses were carried out in positive ion mode APPI. A vacuum UV krypton dc discharge photoionization lamp with 10 eV photon energy was used for ionization. A syringe was used to deliver the dopant (toluene (III) or chlorobenzene (IV), flow rate 3.5 μL/min) to the auxiliary gas line. The repeller voltage and orifice (declustering) voltages were 1300 and 20 V, respectively. Nitrogen was applied as the auxiliary, curtain, and collision gas. The auxiliary gas flow rate (80 mL/min) was controlled with a mass flow controller. The dopant was delivered coaxially to the auxiliary gas line with a syringe pump. The microchip was heated with dc power supply (2,2 W (III) 3,5 W (IV)) and positioned in front of the curtain plate of the MS. More specific details of the system setup are described in a publication by Haapala et al. in 2007 [163].

In study III, the scan and product ion spectra were measured from *m/z* 50 to 350 with a speed of 1 scan/s. In MS/MS, the collision energy was optimized within offset range 5–70 V and the scan range was set to *m/z* 50–350. In the final GC–μAPPI–MS/MS method the collision energy was 12–38 V, three specific product ions per analyte were monitored in selected reaction monitoring (SRM) mode, and the measurement was divided into four scanning segments. Analyst 1.4.2. software (Applied Biosystems/MSD SCIEX Instruments) was applied to data processing.

In study IV, the measured mass range was extended to also cover the higher mass TMS-derivatives, being from *m/z* 50 to 470. The MS parameters were optimized with repeated analyte peaks generated by running the GC isothermally and making several split injections of standard solutions. Two specific product ions per analyte were monitored with a scheduled SRM algorithm with a 15 s detection window. Analyst 1.5 software was used for data acquisition and processing.

Repeatability (within-day and injection), linearity, LOD, and limit of quantification (LOQ) were investigated in the method validation. A drug-free pool of blank urine samples from five individuals spiked with a mixture of the AAS served as a positive quality control in the analyses. In the study with underivatized steroids (III), the positive controls were spiked within the range of 0.1-500 ng/mL, whereas for derivatized steroids (IV) the range of the positive controls was 0.1–100 ng/mL of each analyte. Methyltestosterone (MTS) was used as an internal standard and spiked at 25 ng/mL and 10 ng/mL in studies III and IV, respectively. The method applicability was tested with excretion urine samples obtained from United Medix Laboratories and the sample preparation was as described above (section 3.4). Calibration curve (0.5–100 ng/mL), reagent blank, and urine blank samples were prepared together with the excretion study samples.

4 RESULTS AND DISCUSSION

4.1 Production of AAS glucuronides

4.1.1 Enzyme assisted synthesis

Enzyme assisted synthesis method was used to produce small amounts of steroid glucuronides. Glucuronidation synthesis was optimized using incubation volumes of 100 µL with respect to substrate (steroid aglycone), co-substrate (UDPGA), and UGT-enzymes (microsomal protein) concentration, in order to rationalize the consumption of expensive starting materials, especially that of UDPGA. The optimization experiments were carried out with 10% methanol, which was a compromise between dissolution of non-polar steroids and denaturation of enzyme protein. All the tested steroid substrates were able to conjugate via enzyme-assisted pathways using rat liver microsomes, and the optimal conditions for the various substrates were almost the same (Table 5). None of the steroids showed any substrate inhibition within the concentration range of 1–1000 µM, but the plateau of glucuronidation was observed at 500–1000 µM, except for 5α-NG, for which it was 250 µM. The first point of the plateau was selected as the concentration level for the bulk synthesis. The increase of UDPGA concentration increased the yield of the glucuronides until the plateau was achieved. The lowest possible concentration was selected for cost-effectiveness of the assay. Optimal concentration of UDPGA was 2.5–5 mM for all the other compounds except for methyltestosterone, which required 7.5 mM concentration of co-substrate for maximal yield of MTG. The formation of the steroid glucuronides was linearly proportional to the concentration of protein up to 0.5–1 mg/mL. The effect of incubation time was tested up to 24 h and observed to reach the plateau after 6 h of incubation at the latest. However, there was no evidence of breakdown of the glucuronide conjugate during that time, which allowed an overnight incubation (12–15 h).

***Table 5**. Optimized conditions of the synthesis of AAS glucuronides.*

Compound	*Substrate (µM)*	*UDPGA (mM)*	*Protein (mg/ml)*	*Compound*	*Substrate (µM)*	*UDPGA (mM)*	*Protein (mg/ml)*
5α-NG	250	5	1	**AG**	500	2.5	1
5β-NG	500	5	0.75	**d_3-TG**	500	5	1
5β-EPIMG	500	5	1	**NG**	500	5	1
5α-MTG	1000	2.5	0.5	**5α-1-MEG**	500	5	1
5β-MTG	1000	2.5	0.5	**MTG**	1000	7.5	0.5
5α-MEG	500-1000	2.5	0.75				

The larger scale enzymatic syntheses of the steroid glucuronides were carried out under optimized conditions (Table 5) using 20 mL reaction volume. The excess of steroid substrate was removed in LLE with dichloromethane. The more polar steroid glucuronide remained in aqueous layer, but also some residue of starting material was found to remain in that fraction. To increase purity of the product, a further purification was performed with SPE. Retention of steroid substrate and glucuronide conjugate in SPE was examined as a function of methanol concentration (1–100%). The polarities of these two compounds were different enough for the complete isolation of the AAS glucuronide from steroid substrate when methanol–water (60:40, v/v) was used in the final elution.

The synthesis method was suitable for producing steroid glucuronides in milligram amounts (1.1–6.5 mg), with yields of 13–78% (Table 6). Steroid substrates with a 4-ene-3-one structure and a sterically free 17β-OH position available for *O*-glucuronidation (NG and d_3-TG) showed superior yields of 77% in comparison to the substrates with 3α-OH and completely saturated or 1-ene A-ring structure (13–28%). Glucuronidation of methyltestosterone was exceptional (14%), as the only possible site for *O*-glucuronidation is the 17β-OH-group, though sterically hindered by the neighbouring 17α-CH_3, which is likely to inhibit the catalysis of the bulky UGT enzymes. Based on the detected formation of MTG, although in low yield, it may be proposed that the structure still allows conjugation to some extent in the absence of a more favorable functionality. Within the group of selected compounds, 5α-androstane-3α,17β-diol and its d_5-labeled analogue were the only ones with sterically non-hindered 3α- and 17β-hydroxyl groups, offering multiple sites for *O*-glucuronidation.

Table 6. *Synthesized steroid glucuronides and their yields.*

Compound	*Yield (mg)*	*Yield (%)*	*Compound*	*Yield (mg)*	*Yield (%)*
5α-NG	1.3	16	**AG**	1.9	16
5β-NG	1.1	13	**d_3-TG**	6.5	77
5β-EPIMG	1.4	25	**NG**	3.5	77
5α-MTG	2.1	22	**5α-1-MEG**	1.7	24
5β-MTG	2.6	26	**MTG**	1.2	14
5α-MEG	2.5	28			

4.1.2 Characterization

The structure of enzymatically synthesized steroid glucuronides was confirmed with MS and NMR. MS gave molecular weight and specific fragmentation information, but NMR was needed in order to confirm the position and configuration of glucuronidation. In positive ion ESI MS spectra the abundant ammonium adduct ion $[M+NH_4]^+$ or protonated molecule $[M+H]^+$ were detected as base peaks and loss of glucuronide moiety was the main fragment in MS/MS spectrum. In negative ion ESI MS spectra, the base peak for all AAS glucuronides was the deprotonated molecule $[M-H]^-$ that was fragmented to an intense deprotonated glucuronic acid $[Glu-H]^-$ as product ions in the MS/MS spectra. The positive and negative ion MS and MS/MS spectra clearly indicate the presence of the glucuronide moiety. However, the MS data does not provide unambiguous information on the site of glucuronidation and for this reason NMR characterization was performed.

One-dimensional ^1H NMR was used to determine the position of the glycosidic linkage and the anomericity of the glucuronic acid moiety in the steroid glucuronides. Each glucuronide spectrum contained the five methine signals arising from the glucuronic acid. The measured vicinal $^3J_{GH1,GH2}$ coupling constants of approximately 7.8 Hz are typical values between two axial protons, and thus confirmed the β-configuration for the glucuronic acid for each glucuronide. Integration of the signals gave a 1:1 glucuronic acid/aglycone ratio indicating the correct stoichiometry.

As the resulting steroid substrates (d_4-5α-NG, 5α-MEG, d_3-TG, NG, d_3-NG, 5α1-MEG, and MTG) contain only one possible site for glucuronidation, there was no ambiguity about the position of the glycosidic linkage in the corresponding glucuronides. In the structures of AG and 5β-EPIMG, there are two possible glucuronidation sites, namely hydroxyl groups in positions 3 and 17. For these compounds ^1H spectra of both steroid aglycone and glucuronide were measured and compared. In 5α-androstane-3α,17β-diol (A, steroid aglycone) H3 and H17 resonate downfield to the steroid bulk region and can be distinguished from each other due to their different coupling patterns. H17 of steroid aglycone is a triplet [219] with a coupling constant of 8.5 Hz while H3 is typically a multiplet resulting from several small couplings [220] Comparison of the steroid aglycone spectrum with its glucuronide-conjugated product (AG) spectrum showed that the H17 signal shifted by 0.25 ppm downfield upon glucuronidation and also that H3 signal did not shift, which suggests that the glucuronide, indeed, is a 17β-*O*-glucuronide.

Comparison of aglycone and glucuronide ^1H spectra also suggested that 5β-EPIMG is a 3-glucuronide. This is because none of the methyl signals, including 17β-methyl, shifted upon glucuronidation while H1, H2, and H3 shifted downfield by 0.09, 0.12, and 0.16 ppm, respectively. NMR-characterization of 5α-NG, 5β-NG, 5α-MTG, and 5β-MTG has been presented previously elsewhere [67].

4.2 Sample preparation

Three different sample clean up procedures, namely LLE, SPE, and LPME were compared previously [94] for AAS glucuronides in urine, concluding that the SPE and LPME methods resulted in cleaner samples than LLE. LPME provided slightly better limits of detection and specificity than SPE. However, the LPME method resulted in poor recoveries (8-19%) and it is not easily automated, and therefore SPE was chosen for future development of the sample clean up procedure. SPE of AAS glucuronides has previously [67,94,187,195] been performed with C8 and C18 sorbents. Within this method development, as well as in earlier research [94], the urine matrix clearly interfered with the ion traces of 5α-MTG and 5β-MTG in authentic urine samples when extracted with C18 cartridge. Also, the recoveries with the C18 column were insufficient. Therefore, various sorbent materials were investigated, and in this application, the best selectivity was obtained with macroporous polymeric sorbent cartridge containing both hydrophilic and lipophilic characters (Oasis HBL 30 mg/1 cc). Methanol:water (1:9, v/v) and methanol:water (6:4, v/v) were optimized as washing and elution steps, respectively.

4.3 Development of LC–ESI-MS/MS method for AAS glucuronides

Separation of AAS glucuronides and matrix components with HPLC was optimized. Ionization in both positive and negative mode ESI was investigated, as well as the fragmentation pattern in MS/MS measurements. Specific product ions and optimal collision energies of each compound were determined. The developed method was validated and the possibility to transfer it to other laboratories was investigated in inter-laboratory comparison.

4.3.1 LC separation of AAS glucuronides

Separation of 5α-MTG and 5β-MTG with a C18 column was found to be insufficient in earlier studies [94]. The HPLC separation was enhanced in order to achieve adequate resolution between 5α-MTG and 5β-MTG, for which also the endogenous interference was the most intense. Four different HPLC columns were tested during the development of the HPLC method. One C18 column (Purospher STAR RP-18, endcapped, 55 mm x 2mm, particle size 3 μm) and three cyano columns (Zorbax 300 SB-CN 150 mm x 2.1 mm (5 μm), Discovery CN endcapped 1.0 x 150 mm (5 μm) and Phenomenex Luna CN endcapped 2.0 x 150 mm (3 μm)). In this application, the best specificity and selectivity was achieved with endcapped Phenomenex Luna CN 2.0 x 150 mm (3 μm) column. Cyano-substituted phase material offers improved selectivity and the length of the column together with narrow diameter and smaller particles provide better chromatographic performance. In a 25-minute gradient run the steroids eluted in 14.1-20.6 min and resulted in fairly narrow and symmetric peaks. The peak widths at half heights were 0.178-0.237 min, and peak symmetry factors were 0.69-1.6 (Table 11). All the examined glucuronide-conjugated steroid metabolites

were chromatographically separated, and the resolution of the most similar pair of compounds (5α-MTG and 5β-MTG) was acceptable (R_s=1.0).

4.3.2 Ionization and MS detection of AAS glucuronides

Previous studies have shown ESI to be more sensitive than APCI or atmospheric pressure photoionization (APPI) for steroid glucuronides and free anabolic steroids [187,189]. AAS glucuronides can be detected in both polarities of ESI-MS detection, and the behavior of the conjugates was investigated in both polarities.

The abundant ammonium adduct ion $[M+NH_4]^+$ was detected in positive ion ESI-MS for the glucuronides with a saturated A-ring system, whereas for d_3-TG, NG, and d_3-NG with 4-ene-3-one structure, protonated molecule $[M+H]^+$ was detected as a base peak. The ratio of protonated molecule and ammonium adduct ion was found to strongly depend on the proton affinity (PA) of the compound, where a higher proton affinity resulted in the formation of a stronger protonated molecule. Proton affinity of the 4-ene-3-one structure in the steroid ring results in higher proton affinity than a hydroxyl group or carbonyl group without a conjugated double bond [221]. For 5α-1-MEG and MTG, the relative abundance of $[M+NH_4]^+$ and $[M+H]^+$ was approximately equal. Formation of sodium adduct $[M+Na]^+$ was also detected for most of the compounds, although with variable relative abundances. In negative ion ESI, an intensive deprotonated molecule $[M-H]^-$ was observed as the base peak for all AAS glucuronides (Table 7). In negative ion ESI, the most potential site for deprotonation is the carboxylic acid moiety of the glucuronic acid, as the steroid aglycones are not ionized in negative ion ESI at all. These results are in good agreement with earlier studies by Bowers and Sanaullah [105], Williams et al. [222], and Kuuranne et al. [187] and [94], and gave evidence on the correct molecule weights of the synthesized steroid glucuronides.

***Table** 7. Positive and negative ion ESI-MS of AAS glucuronides.*

	POS ESI			NEG ESI
Compound	$[M+H]^+$	$[M+NH_4]^+$	$[M+Na]^+$	$[M-H]^-$
5α-NG	n.d.	470 (100)	n.d.	451 (100)
d_4-5α-NG	n.d.	474 (100)	n.d.	455 (100)
5β-NG	n.d.	470 (100)	475 (43)	451 (100)
5β-EPIMG	n.d.	498 (100)	n.d.	479 (100)
5α-MTG	n.d.	500 (100)	505 (36)	481 (100)
5β-MTG	n.d.	500 (100)	505 (38)	481 (100)
5α-MEG	479 (2)	496 (57)	501 (100)	477 (100)
AG	n.d.	486 (100)	491 (6)	467 (100)
d_5-AG	n.d.	491 (100)	496 (10)	472 (100)
d_3-TG	468 (100)	485 (41)	490 (66)	466 (100)
NG	451 (100)	n.d.	473 (50)	449 (100)
d_3-NG	454 (100)	471 (2)	476 (6)	452 (100)
5α-1-MEG	479 (89)	496 (100)	501 (11)	477 (100)
MTG	479 (94)	496 (100)	501 (15)	477 (100)

The positive ion MS/MS spectra showed abundant product ions formed by loss of glucuronide moiety without or with loss of one or two water molecules. The positive charge stayed predominantly on the aglycone side, and only weak fragments were detected from the glucuronic acid side, e.g. $[Glu+H]^+ = 177$, $[Glu+H-H_2O]^+ = 159$, $[Glu+H-2H_2O]^+ = 141$ (Table 8). The negative ion MS/MS spectra of $[M-H]^-$ showed intense product ions derived from the deprotonated glucuronic acid $[Glu-H]^-$ (Table 9). The spectra also showed weak product ions formed by the cleavage of a glucuronic acid moiety $[M-H-Glu]^-$, followed by the abstraction of two hydrogens $[M-H-Glu-2H]^-$, and losses of water $[M-H-Glu-nH_2O]^-$.

Table 8. *Positive ion ESI-MS/MS of AAS glucuronides. Collision offset voltages indicated separately for each steroid glucuronide.*

Ions	5α-NG 20 V	5β-NG 20 V	5β-EPIMG 20 V	5α-MTG 20 V	5β-MTG 20 V	5α-MEG 20 V	AG 20 V	d_3-TG 25 V	NG 30 V	5α-1-MEG 30 V	MTG 30 V
Precursor											
$[M+NH_4]^+$	470 (11)	470 (100)	498 (5)	500 (13)	500 (9)	496 (7)	486 (4)			496 (1)	496 (2)
$[M+H]^+$								468 (100)	451 (100)		
Product ions											
$[M+NH_4-NH_3]^+$	453 (6)	453 (23)	481 (4)	n.d.	n.d.	479 (1)	n.d.			479 (100)	479 (100)
$[M+NH_4-H_2O]^+$	n.d.	n.d.		482 (4)	482 (4)	n.d.	n.d.			n.d.	n.d.
$[M+H-H_2O]^+$	435 (28)	435 (21)	n.d.	465 (15)	465 (13)	461 (6)	451 (7)	n.d.	n.d.	n.d.	461 (2)
$[M+H-2H_2O]^+$	417 (74)	41 (92)	n.d.	447 (5)	447 (7)	443 (2)	433 (1)	n.d.	n.d.	n.d.	n.d.
$[M+H-Glu]^+$	277 (60)	277 (67)	305 (3)	n.d.	n.d.	303 (8)	n.d.	292 (45)	275 (71)	303 (18)	303 (32)
$[M+H-Glu-H_2O]^+$	259 (100)	259 (42)	287 (4)	289 (44)	289 (58)	285 (100)	275 (34)	274 (13)	257 (24)	285 (11)	285 (71)
$[M+H-Glu-2H_2O]^+$	241 (14)	241 (13)	269 (100)	271 (100)	271 (100)	267 (10)	257 (100)	256 (8)	239 (14)	267 (4)	267 (34)
$[Glu+H]^+$	177 (22)	177 (4)	177 (2)	177 (19)	177 (6)	n.d.	177 (13)	177 (5)	n.d.	177 (1)	177 (9)
$[Glu+H-H_2O]^+$	159 (16)	159 (4)	159 (1)	159 (7)	159 (3)	159 (1)	159 (7)	159 (8)	159 (12)	159 (4)	159 (15)
$[Glu+H-2H_2O]^+$	141 (16)	141 (5)	n.d.	141 (5)	141 (4)	141 (1)	141 (6)	141 (5)	141 (6)	141 (5)	141 (4)

Table 9. *Negative ion ESI-MS/MS of AAS glucuronides at applied collision offset voltage of 40 V.*

Ions	5α-NG	5β-NG	5β-EPIM	5α-MTG	5β-MTG	5α-MEG	AG	d_3-TG	NG	5α-1-MEG	MTG
Precursor											
$[M-H]^-$	451 (52)	451 (57)	479 (100)	481 (89)	481 (100)	477 (56)	467 (26)	466 (10)	449 (11)	477 (14)	477 (15)
Product ions											
$[M-H-H_2O]^-$	433 (5)	433 (3)	461 (8)	463 (6)	463 (6)	459 (7)	449 (1)	n.d.	431 (1)	459 (1)	459 (2)
$[M-H-Glu]^-$	n.d.	n.d.	303 (9)	305 (1)	n.d.	301 (5)	291 (6)	290 (6)	273 (32)	301 (26)	301 (15)
$[M-H-Glu-2H]^-$	273 (12)	273 (3)	301 (1)	303 (1)	303 (4)	n.d.	289 (11)	n.d.	271 (23)	299 (22)	n.d.
$[M-H-Glu-H-D]^-$								n.d.			
$[M-H-Glu-2D]^-$								286 (3)			
$[Glu-H]^-$	175 (2)	n.d.	175 (6)	175 (28)	175 (15)	175 (4)	175 (2)	175 (3)	175 (4)	175 (2)	175 (2)
$[Glu-H-H_2O]^-$	157 (11)	157 (9)	157 (9)	157 (36)	157 (18)	157 (11)	157 (6)	157 (6)	157 (9)	157 (5)	157 (4)
$[Glu-H-H_2O-CO]^-$	n.d.	129 (6)	129 (9)	129 (16)	129 (9)	129 (5)	129 (6)	129 (5)	129 (6)	129 (6)	129 (5)
***m/z* 113**	(75)	(55)	(57)	(90)	(59)	(100)	(47)	(82)	(75)	(55)	(48)
***m/z* 99**	(6)	(6)	(9)	(4)	(4)	(5)	(5)	(3)	(8)	(9)	(9)
***m/z* 97**	(1)	n.d.	(4)	(1)	(1)	n.d.	(4)	n.d.	(4)	(4)	n.d.
***m/z* 95**	(12)	(7)	(8)	(10)	(6)	(8)	(18)	(11)	(11)	(15)	(12)
***m/z* 85**	(100)	(100)	(61)	(100)	(41)	(55)	(100)	(100)	(100)	(100)	(100)
***m/z* 75**	(82)	(82)	(40)	(89)	(49)	(85)	(60)	(72)	(77)	(70)	(63)
***m/z* 59**	(6)	(5)	(4)	(12)	(3)	(18)	(6)	(5)	(6)	(7)	(6)

The behavior of the AAS glucuronide conjugates is relatively uniform in both polarities of ESI-MS detection. Negative ionization mode usually results in lower background, but the negative ion mode fragmentation leads to formation of non-specific ions from the glucuronide moiety, and therefore, LC–MS/MS method development was carried out in positive ion mode. In positive ion mode MS, the base peak was most often the ammonium adduct $[M+NH_4]^+$, indicating relatively low proton affinities (PA) of the compounds. The protonated molecule $[M+H]^+$ was the base peak for compounds containing conjugated double bonds (d_3-NG, NG, d_3-TG, 5α-1-MEG, and MTG). These results suggest that the presence of the 4-ene-3-one structure in the aglycone moiety of steroid glucuronides favors the formation of an abundant $[M+H]^+$ ion and are in good correlation with earlier studies [105,187,221,223].

The most abundant ion, ammonium adduct, or protonated molecule was selected as a precursor ion for following MS/MS studies, which were carried out for determination of specific product ions and optimal collision energies of each compound. Some SRM ion transitions were strongly interfered by the background ions at collision offset voltages of 20-25 V, which produced product ions formed by the losses of one or several water molecules and cleavage of the glucuronide moiety, followed by the loss of water from the steroid aglycone. Because urine is generally rich of endogenous steroid glucuronides and glucuronide-conjugated compounds, which are fragmented similarly to the target compounds, specificity problems were encountered. Therefore, the collision energy in the MS/MS analysis was increased up to 45-70 V to produce specific product ions derived from the dissociation of the aglycone moiety. The results showed significantly better specificity than those produced by lower collision energy. Precursor-product ion pairs of the SRM method with corresponding collision energies are listed in Table 10.

In the final LC–ESI-MS/MS method, the detection was performed by following three specific ions per analyte in SRM (Table 10). The collision energy was 15-70 V and due to the technical characteristic of the instrument, analytical run was divided between three segments. The first segment (0-15.80 min) included characteristic SRM ions for d_3-NG, NG and d_3-TG with dwell time of 200 ms. The second segment (15.80-19.35 min) containing ions for 5α-1-MEG, MTG, d_4-5α-NG, 5α-NG, 5β-MTG, 5α-MTG, d_5-AG, and 5α-MEG were monitored with dwell time of 90 ms. 5β-EPIMG was the only analyte in the last segment (19.35-25 min) with the dwell time of 450 ms.

Table 10. *Precursor-product ion pairs of AAS glucuronides in SRM method. CE = collision energy, expressed as offset voltage, Tgt = Target, Q1 = qualifier1, Q2 = Qualifier 2. Dwell time (msec)*

Compound		***Precursor ion (m/z)***	***Product ion (m/z)***	***CE***	***Dwell time***	***Segment***
NG	Tgt	$[M+H]^+ = 451$	m/z = 73	70 V	200	1
	Q1	$[M+H]^+ = 451$	m/z = 113	40 V	200	1
	Q2	$[M+H]^+ = 451$	$[M+H\text{-}Glu]^+ = 275$	25 V	200	1
d_3-NG	Tgt	$[M+H]^+ = 454$	$[M+H\text{-}Glu]^+ = 278$	25 V	200	1
	Q1	$[M+H]^+ = 454$	m/z = 73	70 V	200	1
	Q2	$[M+H]^+ = 454$	m/z = 113	40 V	200	1
d_3-TG	Tgt	$[M+H]^+ = 468$	m/z = 97	45 V	200	1
	Q1	$[M+H]^+ = 468$	$[M+H\text{-}Glu]^+ = 292$	25 V	200	1
	Q2	$[M+H]^+ = 468$	$[M+H\text{-}Glu\text{-}H_2O]^+ = 274$	30 V	200	1
5α-1-MEG	Tgt	$[M+H]^+ = 479$	$[M+H\text{-}Glu]^+ = 303$	20 V	90	2
	Q1	$[M+H]^+ = 479$	$[M+H\text{-}Glu\text{-}H_2O]^+ = 285$	20 V	90	2
	Q2	$[M+H]^+ = 479$	$[M+H\text{-}Glu\text{-}2H_2O]^+ = 267$	25 V	90	2
MTG	Tgt	$[M+H]^+ = 480$	$[M+H\text{-}Glu\text{-}H_2O]^+ = 286$	30 V	90	2
	Q1	$[M+H]^+ = 480$	$[M+H\text{-}Glu\text{-}2H_2O]^+ = 268$	35 V	90	2
	Q2	$[M+H]^+ = 480$	$[M+H\text{-}Glu]^+ = 304$	25 V	90	2
5α-NG	Tgt	$[M+NH_4]^+ = 470$	$[M+H\text{-}Glu\text{-}H_2O]^+ = 259$	20 V	90	2
	Q1	$[M+NH_4]^+ = 470$	$[M+H\text{-}Glu\text{-}2H_2O]^+ = 241$	30 V	90	2
	Q2	$[M+NH_4]^+ = 470$	$[M+H\text{-}Glu]^+ = 277$	15 V	90	2
d_4-5α–NG	Tgt	$[M+NH_4]^+ = 474$	$[M+H\text{-}Glu]^+ = 281$	15 V	90	2
	Q1	$[M+NH_4]^+ = 474$	$[M+H\text{-}Glu\text{-}2H_2O]^+ = 245$	30 V	90	2
	Q2	$[M+NH_4]^+ = 474$	$[M+H\text{-}Glu\text{-}H_2O]^+ = 263$	20 V	90	2
5β-MTG	Tgt	$[M+NH_4]^+ = 500$	$[M+H\text{-}Glu\text{-}2H_2O]^+ = 271$	20 V	90	2
	Q1	$[M+NH_4]^+ = 500$	$[M+H\text{-}Glu\text{-}H_2O]^+ = 289$	20 V	90	2
	Q2	$[M+NH_4]^+ = 500$	m/z = 161	50 V	90	2
5α-MTG	Tgt	$[M+NH_4]^+ = 500$	$[M+H\text{-}Glu\text{-}2H_2O]^+ = 271$	20 V	90	2
	Q1	$[M+NH_4]^+ = 500$	$[M+H\text{-}Glu\text{-}H_2O]^+ = 289$	20 V	90	2
	Q2	$[M+NH_4]^+ = 500$	m/z = 161	50 V	90	2
d_5-AG	Tgt	$[M+NH_4]^+ = 491$	$[M+H\text{-}Glu\text{-}2H_2O]^+ = 262$	30 V	90	2
	Q1	$[M+NH_4]^+ = 491$	$[M+H\text{-}Glu\text{-}H_2O]^+ = 280$	15 V	90	2
	Q2	$[M+NH_4]^+ = 491$	m/z = 92	70 V	90	2
5α-MEG	Tgt	$[M+NH_4]^+ = 496$	$[M+H\text{-}Glu\text{-}H_2O]^+ = 285$	25 V	90	2
	Q1	$[M+NH_4]^+ = 496$	m/z = 95	50 V	90	2
	Q2	$[M+NH_4]^+ = 496$	$[M+H\text{-}Glu\text{-}2H_2O]^+ = 267$	30 V	90	2
5β-EPIMG	Tgt	$[M+NH_4]^+ = 498$	m/z = 201	60 V	450	3
	Q1	$[M+NH_4]^+ = 498$	$[M+H\text{-}Glu\text{-}2H_2O]^+ = 269$	30 V	450	3
	Q2	$[M+NH_4]^+ = 498$	$[M+H\text{-}Glu\text{-}H_2O]^+ = 286$	20 V	450	3

4.3.3 Validation

The recovery and repeatability of the analysis was determined with spiked urine samples (200 ng/mL). Recovery of the SPE was good for all glucuronides (89-100%). Within-day repeatability of the LC–ESI-MS/MS method was 2-10% (relative standard deviation (RSD)) and between-day repeatability was below 16% (RSD) for most of the compounds (except for d_3-NG, NG and MTG), which could be considered as suitable for trace analysis of compounds in a complex biological matrix. The repeatability of retention time was good (0.1% within day and 0.8-1% between days). The relationship between peak areas and concentrations of AAS glucuronides in urine is best described using a quadratic regression model. The regression coefficient (R) of the quadratic curve for all the AAS glucuronides was over 0.994 within the range of 1-60 ng/mL (Table 11). For most of the AAS glucuronides, the detection limits with one SRM-transition were from 1 to 10 ng/mL, but 40 ng/mL for 5α-1-MEG and MTG, due to the complex nature of urine as matrix in analysis of exogenous and endogenous AAS glucuronides.

Table 11. *Recovery, repeatability, and detection limits of the method. Peak widths at half height, peak symmetry factors and regression coefficients of quadratic fit for concentration versus peak area.*
*W_h = Peak width at half height **R = Regression coefficient. Quadratic fit in urine.*

Compound	*Recovery of SPE (%)*	*Repeatabilities (RSD%)*			*LOD (ng mL^{-1})*	*W_h**	*Peak symmetry factor*	*R***
		Within-day	*Between-day*	*Injection*				
d_3-NG	95	2.8	32	5.3	5	0.198	1.0	0.9968
NG	95	3.0	24	3.8	10	0.189	0.71	0.9942
d_3-TG	95	3.3	16	3.4	5	0.187	0.96	0.9963
5α-1-MEG	93	7.0	14	3.5	40	0.181	1.3	-
MTG	105	9.6	28	3.8	40	0.178	1.2	-
d_4-5α-NG	98	2.7	13	3.6	5	0.197	1.0	0.9949
5α-NG	95	2.7	12	3.4	5	0.204	1.0	0.9955
5β-MTG	98	2.3	13	3.3	10	0.205	0.69	0.9998
5α-MTG	103	3.8	7.7	2.8	10	0.212	1.6	0.9966
d_5-AG	100	4.2	13	4.2	1	0.192	1.2	0.9970
5α-MEG	95	4.9	9.3	2.7	5	0.237	1.3	0.9996
5β-EPIMG	89	2.1	16	3.4	1	0.207	1.1	0.9985

The results obtained indicate that the developed LC–MS analysis provides a reliable method for the detection of AAS glucuronides. However, the limits of detection measured with standards prepared in pure solvent were one to two orders of magnitude lower than those obtained with urine samples. This reveals that the selectivity of the method is limited due to endogenous compounds in urine samples, despite the use of tandem mass spectrometry. The number of compounds possessing identical or similar ion transitions in SRM and the limited chromatographic resolution (due to a similar constitution of AAS glucuronides) is a considerable problem with the analysis of AAS from urine.

4.3.4 Inter-laboratory comparison

Seven laboratories participated in the inter-laboratory comparison to evaluate the performance and transferability of the method, as well as to assess the fitness-for-purpose. Laboratories were requested to analyze individual and authentic urine samples resulting from excretion studies with nandrolone, methenolone, and methyltestosterone. Samples were provided at low (20 ng/ml) and high (200 ng/ml) concentration and the laboratories performed the analysis of both levels as triplicates. Sample preparation was performed with SPE as described in chapter 3.4. Six out of seven participating laboratories utilized triple quadrupole mass spectrometers and one laboratory employed an ion trap analyzer. The summary of instrumental parameters and metabolites identified in excretion study urine samples is given in Table 12. Identification of prohibited compounds was based on the WADA guidelines [218] and an independently used matrix for result evaluation by calculation of identification (ID) points. A successful identification of one transition of an analyte in the sample resulted in one ID-point, the maximum being 18 points per one steroid glucuronide (three transitions per sample, samples at two concentration levels as triplicates). Altogether 68% of urine samples containing 5α-NG, 71% containing 5β-MTG, and 95% containing 5α-MEG were successfully identified. In addition, two laboratories succeeded in determining 5α-MTG at low concentration levels in excretion sample. Two laboratories experienced problems with respect to the chromatographic separation: one due to signals interfering with the 5α-NG; the other due to insufficient resolution of the methyltestosterone metabolites (5α-MTG and 5β-MTG). The results obtained with the ion trap mass spectrometer and triple quadrupole mass spectrometers were similar when the precursor ion was a protonated molecule, e.g. in the case of methenolone metabolite 5α-MEG. However, the limited stability of ammonium adducts with 5α-NG, 5α-MTG, and 5β-MTG during the isolation of the precursor ion at unit resolution resulted in poor sensitivity of MS/MS experiments in the ion trap mass spectrometer. Both ESI and APCI were equally employed for the ionization of steroid glucuronide conjugates, and the main reasons for inconclusive results were observed to relate to chromatographic issues. Based on the results in Table 12, the developed method is transferable to other laboratories when triple quadrupole mass spectrometers are used.

***Table 12**. Interlaboratory test: Summary of instrumental parameters and detected compounds*

	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7
Column	Zorbax CN 2.1x150mm	Luna CN endcapped 1.0x150mm	Luna CN 2.0x100mm	Zorbax CN 2.1x150mm	Zorbax CN 2.1x150mm	Zorbax CN 2.1x150mm	Zorbax CN 2.1x150mm
MS	Triple Quadrupole	Triple Quadrupole	Triple Quadrupole	Triple Quadrupole	Triple Quadrupole	Ion Trap	Triple Quadrupole
Ionization	ESI (350°C)	ESI (350°C)	ESI (350°C)	ESI (270°C)	APCI (400°C)	ESI (240°C)	ESI (350°C)
Collision gas	Argon	Nitrogen	Argon	Argon	Nitrogen	Helium	Nitrogen
Ion transitions	2	3	3	3	3	2	3
Metabolite	**Identification points (max 18)**						
5α-NG	13	18	18	0	18	0	18
5α-MTG	0	0	18	18	0	0	0
5β-MTG	0	18	18	18	18	0	18
5α-MEG	12	18	18	18	18	18	18

4.4 Development of GC–μAPPI-MS/MS method for AAS

Gas chromatography–mass spectrometry with electron ionization is still widely used in the analysis of AAS. GC provides good chromatographic resolution, but the relative abundance of the molecular ion may be low and the analytes are strongly fragmented in the electron ionization process, which decreases the overall sensitivity. GC can also be connected to MS with softer ionization techniques operating at atmospheric pressure. A heated nebulizer (HN) microchip offers feasible interfacing of GC to API-MS. The HN microchip using APCI (μAPCI) or APPI (μAPPI) provides high ionization efficiency and produces abundant protonated molecules or molecular ions with minimal fragmentation. GC–μAPPI-MS/MS method was developed for the determination of selected anabolic steroids in human urine. The method was validated, and the proof of principle was tested with two authentic excretion urine samples, and the results were compared with those obtained with conventional GC–EI-MS.

4.4.1 Combination of GC to API-MS with HN microchip using APPI

The positioning of the microchip and the krypton discharge lamp were the most critical parameters, but the flow rates of the dopant and auxiliary gas also influenced the ionization efficiency. All the parameters were optimized to achieve maximum sensitivity and stability. The microchip nozzle was set at the right side of the orifice and the narrow sample jet was directed about 1 mm left from the orifice to maximize stability and to minimize background interference (Figure 6). The optimal position of the HN microchip nozzle was about 10-13 mm from the MS orifice for derivatized steroids but much shorter for underivatized steroids (4.9 mm). Shorter distances generally decreased stability, whereas longer distances decreased sensitivity but favored the formation of a radical cation. The vacuum UV lamp was placed perpendicularly to the nozzle of the HN microchip and as close to the vaporized sample jet as possible to achieve maximum ionization efficiency. Longer distance between the UV lamp and the jet decreases the intensity of radiation due to absorption of photons by air and resulting in decreased ionization efficiency.

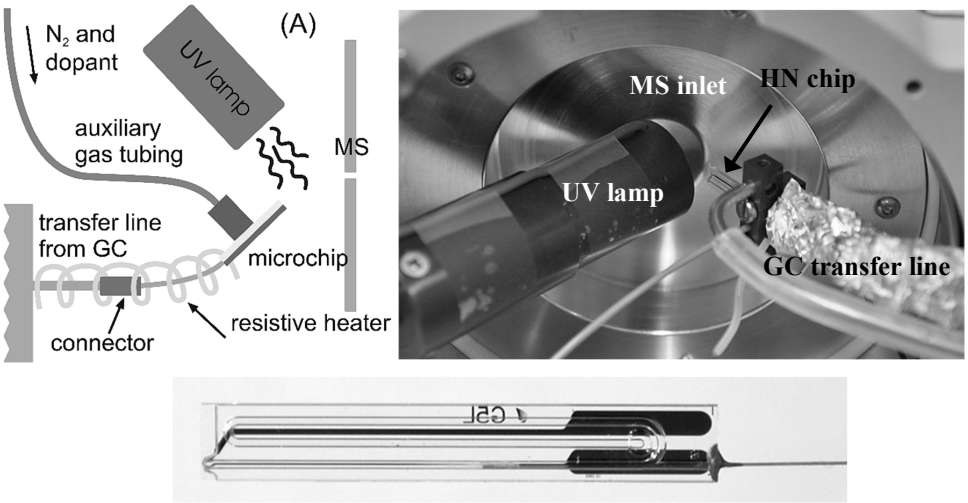


Figure 6. *Schematic (left) and picture (right) of the uAPPI setup and enlarged picture of HN chip (below).*

Sensitivity and stability were optimal with dopant and nebulizer gas flow rates of 3.5 µL/min and 80 mL/min, respectively. The heating power of the nebulizer chip was adjusted to result in temperatures at approximately 300°C (underivatized) or 350 °C (derivatized) in the chip to prevent adsorption and peak broadening. Toluene was chosen as dopant for underivatized steroids, since it enables efficient proton transfer reaction for high proton affinity steroids, and efficient ionization via charge exchange reaction for low proton affinity steroids, which cannot be ionized by proton transfer. On the other hand, chlorobenzene was the best dopant for derivatized steroids, as the ionization energies of TMS-steroids are well below that of chlorobenzene, resulting in efficient charge exchange reactions.

4.4.2 Mass spectrometry

The underivatized AAS are most likely ionized via proton transfer reaction whereas charge-exchange reactions become more favorable when AAS are trimethylsilylated. Positive ion APPI spectra of the underivatized steroids with both carbonyl and hydroxyl groups (NAN, NANm, DNZm, 17MDN, and MTS) showed an abundant $[M+H]^+$ ion, whereas the steroids possessing only hydroxyl functionality (MDNm and MTm) showed $[M-H]^+$ ions and intense radical cations ($M^{+\bullet}$), as well as fragments formed by the loss of one or two water molecules (Table 13). In compounds with a carbonyl group, the proton affinity (PA) is high enough for the proton transfer, but for the compounds possessing only hydroxyl functionality this route is not possible. Abundant $[M-H]^+$ ion was formed from MDNm and MTm most likely via hydride abstraction but oxidation of a hydroxyl group to a carbonyl group followed by proton transfer reaction is also possible.

Table 13. *µAPPI mass spectra of underivatized anabolic androgenic steroids; m/z (rel.abund.).*

	$[M+H]^+$	$M^{+\bullet}$	$[M-H]^+$	$[M+H-H_2O]^+$	$[M-H_2O]^+$	$[M-H-H_2O]^+$	$[M+H-2\cdot H_2O]^+$	$[M-2\cdot H_2O]^+$
NANm	277 (100)			259 (76)				
MDNm		304 (47)	303 (97)	287 (45)	286 (100)	285 (98)	269 (79)	268 (34)
MTm		306 (35)	305 (83)	289 (64)	288 (100)	287 (38)	271 (83)	270 (30)
NAN	275 (100)							
MTS	303 (100)							
DNZm	313 (100)							
17MDN	301 (100)			283 (23)				

The APPI–MS spectra of the TMS steroids with chlorobenzene as dopant show an abundant radical cation ($M^{+\bullet}$), which was the base peak for all compounds except MDNm-TMS and MTm-TMS (Figure 7). For MDNm-TMS the most abundant peak was $[M-H]^+$ at *m/z* 447, while for MTm-TMS it was $[M-15]^+$ at *m/z* 435. The APPI spectra of NANm-TMS and MTm-TMS showed, in addition to the radical cation, a strong protonated molecule ($[M+H]^+$) after subtraction of the theoretical relative abundance of the M+1 isotope peak of the $M^{+\bullet}$ ion. With toluene and chlorobenzene as dopants, the APPI spectra were closely similar except for the relative abundances of protonated molecules - the abundance of the

protonated molecule being somewhat higher with toluene than with chlorobenzene. The results show that TMS-derivatized AAS favor charge exchange reaction over proton transfer, which is the predominant ionization reaction of underivatized AAS. Charge exchange will take place if the ionization energy (IE) of the analyte is lower than that of the reactant ion, which can be the radical cation of a dopant. The ionization energies of intact TES, NAN, and MTS are reported to be 8.95, 8.85, and 9.02 eV, respectively [224]. Since the ionization energies of intact AAS are about the same as those of toluene (8.8 eV) and chlorobenzene (9.07 eV), intact AAS are not ionized efficiently via charge exchange but via energetically more favorable proton transfer. Trimethylsilylation of the hydroxyl and carbonyl groups of AAS results in less efficient proton transfer reactions than for intact AAS because the proton affinities of the ether moieties of the TMS derivatives are lower than those of the hydroxyl and carbonyl groups of the corresponding intact AAS. In addition to this, the TMS group causes steric hindrance for the protonation of the ether oxygen. For these reasons, charge exchange reactions become more favorable than proton transfer reactions when AAS are derivatized with TMS, and ionization energies of the derivatized AAS are well below the IE of chlorobenzene. An advantage of the TMS derivatives of AAS being ionized via charge exchange reaction instead of proton transfer is that selectivity is expected to improve when molecular ion $M^{+\cdot}$ is formed. That is because in APPI, most of the compounds (also interfering back-ground compounds) are ionized via proton transfer reaction appearing at odd mass numbers, whereas molecular ions often appear at even mass numbers. Sensitivity is also enhanced compared to EI, as the soft ionization in atmospheric pressure results in more intensive molecular ion.

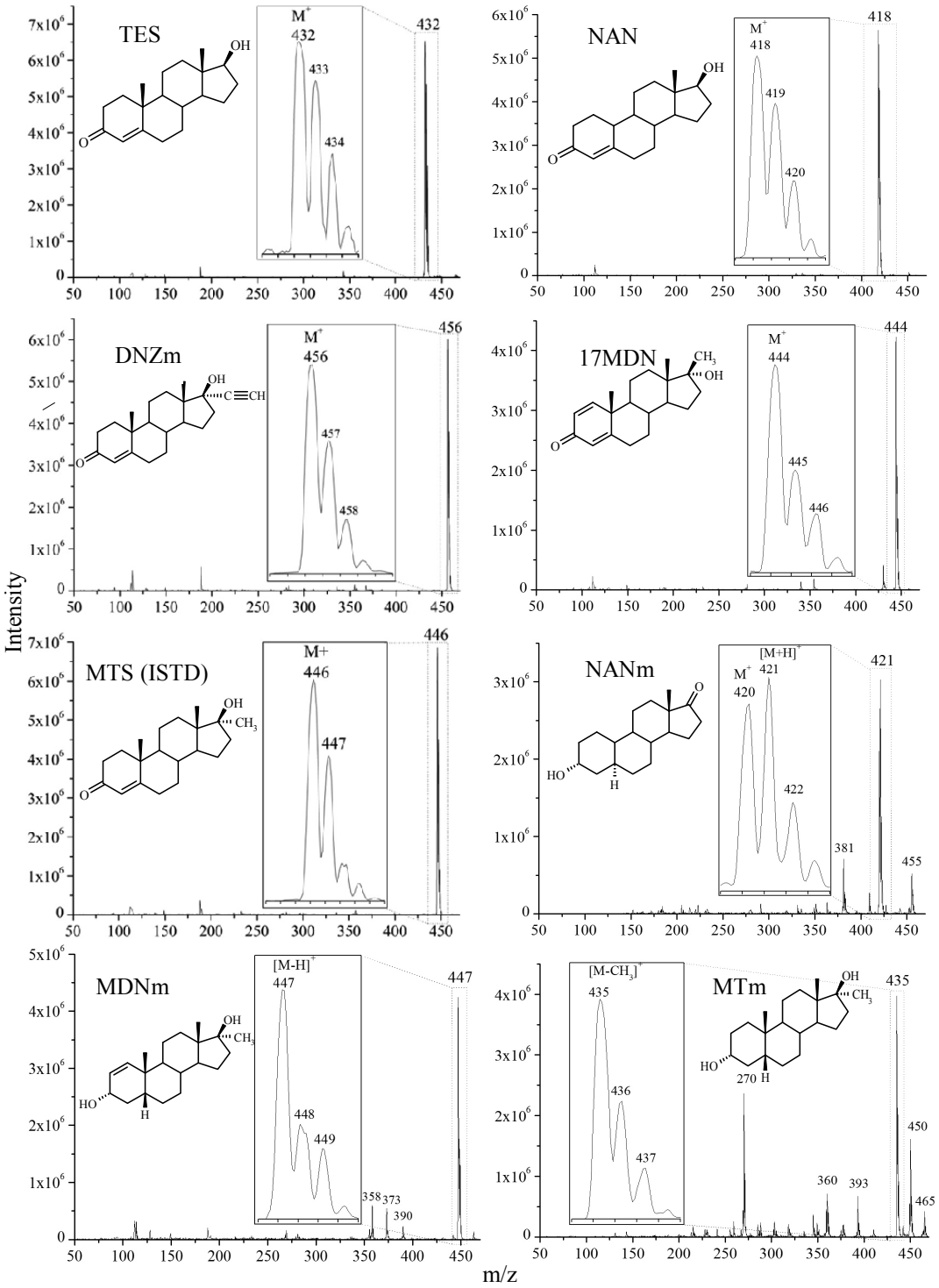


Figure 7. *APPI–MS spectra of the TMS derivatives of the studied AAS.*

The most abundant ion(s) were selected as precursor ion for the GC–μAPPI-MS/MS analysis (Table 14). Orifice (declustering), focusing, and entrance potentials were optimized to maximize the intensity of potential precursor ions, and they were fairly similar for all compounds: namely 20, 200, and 10 V for the underivatized and 10–20, 175, and 4 V for derivatized AAS, respectively. Loss of one or two water molecules was observed in all the MS/MS spectra of the underivatized AAS with numerous structure-specific product ions. The fragmentation patterns of the protonated molecules of DNZm and MTS, which have 4-ene-3-one structure and angular methyl group (in C19) showed the same features: the product ions at m/z 109 and m/z 97 were characteristic and the protonated molecule was relatively stable. A detailed fragmentation mechanism for these ions has been presented earlier by Williams et al. [222]. The protonated molecule of NAN, with 4-ene-3-one structure but without a methyl group at C10, was also relatively stable, and the MS/MS spectra showed an abundant ion at m/z 109. The product ion spectrum of 17MDN was exceptional as it did not show an abundant ion at either m/z 97 or m/z 109 even though it contains both 4-ene-3-one structure and angular methyl group (C-19). The most abundant fragments for 17MDN were m/z 121 and m/z 149, the structures of which have been presented earlier [225] and [226]. The $[M-H]^+$ ion of the steroids containing 3α-hydroxyl group (MDNm and MTm) instead of 4-ene-3-one structure was relatively unstable and the product ions spectra showed abundant ions formed by loss of one or two water molecules. In addition, numerous product ions were observed, originating from the dissociation of the core ring structure.

In the MS/MS spectra of derivatized steroids, product ions formed by the loss of TMS groups were the most abundant ions in the spectra of the TMS ether derivatives (MDNm, NANm, and MTm) and structure-specific product ions formed by the fragmentation of the steroid ring structure were the most abundant ions in the spectra of the TMS 3,5-dienol ether derivatives (TES, NAN, DNZm, 17MDN, and MTS) (Table 14 and Figure 8). The fragmentation characteristics of the TMS derivatives of AAS in EI have been discussed earlier in more detail [202,227]. The fragmentation of the odd-electron molecular ions of TMS derivatized steroids in MS/MS is similar to EI, which could allow for theuse of EI spectral libraries to support the identification of unknown TMS derivatized steroids [176].

Table 14. *Precursor (Q1) and product ions (Q3) with corresponding collision energies (CE) monitored in GC–μAPPI-MS/MS after derivatization (TMS) or without derivatization (non-TMS).*

TMS									
AAS	**Q1**		**Q3**	**CE**	**AAS**	**Q1**		**Q3**	**CE**
TES	432	$M^{+\cdot}$	209	30	**MTS**	446	$M^{+\cdot}$	301	30
	432	$M^{+\cdot}$	196	30	**(ISTD)**	446	$M^{+\cdot}$	169	50
NAN	418	$M^{+\cdot}$	182	35	**NANm**	421	$[M+H]^+$	241	25
	418	$M^{+\cdot}$	194	40		421	$[M+H]^+$	145	35
DNZm	456	$M^{+\cdot}$	208	45	**MDNm**	447	$[M-H]^+$	159	40
	456	$M^{+\cdot}$	316	35		447	$[M-H]^+$	215	30
17MDN	444	$M^{+\cdot}$	206	30	**MTm**	435	$[M-CH_3]^+$	255	30
	444	$M^{+\cdot}$	339	30		435	$[M-CH_3]^+$	173	40
non-TMS									
AAS	**Q1**		**Q3**	**CE**	**AAS**	**Q1**		**Q3**	**CE**
NANm	277	$[M+H]^+$	241	20	**MTS**	303	$[M+H]^+$	109	37
	277	$[M+H]^+$	259	12		303	$[M+H]^+$	285	24
	277	$[M+H]^+$	145	30		303	$[M+H]^+$	97	30
MDNm	303	$[M-1]^+$	201	25	**DNZm**	313	$[M+H]^+$	109	37
	286	$M^{+\cdot}$-H_2O	228	15		313	$[M+H]^+$	295	22
	286	$M^{+\cdot}$-H_2O	150	15		313	$[M+H]^+$	97	33
MTm	305	$[M-1]^+$	269	18	**17MDN**	301	$[M+H]^+$	121	30
	306	$M^{+\cdot}$	230	15		301	$[M+H]^+$	283	17
	306	$M^{+\cdot}$	270	15		301	$[M+H]^+$	149	23
NAN	275	$[M+H]^+$	109	37					
	275	$[M+H]^+$	257	24					
	275	$[M+H]^+$	239	25					

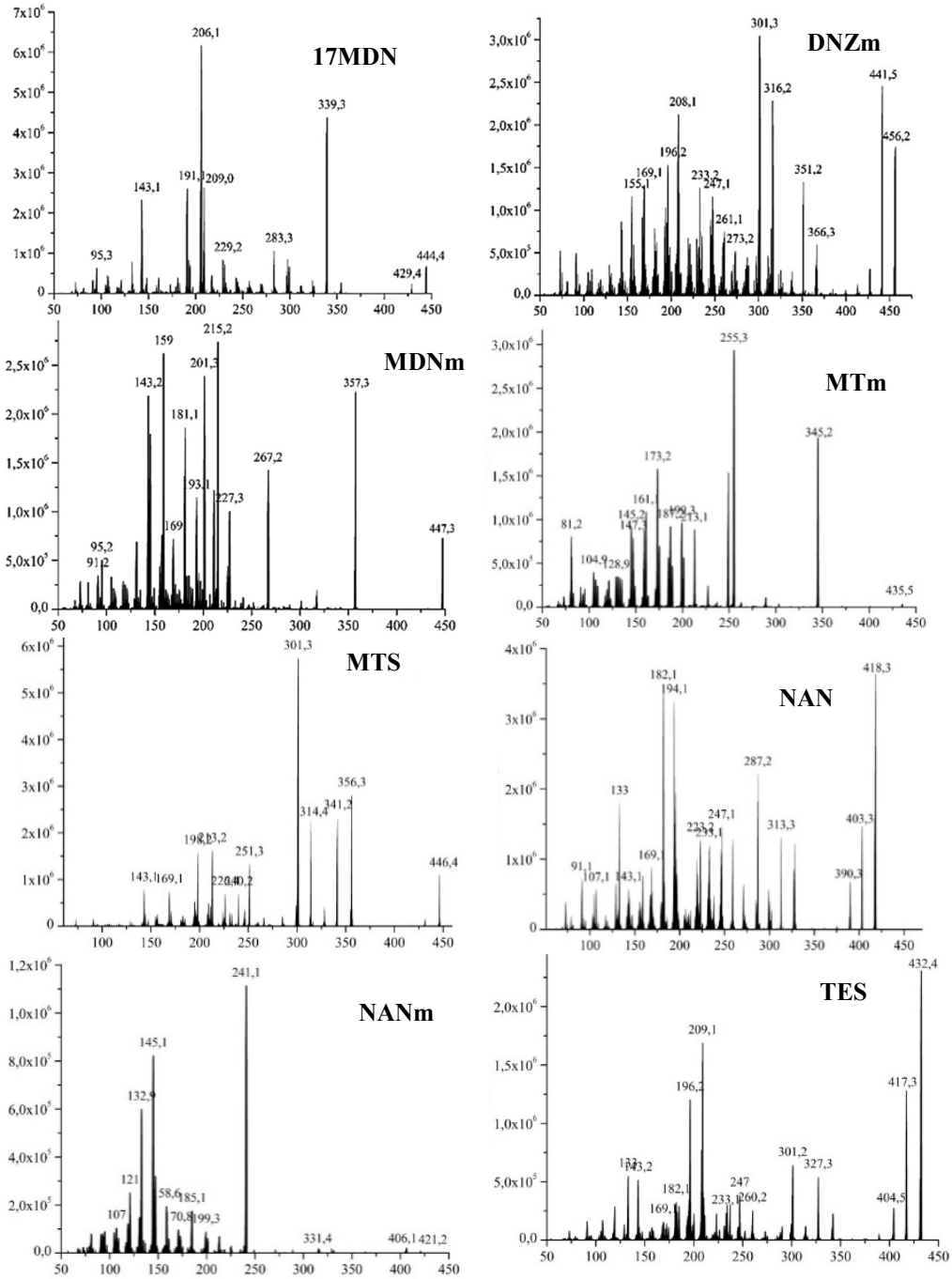


Figure 8. *APPI-MS/MS spectra of the TMS derivatives of the studied anabolic androgenic steroids. CE = 35 V (y-axis Intensity (cps), x-axis m/z)*

4.4.3 Feasibility of GC–μAPPI-MS/MS in analysis of AAS

The GC temperature program in both methods was optimized for fast separation. All the analytes eluted between 4.3 and 6.0 min (derivatized) and 6.4-7.8 min (underivatized). In general, the retention times were proportional to the polar moieties in the chemical structure of the steroid and the size of the molecule. The steroids without a conjugated double bond eluted first and then the steroids with 4-ene-3-one structure (III) The similar elution order was observed with derivatized steroids (IV). TMS ether derivatives (MDNm, NANm, and MTm) eluted first and then the TMS 3,5-dienol ether derivatives (NAN, 17MDN, TES, MTS, and DNZm) (Fig. 2), indicating the later elution of compounds with 3,5-diene structure. The peaks for derivatized steroids were narrow and it could be concluded that the dead volumes of the interface were minimal and adsorption onto the vaporization channel of the chip insignificant (Figure 9).

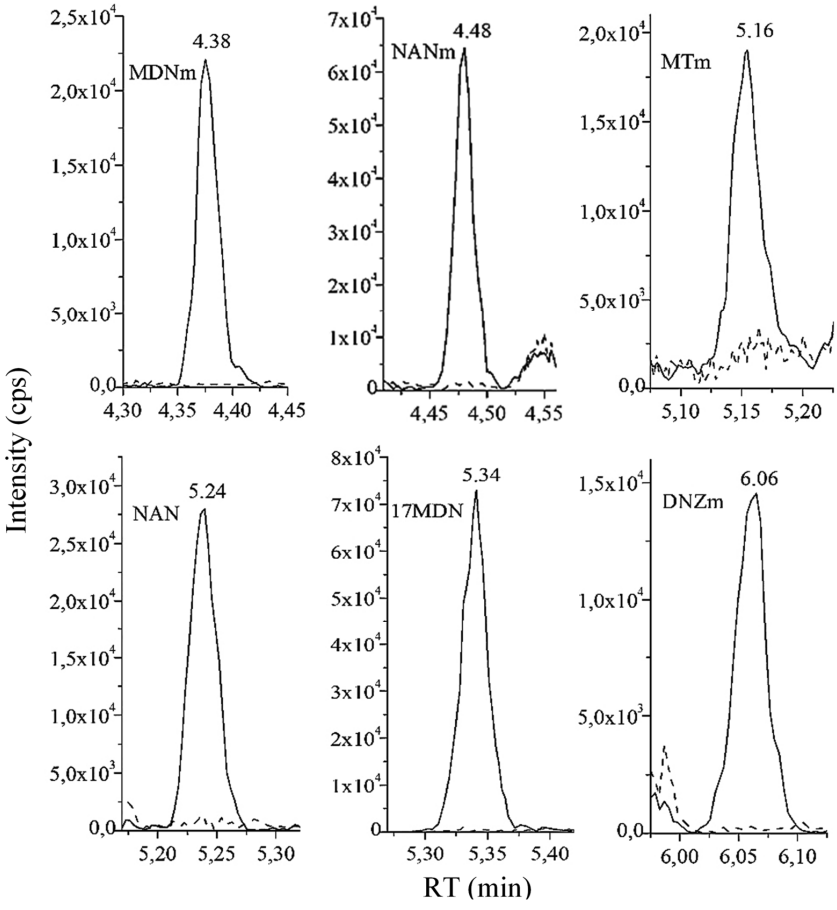


Figure 9. *SRM chromatograms of a derivatized urine sample spiked with AAS at 1 ng/ml (MTm 2 ng/ml) (solid line) and urine blank (dotted line).*

In doping analysis, qualitative determination is sufficient for exogenous anabolic steroids, but quantification is required for steroid profiling of endogenous AAS. The feasibility of GC–μAPPI–MS/MS in quantitative analysis of anabolic steroids in urine was therefore validated with respect to LOD, LOQ, linearity, linear range, and repeatability. The specificity of the method was good, since no interfering peaks were observed in the chromatograms of the urine blank sample pooled from five individuals (Figure 9). The method was repeatable and linear in the range between LOD-100 ng/mL (derivatized compounds) and LOD-250 ng/ml (underivatized compounds), with correlation coefficients (R^2) above 0.996 for all compounds. The repeatability of injection and within-day repeatability were similar and acceptable [228] in both methods (RSD under 10% for all compounds). LODs determined with a signal-to-noise ratio criterion of three, using standard deviation of the analyte peak height vs. background noise, were 0.2-1 ng/mL for underivatized steroids and 0.05-0.5 ng/mL for derivatized steroids (Table 15). The sensitivity is 2–10 times better with TMS-derivatized steroids than for underivatized analytes, and the LOQs of 1 ng/mL were determined for derivatized anabolic steroids. The difference is most likely because the TMS-derivatized steroids are more efficiently ionized by charge exchange reaction than non-derivatized steroids by proton transfer reaction and the background interference is lower with odd number radical cations. However, both methods fulfil the current criteria of the minimum required performance levels of 2 or 5 ng/mL set by WADA [80].

Table 15. *Repeatability of injection (n=6) for derivatized (TMS) at 10 ng/mL and non-derivatized (non-TMS) at 25 ng/mL, LOD (ng/mL), peak symmetry factors (f_s, at 5 ng/mL), and peak widths at half height* W_h *(s, at 5 ng/mL) of the GC–μAPPI-MS/MS methods for AAS.*

Compound	Repeatability (RSD%)		LOD (ng/mL)		f_s (5 ng/mL)		W_h (s) (5 ng/mL)	
	TMS 10 ng/mL	non-TMS 25 ng/mL	TMS	non-TMS	TMS	non-TMS	*TMS*	*non-TMS*
MDNm	**3.7**	9.3	**0.1**	1.0	1.24	**1.10**	**1.22**	1.70
NANm	4.3	**3.2**	**0.1**	0.2	1.14	**0.95**	**1.22**	1.34
MTm	**2.8**	9.4	**0.5**	0.5	1.16	**1.07**	1.44	**0.85**
NAN	4.8	**4.2**	**0.05**	0.2	**1.13**	1.81	**1.49**	1.70
17MDN	5.4	**2.5**	**0.1**	0.3	**0.94**	1.36	**1.43**	1.63
DNZm	3.5	**2.7**	**0.05**	0.3	**1.19**	0.74	1.63	**1.47**

Quantitative performance of the GC–μAPPI-MS/MS method was assessed by analyzing two excretion urine samples and by comparing the results to those obtained by a conventional GC–EI-MS method. Three metabolites of methandienone (17MDN, MTm, and MDNm) were identified and quantified from two authentic urine samples collected from two voluntary male subjects after a single dose of methandione. Considering the qualitative nature of routine analysis methods, the results obtained by both GC–μAPPI-MS/MS methods and the conventional GC–EI-MS method were in good agreement for all three metabolites (Table 16).

Table 16. *Concentrations of metabolites of methandienone in two authentic urine samples measured by GC–EI-MS and GC–μAPPI-MS/MS after derivatization (TMS) or without derivatization (non-TMS).*

Compound	Sample 1 (ng/mL)			Sample 2 (ng/mL)		
	EI TMS	μAPPI TMS	μAPPI non-TMS	EI TMS	μAPPI TMS	μAPPI non-TMS
17MDN	303	309	328	33	34	32
MDNm	388	331	579	86	105	151
MTm	373	309	461	171	161	212

5 CONCLUSIONS

With respect to routine doping control analysis, LC–MS/MS offers a direct and sensitive approach to the analysis of exogenous steroids as their glucuronides without a hydrolysis process and time-consuming sample preparation that conventional GC–MS methods require. The ever-increasing number of prohibited substances with various physico-chemical properties challenges laboratories to revise their analytical strategies to maintain sensitive and cost-effective screening and confirmation methods. Implementation of LC–MS-based methods with various instrument set-ups has become routine, thus allowing the acquisition of data on glucuronide-conjugated prohibited compounds to support and complement the traditional GC–MS data. This might be especially advantageous in the analysis of steroids with completely saturated structures that have low proton affinity and for which the detection as aglycones is difficult with LC–MS.

One significant drawback of the conjugate analysis is the lack of reference material. The *in vitro* enzyme-assisted synthesis method offers a practical pathway for the rapid production of stereochemically pure AAS glucuronides in milligram amounts that are sufficient for the development of analytical methods. Due to a relatively simple reaction mixture and only minor differences between the optimal conditions for the various AAS substrates, the addition of a new structural analogue to the test set should be straightforward and thus also easily applied.

The intact AAS glucuronides can be ionized in both positive and negative ion ESI. The most representative mass spectrometric information on the AAS glucuronide structure was obtained from MS/MS fragmentation studies in positive ion ESI, which is also the method of choice for the routine LC–MS/MS analysis of AAS glucuronides. Glucuronide-conjugated steroid metabolites were extracted by SPE and analyzed by LC–ESI-MS/MS using positive ion mode and selected reaction monitoring. The developed method showed robust sensitivity, quantitative performance, and repeatability, and the inter-laboratory comparison demonstrated that it is transferable to other laboratories equipped with triple-stage quadrupole mass spectrometers.

The resolving power of gas chromatography combined with efficient, but soft, ionization in atmospheric pressure without fragmentation is an attractive combination. The heated nebulizer microchip operated in APPI mode provides efficient ionization of anabolic steroids and a feasible option to couple GC to API-MS. Two sensitive and selective GC–μAPPI-MS/MS methods were developed, validated, and successfully applied to the analysis of anabolic steroids in authentic urine samples. The anabolic steroids were analyzed from urine samples without derivatization or as their TMS derivatives. Both methods showed sensitivity and quantitative performance, demonstrating their potential for the

analysis of biological samples. The TMS derivatives were ionized efficiently via charge exchange reactions resulting in 2-10 times better sensitivity compared to non-derivatized steroids, which were ionized mainly by proton transfer reaction. Furthermore, the fragmentation of the molecular ions of the TMS derivatized steroids in μAPPI-MS/MS is similar to EI, which facilitates the use of EI spectral libraries in the identification of TMS derivatized steroids. The advantage of GC–μAPPI-MS/MS is soft and efficient ionization that produces abundant molecular ions or protonated molecules with only slight fragmentation, ensuring selectivity and sensitivity. Although the results were promising, the usability of the heated nebulizer microchip requires further optimization. System assembly is challenging and it is sensitive to small changes in positioning of the chip and lamp in relation to the MS inlet. The open ion source design may result in repeatability problems as the gases and particles from the surrounding air may enter the ionization region. Novel high sensitivity capillary APPI (cAPPI) could resolve this problem. In cAPPI, the sample is introduced, vaporized, and photoionized inside the extended heated capillary. The surrounding air does not interfere in the ionization process and transmission into MS is maximized resulting in excellent overall sensitivity [158].

Despite the extensive LC–MS/MS method development described here, some compounds were not sufficiently resolved from background interference. Enhanced sensitivity and selectivity is required from the analytical methods due to the similarity of the AAS metabolites and the multiplicity of endogenous compounds in the urine samples. Several compounds can possess identical or similar ion transitions in SRM and the unit resolution resolving power of tandem mass spectrometry is not always sufficient. Also, given the similar chromatographic properties of AAS glucuronides, efficient chromatographic separation is required. Enhanced chromatographic resolution, for example, with UPLC and/or detection with high resolution mass spectrometry (HRMS) are promising techniques for doping control analysis [114,137,211,229]. Furthermore, the full scan measurements in HRMSs offer a retrospective option to re-process the raw data, which is a major advantage in anti-doping, e.g. in case of new designer steroids. Untargeted profiling is an efficient tool for doping analysis, as it provides the possibility of monitoring a large number of analytes in a single experiment with high sensitivity and specificity. The evolution of mass spectrometers has generated a multiplicity of techniques readily available for steroid analytics. The choice of the most suitable analyzer together with the selection of matrix, purification steps and separation methods enables efficient control of AAS misuse.

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