Investigations on Phase I Metabolism of Anabolic Androgenic Steroids and Its Influenceability as Tool to Refine Steroid Detection and Evaluation

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Anna Stoll
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Research of the present study was conducted from 2016 till 2020 under supervision of Prof. Dr. Maria Kristina Parr at the Institute of Pharmacy of the Freie Universität Berlin in collaboration with the Laboratorio Anti-Doping FMSI, Rome.

1st Reviewer: Prof. Dr. Maria Kristina Parr
2nd Reviewer: Prof. Dr. Francesco Botrè

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L'acte le plus courageux reste de penser par vous-même. À haute voix.

- Coco Chanel -

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I ABBREVIATIONS

Steroids

***Table 1**: Names, abbreviations and numbers of steroids mentioned in this work*

Trivial name	Chemical name	Abbreviation	Number
Cholesterol	Cholest-5-en-3β-ol	-	1
Pregnenolone	3β-Hydroxypregn-5-en-20-one	-	2
Progesterone	Pregn-4-ene-3,20-dione	-	3
17α-OH-pregnenolone	3β,17α-Dihydroxypregn-5-en-20-one	-	4
17α-OH-progesterone	17α-Hydroxy-pregn-4-ene-3,20-dione	-	5
Dehydroepiandrosterone	3β-Hydroxyandrost-5-en-17-one	DHEA	6
Androstenedione	Androst-4-ene-3,17-dione	AED	7
Androstenediol	Androst-5-ene-3β,17β-diol	-	8
Testosterone	17β-Hydroxyandrost-4-en-3-one	T	9
5α-Dihydrotestosterone	17β-Hydroxy-5α-androstan-3-one	5αDHT	10
Estrone	3-Hydroxyestra-1,3,5(10)-trien-17-one	E1	11
Estradiol	Estra-1,3,5(10)-triene-3,17β-diol	E2	12
Estriol	Estra-1,3,5(10)-triene-3,16α,17β-triol	E3	13
5α-Androstanediol	5α-Androstane-3α,17β-diol	5αAdiol	14
Androsterone	3α-Hydroxy-5α-androstan-17-one	A	15
5α-Androstanedione	5α-Androstane-3,17-dione	5αAD	16
5β-Dihydrotestosterone	17β-Hydroxy-5β-androstan-3-one	5βDHT	17
5β-Androstanediol	5β-Androstane-3α,17β-diol	5βAdiol	18
Etiocholanolone	3α-Hydroxy-5β-androstan-17-one	Etio	19
5β-Androstanedione	5β-Androstane-3,17-dione	5βAD	20
-	4-Chloro-17β-hydroxymethyl-17α-methyl-18-norandrosta-1,4,13-trien-3-one	20βOH-NorDHCMT	21
Oral-Turinabol	4-Chloro-17β-hydroxy-17α-methylandrosta-1,4-dien-3-one	OT; DHCMT	22
-	4-Chloro-17,17-dimethyl-18-norandrosta-1,4,13-trien-3-one	NorDHCMT	-

Other abbreviations

3D	3-Dimensional
3R	Reduce, Replace, Refine
AAF	Adverse analytical finding
AAS	Anabolic androgenic steroids
ABP	Athlete Biological Passport
ACE	Angiotensin-converting enzyme
ADAMS	Anti-Doping Administration and Management System
AKR	Aldo-keto reductase
APF	Adverse Passport Finding
Arg	Arginine
ASS	Acetylsalicylic acid
AT1	Angiotensin II receptor subtype 1
C18, C19 or C21	Structure with 18, 19 or 21 carbon atoms
C-3, C-17 or C-21	Carbon number 3, 17 or 21 in steroid (Figure 1)
CCB	Calcium channel blocker
COX	Cyclooxygenase
CYP	Cytochrome P450-enzyme
EAAS	Endogenous anabolic androgenic steroids
ED	Endocrine disruptor
EU	European Union
EURL ECVAM	European Union Reference Laboratory for alternatives to animal testing
GC	Gas chromatography
GC-C-IRMS	Gas chromatography-combustion-isotope ratio mass spectrometry
GC-MS	Gas chromatography-mass spectrometry
GC-MS/MS	Gas chromatography-tandem mass spectrometry
GC-QQQ	Gas chromatography-triple quadrupole mass spectrometry
GC-q-ToF	Gas chromatography-time-of-flight-mass spectrometry
hCG	Human chorionic gonadotropin
HLM	Human liver microsomes
HRMS	High-resolution mass spectrometry
HSD	Hydroxysteroid-dehydrogenase
ISL	International Standard for Laboratories
Iso rh enzyme	Isolated recombinant human enzyme
ISTI	International Standard for Testing and Investigations
JRC	Joint Research Centre
LC	Liquid chromatography

LC-MS	Liquid chromatography-mass spectrometry
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
MSTFA	N-methyl-N-(trimethylsilyl)-trifluoroacetamide
NH_4I	Ammonium iodide
NSAID	Non-steroidal anti-inflammatory drug
OECD	Organization for Economic Co-operation and Development
OTC	Over the couter
PPI	Proton pump inhibitor
QQQ	Triple quadrupole mass spectrometry
S.pombe	*Schizosaccharomyces pombe*
S9	9000 *g* Supernatant; detailed explanation on page 18 (Chapter 2.2.2.)
SDR	Short-chain dehydrogenase/reductase
SSP	Suspicious steroid profile
TA	Testing authority
TD	Technical document
UDP	Uridine diphosphate
WADA	World Anti-Doping Agency
Whole-cell BT	Whole-cell biotransformation
WMA	World Medical Association
Δ1 or Δ4	Double bond in position 1 or 4 of steroid

1 INTRODUCTION AND AIM OF THE PROJECT

The knowledge of metabolism in general and steroid metabolism in particular is of high importance for many research fields. Expertise in this field represents a valuable tool to, among others, develop effective detection methods or straightforwardly interpret analytical results. All areas where steroid detection is of importance hence depend on research providing insights in metabolic processes and their influence on steroid balance in humans. In particular, this work aims to illustrate the significance of metabolic studies in the field of anti-doping analysis exemplarily on the metabolism and investigations on metabolic routes of anabolic androgenic steroids (AAS). This example was chosen, as it comprises several challenges in steroid detection which are in accordance with multiple other disciplines. Subsequently the broader relevance will be discussed.

Detection of steroids in anti-doping analysis is of high importance, to defend the spirit of sport. Besides multiple beneficial physical and psychological aspects of sport, the principle of "higher, faster, further" is and probably has always been a key element especially for meritocratic athletes. Factors like training, nutrition, regeneration, mental strength and equipment are of importance to guarantee the best performance an athlete is able to achieve. Still, the spirit of sport must never be forgotten alongside the endeavour to perform better than others. Even though, the majority of athletes respects the rules of fair play, there are, and again have always been, athletes not following these rules by using certain methods or substances to enhance their performance and to derive an unfair benefit from that. To prevent this acting, in the mid-1960s first substances were banned to be used as doping in sports. In 1999 the World Anti-Doping Agency (WADA) was founded "[…] to promote, coordinate and monitor the fight against doping in sport in all its forms" [1].

In this context AAS, used by athletes as performance enhancing drugs, are by far the most detected substances in anti-doping tests [2]. To detect the use of AAS, concentrations of selected steroids, their metabolites and precursors are measured in urine samples collected from the athletes in and out of competition. Testing procedures have considerably improved during the last years and nowadays are and have to be able to detect trace-amounts of analytes (in the lower ng/mL range) in the collected samples [3]. Nonetheless, a continuous improvement of the applied methods is of paramount importance as not only the methods of the laboratories, but also the methods of the dopers are improving. The use of novel designer steroids, meaning steroids which are designed to complicate detection, pseudo endogenous steroids, meaning endogenous steroids administered exogenously, or very minor doses of substances are only some of the challenges anti-doping laboratories have to master.

The results presented in this thesis are aiming to contribute to overcome these challenges and provide valuable insights not only for anti-doping research but also for other fields where steroid detection and analysis is of importance.

2 THEORETICAL BACKGROUND

2.1 Human steroid hormones and their metabolism

The human endocrine system consists of glands excreting chemical messengers (hormones) which regulate distinct functions in the body. Even if characterized through different chemical structures these hormones all act at very low concentrations. The class of human steroid hormones consist of a lipophilic steran body with different modifications. Based on their structure and function, they can be divided into five major categories: two corticosteroidal groups: mineralo- and glucocorticoids and three groups of sexual hormones: progestagens, estrogens and androgens. They all originate from cholesterol with a cholestane backbone and have different backbone structures themselves, as displayed in Figure 1.

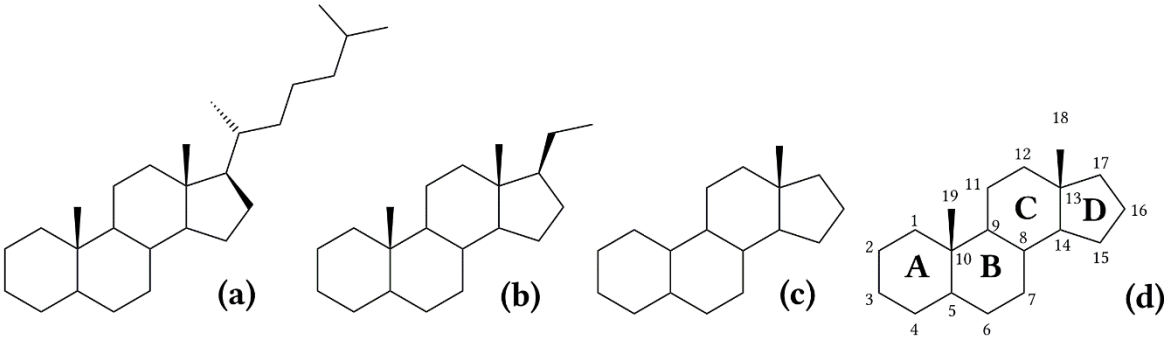

***Figure 1**: Steroid backbone of human steroid hormones and their precursor cholestane (**a**): pregnane (**b**), estrane (**c**) and androstane (**d**) with exemplary numbering of carbon atoms (C1-C19) and rings (A-D)*

Endogenous corticosteroids (mineralo- and glucocorticoids) and progestagens have a pregnane backbone with a C21 structure. Endogenous androgens consist of an androstane backbone with a C19 structure and estrogens have a C18 structure consisting of an estrane base. All of them have cholesterol (structure (**1**) in Figure 2) with a cholestane body as metabolic precursor. The different groups of steroid hormones distinguish themselves not only in their structure and induced effects in the human body but also in their main location of biosynthesis. Besides their principal location of biosynthesis, steroids may be synthesized in minor quantities in other body tissues (e.g. estradiol-formation in postmenopausal women). Mineralo- and glucocorticoids and minor amounts of androgens are produced in the adrenal cortex. Estrogens, progestagens and androgens are mainly produced in the male and female sexual organs. The naturally occurring progestagen progesterone is mainly produced in the corpus luteum and in the placenta of pregnant women. The androgen testosterone is primarily produced in the testis of males and for females in smaller amounts in the ovary and in the adrenal gland [1, 2]. Estrogens in females are mainly synthesized in the ovaries but also in minor amounts in the breast, brain, bone and adipose tissue. In men estrogens are generated from testosterone via aromatization in the testis [4]. The (simplified)

biosynthesis pathway of human steroid hormones from cholesterol without tissue specificity is depicted in Figure 2.

The production of sex hormones is regulated by superordinated hypothalamus hormones via feedback mechanisms [5]. After secretion of sex hormones into the circulation, they bind to transport- or plasma-proteins which function as carrier in the body. These carriers are needed due to the high lipophilicity of steroids. In their target cell, steroid hormones bind to cytosolic steroid receptors, enter the nucleus as steroid-receptor-complex and induce the production of target genes (transcription factors). Due to their lipophilic structure human steroid hormones, as well as synthetic steroids, undergo both, phase I and phase II metabolism before their renal excretion.

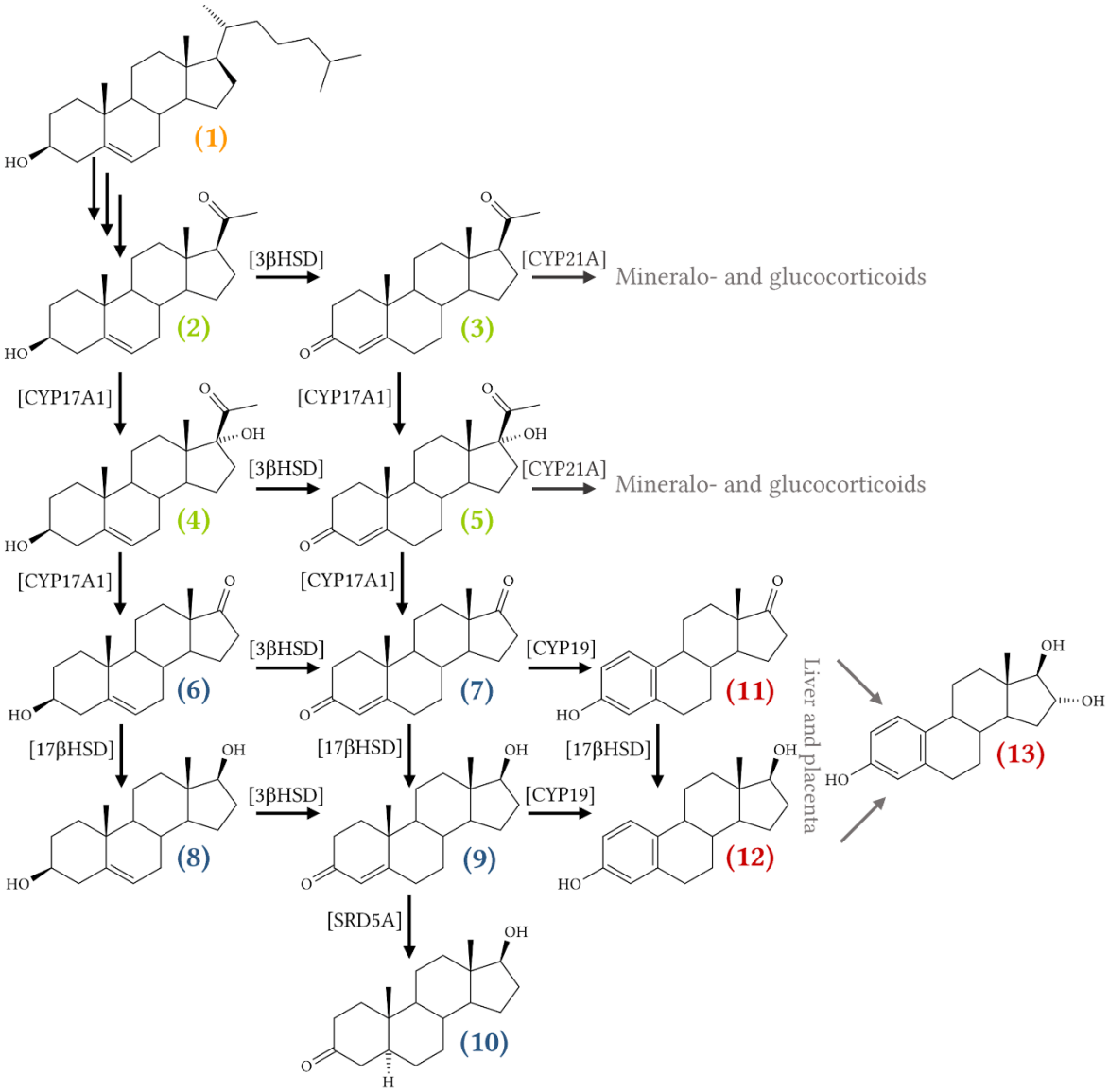

Figure 2: *Simplified biosynthesis of human steroid hormones deriving from cholesterol (**1**). Green numbers indicating progestagens (C21): pregnenolone (**2**), progesterone (**3**), 17α-OH-pregnenolone (**4**) and 17α-OH-progesterone (**5**); Blue numbers indicating androgens (C19): dehydroepiandrosterone (**6**), androstenedione (**7**), androstenediol (**8**), testosterone (**9**) and 5α-dihydrotestosterone (**10**); red numbers indicating estrogens (C18): estrone (**11**), estradiol (**12**) and estriol (**13**). Corresponding enzymes in squared brackets*

2.1.1 Metabolism of androgens

This work investigates predominantly phase I metabolism (reduction, oxidation, hydroxylation and epimerization) of androgens. To briefly mention phase II metabolism, most relevant reactions for androgens are glucuronidation via UDP-glucuronosyltransferases and sulphation via sulfotransferases. Since the complete endogenous steroid metabolism is very complex and involves several tissues, tissue-passages, conjugation and deconjugation steps [6], this chapter wants to give some basic background information on relevant phase I metabolites and their generation in humans. The focus is laid on important structures for this work.

Enzymes involved in phase I metabolism of androgens are cytochrome P450-enzymes (CYPs) and hydroxysteroid-dehydrogenases (HSDs), which can be subdivided into the enzyme super families of short-chain dehydrogenases/reductases (SDRs) and aldo-keto reductases (AKRs) [7].

CYPs mainly catalyse hydroxylations on the steroid backbone which are mostly irreversible. In androgens, they are of relevance in so called minor metabolic pathways, as the excretion of these metabolites into urine (as phase II conjugates) is extremely low [1]. Hence, they do not play an important role in the detection of pseudo endogenous anabolic steroid administration in doping control analysis (later discussed in Chapter 2.3) [3]. CYP enzymes and their minor metabolic pathways are nonetheless important to investigate, as formed metabolites may improve the detectability of synthetic AAS and so-called designer-steroids, meaning steroids designed to complicate detection. This is mostly achieved by structure modifications of already known compounds [8, 9]. Especially in the discovery of new and/or longer detectable metabolites for synthetic AAS, reactions catalysed by CYPs are of relevance [10, 11].

Important HSDs in steroid metabolism are, beside others, 3αHSDs (AKR1C1-AKR1C4), 5α- and 5β-reductase (SDR5A and AKR1D1) and 17βHSDs (AKR1C3) [6]. Selected noteworthy metabolizations catalysed by CYP-enzymes and HSDs are displayed in Figure 3.

In general, exogenous androgenic steroids undergo a comparable metabolism to endogenous steroids.

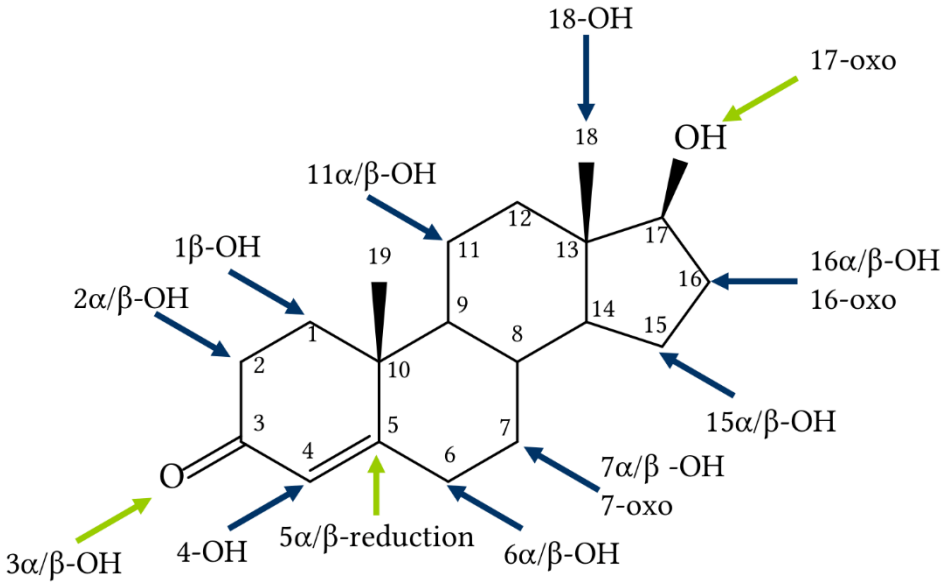

***Figure 3**: Possible phase I androgen metabolizations exemplarily displayed on testosterone (adapted from [6]). Blue arrows indicating modifications mainly catalysed by CYP-enzymes; green arrows indicating modifications mainly catalysed by HSDs. -OH corresponds to possible hydroxylation, -oxo corresponds to oxo-functionalization*

Metabolization by HSDs is of paramount importance for metabolism of endogenous anabolic androgenic steroids (EAAS). Their simplified metabolic pathway is displayed in Figure 4. Focus is laid on steroids playing a key role in anti-doping analysis (discussed in Chapter 2.3) and also in this work (Manuscript III and Manuscript IV).

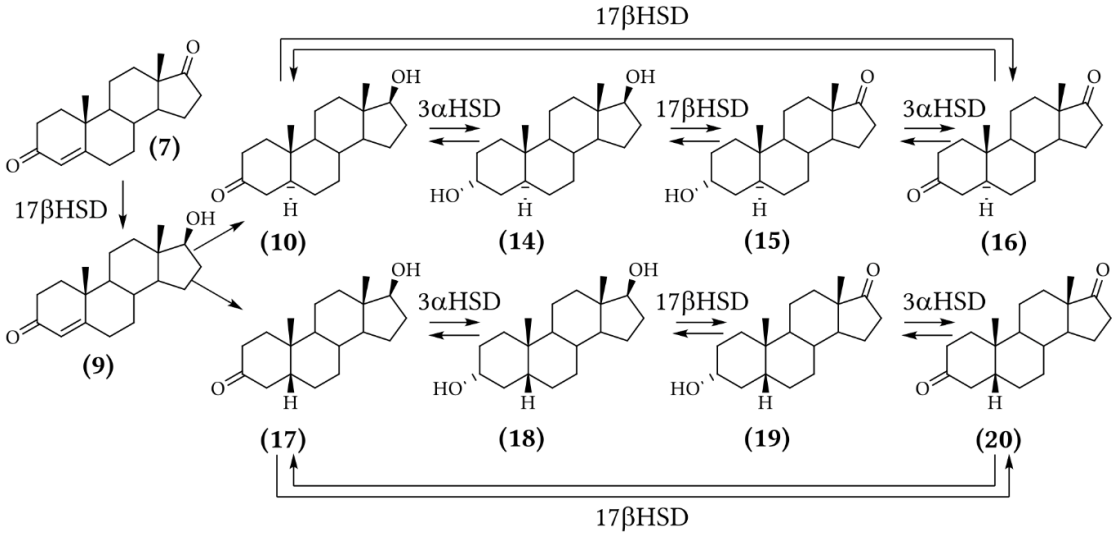

***Figure 4**: Metabolism of endogenous anabolic androgenic steroids (EAAS) with focus on steroids relevant for anti-doping analysis: androstenedione (AED, **7**), testosterone (T, **9**) and 5α-dihydrotestosterone (5αDHT, **10**), 5α-androstanediol (5αAdiol, **14**), androsterone (A, **15**), 5α-androstanedione (5αAD, **16**), 5β-dihydrotestosterone (5βDHT, **17**), 5β-androstanediol (5βAdiol, **18**), etiocholanolone (Etio, **19**) and 5β-androstanedione (5βAD, **20**); with corresponding enzymes indicated next to the arrows*

The male sex hormone testosterone (**9**) is synthesized from its prohormone androstenedione (**7**) and is reduced either by 5α-reductase (mainly located in the endoplasmatic reticulum) or 5β-reductase (mainly located in the cytoplasm) to the corresponding dihydrotestosterone (**10** or **17**). Like a majority of other enzymes, 5α- or 5β-reductases, which play an important role in the biosynthesis of steroids, are not only able to metabolize endogenous steroids but xenobiotics, as well. The structure of the parent steroid herein influences the extend of 5α- or 5β-steroid-formation [12].

2.1.2 Endocrine disruptors

Endocrine disruptors (EDs) are exogenous substances interfering with the endocrine system [13]. They increase the risk of health-related problems (e.g. cancer, metabolic diseases and reproductive impairment) by altering processes involved in the regulation of hormone balance. Exposure with EDs occurs through deliberately or unnoticed contact with these substances in ultimately every situation of daily life. Some examples are the intake over food, pharmaceuticals or drinking water but also leached substances from plastic wrappings or bottles, ingredients of cosmetics and personal care products or toys. As the endocrine system is very complex and hormones act in extremely low concentrations, interfering effects are likely. In an expert consensus statement published in January 2020, La Merrill *et al.* describe 10 key characteristics of EDs [14]. The key characteristics describe points in hormonal generation and their action, where EDs can interfere. One of these key characteristics, which is particularly relevant for metabolism, is the process of "hormone breakdown and clearance". It is characterized by the interference of EDs with metabolic enzymes or mechanisms which play a role in the clearance of hormones in the body. If these processes are inhibited or induced, hormone concentrations are possibly altered and hence their effects in the human body may be changed. As endogenous steroids undergo extensive metabolism, interferences are likely and need further investigation.

Non-steroidal anti-inflammatory drugs as endocrine disruptors

Non-steroidal anti-inflammatory drugs (NSAIDs) have anti-inflammatory, analgetic and mostly anti-pyretic pharmacological effects. Their main therapeutic applications are hence, relieving of mild to moderate pain and inflammatory degenerative diseases like rheumatism or arthrosis. In lower doses (100 mg/day) the NSAID acetylsalicylic acid is also indicated as platelet aggregation inhibitor. Their mode of action can be explained through interference with the prostaglandin synthesis over an inhibition of the cyclooxygenase (COX). While their therapeutic effects as pain killers can be explained through the inhibition of COX2, several adverse drug reactions may mainly be explained by the simultaneous inhibition of COX1 [5].

According to the "Barmer Arzneimittelreport 2020" ibuprofen was the drug prescribed to most patients assured at Barmer health insurance in Germany (Figure 5) [15]. Together with diclofenac

and acetylsalicylic acid there are three NASIDs in the Top 20 drugs prescribed among the assured. In addition, several NSAIDs are available as over the counter (OTC) drugs without prescription in Germany. Hence, it can be assumed that NSAIDs are the most frequently used drugs in the German population. A similar picture is expected for other European and international countries.

It had been demonstrated by several publications that selected substances from the class of NSAIDs show endocrine disrupting effects in different experimental settings [16-23]. These findings are also supported by a publication from Kristensen *et al.* which evaluates the fact that "many putative endocrine disruptors inhibit the prostaglandin synthesis" [24].

Due to their high usage rate and above-mentioned findings from literature, further investigations on the endocrine disrupting potential of NSAIDs and a subsequent re-evaluation of their risk profile may be reasonable.

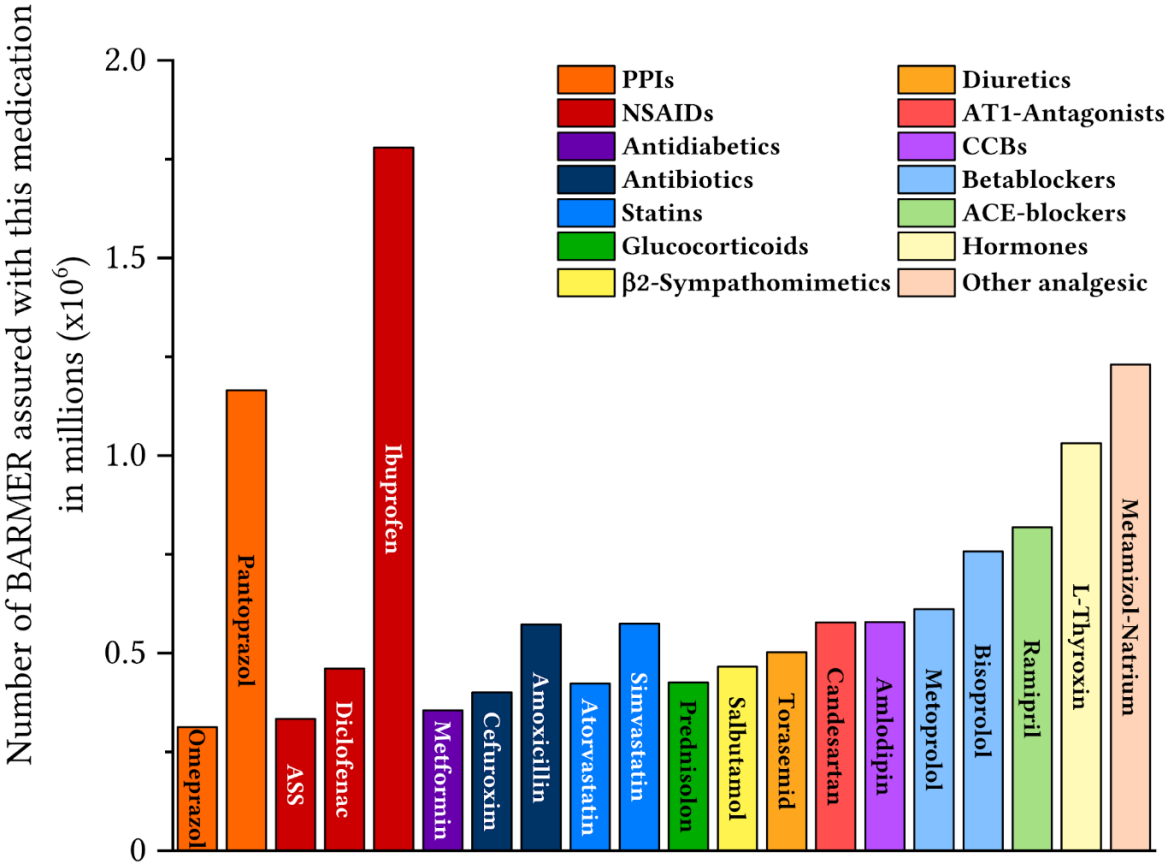

Figure 5: *Top 20 prescribed drugs among BARMER assured in 2019 adapted from [15]. PPI: proton pump inhibitor, NSAID: non-steroidal anti-inflamatory drug; AT1: angiotensin II receptor subtype 1; CCB: calcium channel blocker; ACE: angiotensin-converting-enzyme; ASS: acetylsalicylic acid*

2.2 Metabolic studies

Most anabolic androgenic steroids are metabolized completely in humans and little or no parent compound is detectable in urine, used as analytical matrix in doping control analysis (as discussed in Chapter 2.3). Thus, metabolic studies are important to identify these metabolites and to uncover their formation routes. This knowledge builds the basis for the development of reliable methods to detect and analyse steroids and their metabolites in biological samples [25]

Generally, an evaluation of metabolic processes in humans would be the most enlightening method. However, in anti-doping research, as also for the general field of (forensic) toxicology there are some concerns of metabolic studies in humans. First, the substances themselves are toxic and there are ethical concerns to administer them to a large population of healthy volunteers. This aspect becomes even more relevant for multiple or higher doses applied, which is usually practiced by athletes using performance enhancing substances. In addition, the aspect of multiple drug administration plays an important role in anti-doping research [26, 27]. Hence, *in vitro* and *in silico* studies gain more and more importance in anti-doping research [28]. They can provide a solid and very valuable knowledge basis to plan and perform potential subsequent administration trials. Unfortunately, every model aiming to reproduce human metabolic processes has its drawbacks, which is why their strengths and weaknesses are crucial to consider for data evaluation.

This chapter is giving some background information on possibilities to perform metabolic studies with special focus on *in vitro* and *in vivo* methods relevant in this work.

2.2.1 *In vivo* studies on metabolism

As already mentioned, the most conclusive approach for metabolic studies would be the administration of larger amounts of substances to volunteers, followed by subsequent evaluation of excreted compounds. If metabolism of endogenous substances is of interest, the application of radiolabelled substances or substances labelled with stable isotopes with subsequent analysis of the matrix of interest, can be helpful to evaluate the whole metabolic profile [29]. Even if *in vivo* application trials were performed in the early years of doping control analysis and are still practiced today [9, 11, 25, 30-35], they may raise some critical aspects which should be assessed carefully. Nowadays the WADA research ethics policy, which is applied for all research projects founded by WADA, requests an adequate ethics review for all submitted research proposals [36]. The adherence to the Helsinki Declaration, which is issued by the World Medical Association (WMA) as a statement of ethical principles for medical research involving human subjects [37], is recommended by WADA for "research with international scope" in anti-doping research [36]. However, there are some concerns on applying this declaration in anti-doping research [38]. One example for a critical aspect discussed by Sanchini *et al.* is the fact that the risk of some doping agents (e.g. designer steroids) is not known or even worse, known to be harmful. Nonetheless, their

metabolism is still important to investigate as athletes are using these compounds. Therefore, further investigations on ethics in anti-doping research are necessary, as discussed by Sanchini *et al.* Today, application of drugs to a small group of mostly male and healthy volunteers and retro perspective studies are of relevance in the field.

Application trials on small groups of volunteers are raising two further considerations. First, application trials mostly include only male volunteers, which leaves the question open if and how metabolism would differ in females. Second, trials are performed on small groups of volunteers or even just individual persons. This is on the one hand reasonable due to the potential low or missing information on the health risk of the drug, but on the other hand this study design causes the question of transferability of the results to a larger population. It is known, that the extend of metabolism varies with e.g. age, sex, ethnicity or genetic variations. Furthermore, exogenous factors (e.g. medication, training, consumption of food or dietary supplements) can influence reactions involved in the metabolism. Hence, the excretion and subsequent detection of metabolites can be influenced [39, 40]. The influenceability of metabolic processes is also discussed in more detail in Chapter 2.3.4. Due to these individual variations and the influenceability through exogenous factors, the interpretation of metabolic studies and extrapolation to a large population is challenging and needs very careful evaluation of data.

As mentioned above, detection of new metabolites can also be achieved in retro perspective studies by re-evaluation of already tested samples [41, 42] or from samples collected during observation studies on patients or persons using the compounds of interest [43]. This type of study is giving the opportunity to predefine samples of a specific population (e.g. ethnicity, sex, age). It is furthermore possible to evaluate a rather big number of samples, which do not need to be "generated" from the scratch. Generally, results from retro perspective studies should be treated carefully. This is especially important if samples from uncontrolled trials or patient samples are used. In this case only limited information may be available on the background of the samples. Some factors which may be unknown, but which are relevant for the interpretation of metabolic profiles are concurrent medication of patients, age, sex or dietary habits (Chapter 2.3.4). Another critical point of retro perspective studies is related to samples deriving from an existing sample previously tested positive for a certain compound. Re-evaluation of this sample may reveal misleading results as the metabolites detected may have another origin than the compound they were originally declared as positive for. Hence, evaluation of samples from retro perspective studies shall be performed very carefully and as much as possible background information should be gathered to draw conclusions as precise as possible.

In addition to application trials or retro perspective evaluations on humans the picture of *in vivo* methods shall be concluded by animal experiments. Animal models are mainly used in the early drug development and in toxicological studies to gain data on chemical and metabolic properties and potential risks of a substance, before proceeding with clinical trials on humans.

In anti-doping research there are for example animal studies using baboons [44, 45], rats [46] or zebrafish [47]. All of them having advantages and disadvantages. The difficulty with animal models is, among others, the requirement of an approval from an animal welfare committee and the transferability to humans. Enzymes and non-enzymatic processes involved in biotransformation (e.g. gut metabolome, transport mechanisms, binding of metabolites and excretion mechanisms) in animal models may differ from these in humans. They hence possibly lead to divergent findings [48]. In addition to the already mentioned methods, there are studies on chimeric animals, which can be a promising tool, but still show differences to known human metabolic profiles [49, 50]. In conclusion every (animal) model remains a model and it is important to know the strengths and limitations of this model and to not generalize made findings.

Due to the often-unsatisfying transferability of animal studies to humans and to preserve the animal wellbeing, several research societies, organisations and researchers work on so called 3R (**R**educe, **R**eplace, **R**efine) methods. This is also regulated by the directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes [51]. The goal of 3R method development is to **R**educe the number of animals per test to a minimum while obtaining the same amount of information; **R**eplace as many animal tests as possible with other methods (e.g. *in vitro* or *in silico*) and **R**efine those tests which cannot be replaced, to guarantee the animal welfare. The Organisation for Economic Co-operation and Development (OECD) as international institution and the European Union Reference Laboratory for alternatives to animal testing (EURL ECVAM) as part of the Joint Research Centre (JRC) of the European Commission are just two organizations engaging in this field. This movement is especially important in the field of pharmacology and toxicology and early drug development but may also be transferred to metabolic studies in the field of anti-doping research and other areas.

2.2.2 *In vitro* studies on metabolism

Human trials may raise multiple concerns mentioned above and animal models often have ethical concerns while potentially not leading to completely satisfying results in terms of comparability to humans. Hence *in vitro* studies are a cost efficient and convenient method to collect information about metabolism.

Not only in doping control analysis important *in vitro* models for the analysis of metabolism are recombinant human enzymes or liver preparations, as enzymatic concentrations are especially high in this organ [29, 52].

- **Hepatocytes** are cells deriving from the liver. They are available as primary hepatocytes isolated directly from the liver or as tumour cell-lines with the advantage of self-renewal and longer life than primary cells. Tumour cell-lines have the disadvantage of lower metabolic activities (especially when number of performed passages is high) and hence minor or even no suitability for metabolic studies. Hepatocytes can also be cultivated in

3D-cell cultures as spheroids, organoids or even in multi-organ models to study the influence of surrounding tissues.

- **Human liver microsomes** (HLM): "Artefactual spherical particles, not present in the living cell, derived from pieces of the endoplasmic reticulum present in homogenates of tissues or cells" [53]. They sediment from S9-fraction and are often used as a source of membrane bound monooxygenase enzymes,
- **S9-fraction**: "Supernatant fraction obtained from an organ (usually liver) homogenate by centrifuging at 9000 *g* for 20 min in a suitable medium" [53]. The S9-fraction contains enzymes in lower concentrations compared to HLMs leading to generally lower turnover rates. Due to the presence of microsomal as well as cytosolic proteins the whole metabolic profile can be observed [29].
- **Recombinant human enzymes** are available either as isolated preparations or as recombinantly expressed proteins in host cells.

As isolated recombinant human enzymes and *Schizosaccharomyces pombe* (*S.pombe*) strains recombinantly expressing human CYP-enzymes and are mainly used in this work to study steroid metabolism, these two models are described in more detail.

Recombinant enzymes expressed in *Schizosaccharomyces pombe* as metabolic catalyst

S.pombe is well suited to be used as a host for the expression of functional human enzymes [54]. Whole cell biotransformations using *S.pombe* strains were performed using genetically modified fission yeast strains provided by the group of Prof. Dr. Matthias Bureik (School of Pharmaceutical Science and Technology, Tianjin University, China). The approach is quite straightforward: fission yeast cells recombinantly expressing the enzyme of interest are cultivated and the substrate in question is added. After an appropriate incubation time reaction products are isolated from the incubation broth and subsequently analysed and/or identified.

In this work whole cell biotransformation assays with *S.pombe* strains, recombinantly expressing human CYPs were used to evaluate metabolic products and to generate minor amounts of metabolites [55, 56]. Besides the chemical synthesis of metabolites as reference material also the biotechnological production (e.g. using *S.pombe*) or a combination of both approaches plays a role and has been performed successfully in the past [9, 54, 57-59]. The generation and availability of reference substances is a crucial topic as it is sometimes very difficult to acquire suitable material. Nonetheless, accurate quantitative analysis and unambiguous metabolite identification in proper samples can only be performed, when reference compounds are available [60].

Besides performing whole cell biotransformations, the group of Prof. Dr. Bureik developed an assay using permeabilized fission yeast (so called enzyme bags) to study metabolism [56, 61]. Some

major advantages and disadvantages and main application areas of whole cell biotransformation assays and enzyme bags compared to isolated recombinant human enzymes are listed in Table 2.

Isolated recombinant enzymes for metabolic studies

Rosano and Ceccarelli state in their review article: "At the theoretical level, the steps needed for obtaining a recombinant protein are pretty straightforward. You take your gene of interest, clone it in whatever expression vector you have at your disposal, transform it into the host of choice, induce and then, the protein is ready for purification and characterization. In practice, however, dozens of things can go wrong" [62]. This describes the basic steps and already mentions some difficulties in recombinant enzyme production. As the purpose of this work is the use of recombinant human enzymes and not their production, preparation shall not be commented in more detail here. Nonetheless, this quote is mentioning the three major steps of enzyme production: expression, isolation and purification. These stages also play a role e.g. in the quality assessment of an enzymatic preparation, which is crucial for metabolic experiments.

For metabolic studies it is important that the enzyme of interest is functional, meaning it catalyses the desired reaction; pure, meaning the preparation only contains the desired protein and that it is soluble in the assay system and the storage buffer. For the presented work commercial enzymatic preparations were used. The functional quality of these commercially available preparations can be estimated from the accompanying data sheet. The actual functionality can for example be evaluated by incubation of the enzyme with a well characterized substrate and the evaluation of the product(s) formed. Cytosolic and small enzymes (< 60 kDa) are generally easier to produce than membrane bound or large enzymes (> 60 kDa) [62]. In general, metabolic studies using isolated recombinant enzymes are a convenient method, as they are relatively easy in handling compared to all other methods described above. A detailed overview of advantages and drawbacks and some main application areas are reported in Table 2.

Table 2: *Comparison between S.pombe based whole-cell biotransformation assays (whole-cell BT), enzyme bag assays and isolated recombinant human enzymes (Iso rh enzymes) for metabolic studies with main application areas relevant for this work*

	Whole-cell BT	Enzyme bag	Iso rh enzymes
Main application area	Production of reference material Metabolic studies with subsequent product characterization (several mg of substance needed)	Evaluation of metabolic routes	Evaluation of metabolic routes and kinetic characterization of enzymes
Advantages	Product-generation in mg-scale (production of reference material) No addition of co-factor needed (if corresponding gene is co-expressed in yeast) Regrowing (cost efficient) Enzymes are membrane bound (no modification or tag needed for isolation or purification)	Faster compared to whole-cell BT Low substrate amounts needed Regrowing (cost efficient) Enzymes are membrane bound (no modification or tag needed for isolation or purification)	Fast from experiment to result Low substrate amounts needed
Disadvantages	Substrate (and product) need to pass through cell-wall Relatively high substrate amounts needed (µg-mg range) Longer incubation times compared to enzyme bags and iso rh enzymes Need to grow cells before assay	Addition of co-factor necessary (needs to be purchased) Need to grow cells before assay	Addition of co-factor necessary (needs to be purchased) Enzyme must be purchased if not produced in-house (which is currently not the case for us) Modifications and/or tags needed for isolation from host, purification or stabilization in solution (missing or additional amino acids in protein) → no "wild-type" enzyme

2.2.3 *In silico* studies on metabolism

This chapter very briefly mentions the third pillar of metabolic studies. Besides *in vivo* and *in vitro* studies *in silico* experiments are of relevance in the context of metabolic studies. The term "*in silico"* captures approaches developed to estimate an experimental outcome with the help of mathematical models and computer simulations. One example are molecular modelling experiments which were performed by Alexanda Naß from the group of Prof. Dr. Gerhard Wolber in the context of Manuscript I and by David Machalz also from the group of Prof. Dr. Wolber for Manuscript II. Besides other *in silico* methods, which shall not be discussed in more detail, docking experiments can help to better understand mechanisms and specific sequences of metabolic processes. With 3D-visualization of enzymatic structures, substrates, co-factors and inhibitors it is possible to, among other things, better explain and interpret results obtained during *in vitro* and *in vivo* experiments.

2.3 Doping control analysis

"The purposes of the World Anti-Doping Code and the World Anti-Doping Program which supports it are:

- To protect the Athletes' fundamental right to participate in doping-free sport and thus promote health, fairness and equality for Athletes worldwide, and
- To ensure harmonized, coordinated and effective anti-doping programs at the international and national level with regard to detection, deterrence and prevention of doping" [63].

To assure that these intentions of the "World Anti-Doping Code" can be met, doping control analysis performed by anti-doping laboratories plays a central role. Besides the superordinated "World Anti-Doping Code", the International Standard for Laboratories (ISL), one of several International Standards published by WADA, describes regulations and procedures important for anti-doping laboratories [64]. An example for regulations described in the ISL are the technical documents (TD) which regulate procedures for the analysis of prohibited substances. TDs relevant for the detection of AAS are mentioned in more detail in Chapter 2.3.2.

This chapter is giving a short introduction on doping control analysis and how doping control is performed and organized nowadays. A simplified testing process with focus on analysis of urine samples according to the International Standard for Testing and Investigations (ISTI) is depicted in Figure 6 [65]. A key goal of the whole testing process should be to declare as less as possible samples as false negative, meaning an athlete doped but doping is not detected and even more important do not produce false positive testing results, meaning an athlete has not doped but the result of analysis reported an adverse analytical finding (AAF).

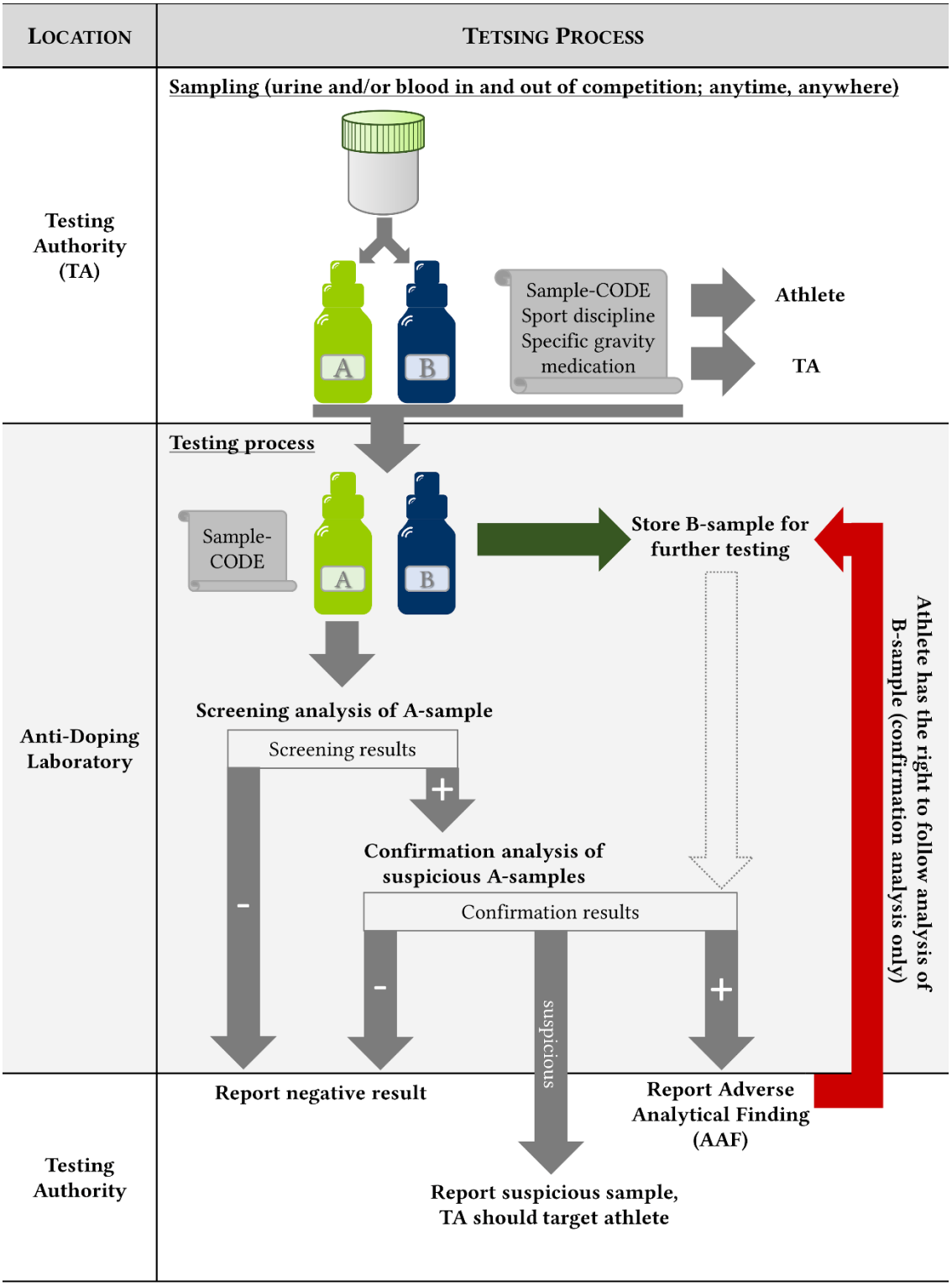

Figure 6: *Simplified testing process of anti-doping samples: Testing authority (TA) selects athlete to be tested; athlete provides urine (and/or blood) sample, divides it into A- and B-sample and fills accompanying form; one copy of this form is send to each the athlete and TA and one anonymized form (including: sample-code, sport-discipline, measured specific gravity and any medication taken) and the A- and B-samples are send to the laboratory; laboratory performs analysis (screening and possibly confirmation) on the A-sample according to the flowchart and reports the results to TA. In case of an AAF, athlete has the right to request supervised analysis of B-sample [65]*

2.3.1 World Anti-Doping Agency and the Prohibited List

"The Code is the fundamental and universal document upon which the World Anti-Doping Program in sport is based" [63]. It regulates all fields connected to anti-doping with the goal to "keep the spirit of sport" and hence to minimize doping in sport, which "is fundamentally contrary to the spirit of sport" [63]. This includes, but is not limited to, doping control, education and research in the field of doping and its prevention. The WADA Prohibited List regulates all substances and methods which are regarded as doping and hence prohibited and controlled [66]. The Prohibited List is divided in three sections:

- substances and methods prohibited at all times (in and out of competition) including
  - substance categories: **S0** non-approved substances; **S1** anabolic agents; **S2** peptide hormones, growth factors, related substances and mimetics; **S3** beta-2-agonists; **S4** hormone and metabolic modulators; **S5** diuretics and masking agents and
  - methods: **M1** manipulation of blood and blood components; **M2** chemical and physical manipulation and **M3** gene and cell doping,
- substances and methods prohibited in-competition including
  - substance categories: **S6** stimulants; **S7** narcotics; **S8** cannabinoids and **S9** glucocorticoids and
- substances prohibited in particular sports
  - class **P1** beta-blockers.

In addition to this list, the monitoring list contains "substances which are not on the Prohibited List, but which WADA wishes to monitor in order to detect patterns of misuse in sport" [67].

This work is focussing on the metabolism of AAS and its influenceability by non-prohibited drugs. Hence, class **S1** of the Prohibited List plays an outstanding role. This class includes AAS and "other anabolic substances" and lists substances, which are prohibited when administered exogenously. Furthermore the number of prohibited compounds is extended by "other substances with a similar chemical structure or similar biological effect(s)" [66] to also include potentially new structures and so called "designer-steroids".

2.3.2 Steroid determination in anti-doping analysis

Since the Olympic games in Los Angeles in 1984 gas chromatography coupled to mass spectrometry (GC-MS) is used for AAS detection in anti-doping analysis [68]. Due to the poor ionization of the majority of AAS and their metabolites in liquid chromatography (LC), GC is the method of choice to detect unconjugated steroids. Currently, routine protocols for anti-doping tests determine steroid concentrations as combined free (unconjugated) and glucuronide fraction. Analysis is generally done after hydrolysis of glucuronide conjugates in the urine samples using β-glucuronidase. After liquid-liquid extraction of free steroids and the cleaved aglycons,

trimethylsilyl-derivatization is generally performed using a mixture of N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA), ammonium iodide (NH_4I) and ethanethiol or mercaptoethanol [3, 69]. The trimethylsilylation of polar hydroxy- and oxo groups leads to the formation of the corresponding ethers or enol-ethers which allows for more sensitive detection of the analytes [70]. For oxo-groups it is mentionable that derivatization isomers may be formed, which usually appear as distinct peaks in the chromatogram.

The analysis and detection of AAS is performed by WADA accredited laboratories according to the technical documents provided by WADA [71], mainly the following:

- TD2018EAAS ("Endogenous Anabolic Androgenic Steroids - Measurement and Reporting") for the detection of most pseudo-endogenous and synthetic steroids [3],
- TD2019IRMS ("Detection of Synthetic forms of Endogenous Anabolic Androgenic Steroids by GC-C-IRMS") for confirmation analysis via gas chromatography-combustion-isotope ratio mass spectrometry [72] and
- TD2019NA ("Harmonization of Analysis and Reporting of 19-Norsteroids Related to Nandrolone") for the "confirmation procedure for the analysis and reporting of findings for 19-norsteroids related to nandrolone" [73].

In case of administration of pseudo-endogenous AAS, meaning steroids, which are identical to endogenously occurring steroids (e.g. exogenous testosterone), or long-term administration of synthetic AAS selected concentrations of EAAS and concentration ratios are altered. Consequently, these concentrations and concentration ratios of EAAS are determined following the technical document TD2018EAAS [3]. This is usually done by gas chromatography coupled to triple quadrupole mass spectrometers (GC-QQQ). For years fixed limits of steroid ratios, based on population-based reference values, were used to classify a specific result as an AAF, meaning a positive testing result. Over the years it had been found that individual limits show more accurate results as for example individual enzymatic variations may alter e.g. the T/E ratio to "untypical" low or "untypical" high values [74]. As it was found that specific concentration ratios of EAAS are stable over months and even years in individuals [75-78], WADA initiated the athlete biological passport (ABP) steroidal module in 2014 [79]. Additionally, to detect the administration of synthetic steroids, precursors and reported (long-term) metabolites are monitored. A comprehensive overview of long-term metabolites used for the detection of synthetic steroids in the Cologne anti-doping laboratory is given by Geyer *et al.* [80].

Besides the screening procedures predominantly using Gas chromatography-tandem mass spectrometry (GC-MS/MS) instrumentation, a confirmation analysis using gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS) is performed in case of a positive screening result for EAAS (Figure 6). This method is able to discriminate between endogenous and exogenous steroids by measuring the ratio of the stable isotopes ^{13}C and ^{12}C [81-83]. In case of a

positive testing in the screening for synthetic steroids a confirmation using GC-MS/MS is performed.

Despite the predominance of GC-MS analysis in steroid detection, liquid chromatography coupled to mass spectrometry (LC-MS) builds a powerful technique for analytes showing poor chromatographic behaviour in GC or those causing problems during derivatization [68].

Furthermore, several studies had been published on the detection of intact phase II steroid metabolites (glucuronides and/or sulphates) using GC-MS(/MS) or LC-MS(/MS) [84-87]. The detection of phase II metabolites may be of interest to extend the detection window of steroid metabolites.

A further perspective is use of high-resolution mass spectrometry (HRMS). HRMS measurements enable the tentative detection of potential new designer steroids or new metabolites of already known substances through accurate mass measurements. An example for HRMS is the use of gas chromatography coupled to time-of-flight mass spectrometry (GC-q-ToF) as a screening application [88]. The operation of this instrument in full scan mode may open the opportunity of retro perspective analysis for additional compounds of already analysed samples. This is not possible with QQQ data, as only preselected ion-transition and hence only preselected analytes are considered.

2.3.3 Athlete Biological Passport

The athlete biological passport (ABP) is a tool to monitor longitudinal changes of each individual athlete in anti-doping testing. Already in 2009, the haematological module has been implemented in the ABP, in 2014 the steroidal module followed. An additional endocrinological module is in preparation and in future also a forensic module and a performance module may be planned [89]. The focus of this chapter is on the steroidal module of the ABP. Its goal is the detection of EAAS administration and long-time use of synthetic AAS, which are resulting in alterations of the monitored markers in the ABP. The steroidal module currently includes six endogenous steroid concentrations (T, E, A, Etio, 5αAdiol and 5βAdiol) and five ratios of EAAS-concentrations (T/E, A/T, A/Etio, 5αAdiol/5βAdiol and 5αAdiol/E). The concentrations are measured by WADA-accredited laboratories according to the WADA technical document TD2018EAAS in urine samples collected from the athletes in and out of competition [3]. Following the WADA Athlete Biological Passport Operating Guidelines, all measured concentrations and concentration ratios shall be reported in the ABP module of the Anti-Doping Administration and Management System (ADAMS) [79]. This module automatically applies an “adaptive model” on all entries for this specific athlete (starting at the addition of the third sample) [74]. Individual limits are determined for each athlete and upon each new reporting by applying a Bayesian fit to the datapoints [90]. Based on these results, the sample is declared as negative, as adverse passport finding (APF) or as suspicious steroid profile (SSP) and further steps are initiated as depicted in Figure 6. In addition

to the screening analysis and if needed the confirmation analysis, the laboratory shall confirm the presence of confounding factors (Chapter 2.3.4) [3]. If the screening results are confirmed in the confirmation method, the B-sample may be analysed. For this procedure, the athlete or his/her representative is allowed to attend the analysis in the laboratory. Only in the case of a confirmation of the positive findings from the A-sample, a positive testing result is reported.

2.3.4 Factors influencing the steroid profile

Especially the ratios, which are part of the steroidal module of the ABP, haven been shown to be very stable in individual adult humans [75-78]. Nonetheless, the steroid metabolism in humans is very complex and many enzymes are involved. Consequently, influencing of these enzymatic processes might change the metabolism and hence the excretion of EAAS and the steroidal profile. Marck-Engelke *et al.* did a lot of research on the stability of steroid profiles in males and females and described various factors influencing the steroid profile [76-78, 91, 92]. It is important to perform these investigations in males as well as in females, as metabolic routes and hence steroidal profiles differ in males and females. As concentrations of EAAS in females are much lower than in males [3], the detection requires very sensitive methods and hence, interpretation of steroid profiles especially in females is challenging [78, 92-94]. In general, changes in steroid profile markers induced by endogenous factors shall be covered by the use of the ABP steroidal module. A collection of some examples for endogenous factors possibly influencing the steroid profile in a general or more individual manner are listed hereafter:

- Sex, age, genetic polymorphisms and ethnicity (among which similar genetic variations are often observable) [40, 75, 93, 95-97],
- Menstrual cycle (discussed contrarily) [78, 94, 98, 99], and
- Time during day [78, 98].

In addition to interindividual variations in the steroid profile caused by endogenous factors, there are some exogenous aspects able to influence the steroid profile [100]:

- Pregnancy or administration of human chorionic gonadotropin (hCG) in males (and females),
- Psychological effects like stress (e.g. in and out of competition testing; or testing after a victory or a loss) are still discussed contrarily [101-103], and
- Intake of (non-prohibited) drugs; including, but not limited to [3]:
    - Oral contraceptives [91, 98], ethanol [104], antifungals [105-107], aromatase-inhibitors, antiestrogens and 5α-reductase inhibitors, other AAS, masking agents (e.g. probenecid) or diuretics.

Besides these personal factors there are also analytical factors like bacterial contamination, hydrolysis, coelution and matrix effects, which can confound the steroidal profile and hence the detection of AAS [100].

As these confounding factors can lead to significant changes in the steroid profiles and to divergent testing results during an anti-doping test, the presence of already known confounding factors should be monitored during an anti-doping analysis [3]. To consistently improve the analytical performance, it is furthermore of relevance to additionally keep investigating the potential of other substances acting as confounding factors.

The knowledge of substances interfering with the steroid metabolism may help to better interpret anti-doping testing results and hence reduce the number of false negative and even more important, false positive testing results.

In addition to the relevance in the field of anti-doping research, the knowledge of compounds interfering with steroid metabolism may be of value in the detection and investigation of potential endocrine disrupting substances (Chapter 2.1.2) and hormone related disorders.

Non-steroidal anti-inflammatory drugs and doping

Until now NSAIDs are neither included in the WADA Prohibited List, nor in the list of confounding factors monitored by anti-doping laboratories. However, just recently a German TV-documentary with special focus on the use of NSAIDs in soccer players came out [108]. The documentary describes the extensive use of pain killers among soccer players at all performance levels. Finally, the justification of extensive NSAID use in sports is discussed. The WADA Anti-Doping Code states that a substance which meets at least two of the following points shall be considered for inclusion to the Prohibited List [63]:

- Potential to enhance or enhancement of sport performance,
- Actual or potential health risk to the athlete or
- Use violates the spirit of sport.

In the documentary Hans Geyer from the Cologne anti-doping laboratory argues, that in the case of NSAIDs all three of these points are met.

The fact that NSAIDs are the most commonly used drugs in sports has been known for years [26, 27, 109-111]. In addition, it was shown *in vitro*, that the NSAIDs ibuprofen and indomethacin inhibit the enzymes AKR1C2 and AKR1C3, which play an important role in the metabolism of EAAS (Chapter 2.1.1) [112-114]. In consequence, an influence of the intake of indomethacin or ibuprofen on the steroid profile was thinkable. Results of investigations on this are presented in Manuscript III and in Manuscript IV and are discussed in Chapter 5.

3 MANUSCRIPTS

3.1 Manuscript I: "Combined chemical and biotechnological production of 20βOH-NorDHCMT, a long-term metabolite of Oral-Turinabol (DHCMT)"

Jiaxin Liu, Lei Chen, Jan Felix Joseph, Alexandra Naß, Anna Stoll, Xavier de la Torre, Francesco Botrè, Gerhard Wolber, Maria Kristina Parr and Matthias Bureik

Journal of Inorganic Biochemistry; 183 (2018) 165-171
https://doi.org/10.1016/j.jinorgbio.2018.02.020

Abstract: Anabolic androgenic steroids (AAS) are misused very frequently in sport competitions as performance enhancing agents. One of the doping compounds that has been detected with increased frequency in the last few years is dehydrochloromethyltestosterone (DHCMT, 4-chloro-17β-hydroxy-17α-methylandrosta-1,4-dien-3-one; brand name Oral Turinabol). The long-term DHCMT metabolite 20βOH-NorDHCMT (4-chloro-17β-hydroxymethyl-17α-methyl-18-norandrosta-1,4,13-trien-3-one) was reported earlier to be detectable in urine samples for more than 22 days after DHCMT administration; however, purified reference material was not available so far. In this study we demonstrate a successful combination of Wagner-Meerwein rearrangement of DHCMT to NorDHCMT (4-chloro-17,17-dimethyl-18-norandrosta-1,4,13-trien-3-one) and subsequent whole-cell biotransformation with a recombinant fission yeast strain expressing the human cytochrome P450 enzyme (CYP or P450) CYP21A2 for the synthesis of mg amounts of this metabolite. It was then used as reference for the analysis of a post administration urine of DHCMT. The availability of this reference compound will provide an incontestable proof for DHCMT abuse.

3.2 Manuscript II: "Fine-mapping of the substrate specificity of human steroid 21-hydroxylase (CYP21A2)"

Anna Stoll, Steffen Loke, Jan Felix Joseph, David Machalz, Xavier de la Torre, Francesco Botrè, Gerhard Wolber, Matthias Bureik and Maria Kristina Parr

Journal of Steroid Biochemistry and Molecular Biology; 194 (2019) 105446
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Abstract: Cytochrome P450 enzymes (CYPs) are capable of catalysing regio- and stereo-specific oxy functionalization reactions, which otherwise are major challenges in organic chemistry. In order to make the best possible use of these biocatalysts it is imperative to understand their specificities. Human CYP21A2 (steroid 21-hydroxylase) acts on the sidechain attached to C-17 in ring D of a steroid substrate, but the configuration of ring A also plays a prominent role in substrate cognition. Here, we comprehensively investigated this relationship using sixteen 17,17-dimethyl-18-nor-13-ene steroids with different arrangements of hydroxy-, oxo-, fluoro- and chloro-groups and in the presence or absence of double bonds (Δ1 and/or Δ4) and heteroatoms in ring A. The results show that presence of a 3-oxo group is a strict requirement for a CYP21A2 substrate, while the other configurations tested were all tolerated. This was also confirmed by control experiments using endogenous steroids. While progesterone and 17-hydroxyprogesterone were hydroxylated at C-21, (17-hydroxy-) pregnenolone did not react. Molecular docking experiments indicate that the interaction of the carbonyl group at C-3 to the sidechain Arg234 of the enzyme is indispensable.

3.3 Manuscript III: "Influence of Pain Killers on the Urinary Anabolic Steroid Profile"

Anna Stoll, Michele Iannone, Giuseppina De Gregorio, Xavier de la Torre, Francesco Molaioni, Francesco Botrè and Maria Kristina Parr

Journal of Analytical Toxicology; 44 (2020) 871-879
https://doi.org/10.1093/jat/bkaa049

Abstract: Anabolic androgenic steroids (AAS) are prohibited as performance-enhancing drugs in sports. Among them, testosterone and its precursors are often referred to as "pseudo endogenous" AAS, that is, endogenous steroids that are prohibited when administered exogenously. To detect their misuse, among other methods, the World Anti-Doping Agency-accredited laboratories monitor the steroid profile (concentrations and concentration ratios of endogenous steroids, precursors and metabolites) in urine samples collected from athletes in and out of competition. Alterations in steroid profile markers are used as indicators for misuse of anabolic steroids in sports. Therefore, especially their metabolic pathways with possible interactions are crucial to elucidate. As steroid metabolism is very complex and many enzymes are involved, certain non-prohibited drugs may influence steroid metabolite excretion. One important group of steroid-metabolizing enzymes is aldo–keto reductases (AKRs). An inhibition of them by non-steroidal anti-inflammatory drugs (NSAIDs), which are neither prohibited nor monitored, but frequently used drugs in sports, was demonstrated *in vitro*. Thus, this work aims to investigate the influence of NSAID intake on the urinary steroid profile. Kinetic and inhibitory studies were performed using 5α-dihydrotestosterone as substrate. The results obtained from *in vitro* experiments show that ibuprofen inhibits AKR1C2 and thus influences steroid biotransformation. For *in vivo* investigations, urine samples prior, during and post administration of ibuprofen were analysed using routine methods to monitor the steroid profile. Changes in markers of the steroid profile of volunteers were observed. The combination of *in vitro* and *in vivo* results suggests that monitoring of ibuprofen may be useful in doping control analysis. The presented work illustrates the importance to consider co-administration of (non-prohibited) drugs during anti-doping analysis. Intake of multiple substances is likely leading to interfering effects. Divergent results in anti-doping analysis may therefore be observed and misinterpretation of analytical data is likely to occur. Similar considerations are appropriate for other fields of forensic applications.

3.4 Manuscript IV: "Influence of Indomethacin on Steroid Metabolism: Endocrine Disruption and Confounding Effects in Urinary Steroid Profiling of Anti-Doping Analyses"

Anna Stoll, Michele Iannone, Giuseppina De Gregorio, Xavier de la Torre, Francesco Molaioni, Francesco Botrè and Maria Kristina Parr

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Abstract: Anabolic androgenic steroids (AAS) are prohibited as doping substances in sports by the World Anti-Doping Agency. Concentrations and concentration ratios of endogenous AAS (steroid profile markers) in urine samples collected from the athletes are used to detect their administration. Certain (non-prohibited) drugs have been shown to influence the steroid profile and thereby sophisticate anti-doping analysis. It was shown *in vitro* that the non-steroidal anti-inflammatory drug (NSAID) indomethacin inhibits selected steroid-biotransformations catalysed by the aldo-keto reductase (AKR) 1C3, which plays a key role in the endogenous steroid metabolism. Kinetic parameters for the indomethacin-mediated inhibition of the AKR1C3 catalysed reduction of etiocholanolone were determined *in vitro* using two comparing methods. As NSAIDs are very frequently used (not only) by athletes, the inhibitory impact of indomethacin intake on the steroid metabolism was evaluated and steroid profile alterations were detected *in vivo* (one male and one female volunteer). Significant differences between samples collected before, during or after the intake of indomethacin for selected steroid profile markers were observed. The presented results are of relevance for interpretation of results from doping control analysis. Additionally, the administration of NSAIDs should be carefully reconsidered due to their potential as endocrine disruptors.

metabolites

MDPI

Article

Influence of Indomethacin on Steroid Metabolism: Endocrine Disruption and Confounding Effects in Urinary Steroid Profiling of Anti-Doping Analyses

Anna Stoll [1], Michele Iannone [2], Giuseppina De Gregorio [2], Francesco Molaioni [2], Xavier de la Torre [2], Francesco Botrè [2,3] and Maria Kristina Parr [1,*]

1 Institute of Pharmacy (Pharmaceutical and Medical Chemistry), Freie Universität Berlin, 14195 Berlin, Germany; anna.stoll@fu-berlin.de
2 Laboratorio Antidoping Federazione Medico Sportiva Italiana, 00197 Rome, Italy; micheleiannone14@gmail.com (M.I.); degregorio.giuseppina@gmail.com (G.D.G.); molaioni@gmail.com (F.M.); xavier.delatorre@gmail.com (X.d.l.T.); Francesco.Botre@unil.ch (F.B.)
3 Synathlon—Quartier Centre, ISSUL—Institut des Sciences du Sport, Université de Lausanne, 1015 Lausanne, Switzerland
* Correspondence: maria.parr@fu-berlin.de; Tel.: +49-30-838-51471

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Abstract: Anabolic androgenic steroids (AAS) are prohibited as doping substances in sports by the World Anti-Doping Agency. Concentrations and concentration ratios of endogenous AAS (steroid profile markers) in urine samples collected from athletes are used to detect their administration. Certain (non-prohibited) drugs have been shown to influence the steroid profile and thereby sophisticate anti-doping analysis. It was shown in vitro that the non-steroidal anti-inflammatory drug (NSAID) indomethacin inhibits selected steroid-biotransformations catalyzed by the aldo-keto reductase (AKR) 1C3, which plays a key role in the endogenous steroid metabolism. Kinetic parameters for the indomethacin-mediated inhibition of the AKR1C3 catalyzed reduction in etiocholanolone were determined in vitro using two comparing methods. As NSAIDs are very frequently used (not only) by athletes, the inhibitory impact of indomethacin intake on the steroid metabolism was evaluated, and steroid profile alterations were detected in vivo (one male and one female volunteer). Significant differences between samples collected before, during or after the intake of indomethacin for selected steroid profile markers were observed. The presented results are of relevance for the interpretation of results from doping control analysis. Additionally, the administration of NSAIDs should be carefully reconsidered due to their potential as endocrine disruptors.

Keywords: NSAID; inhibition; doping; aldo-keto reductases; endocrine disruption

1. Introduction

Anabolic androgenic steroids (AAS) are very frequently used drugs in sports [1]. Their use as doping agents is prohibited in and out of competition by the World Anti-Doping Agency (WADA; class S1 in the WADA prohibited list) [2]. The analytical detection is challenging, especially if so-called pseudo endogenous AAS (e.g., exogenous testosterone) are used as performance enhancing substances, due to their high similarity to the naturally occurring endogenous AAS (EAAS). To detect the misuse of those pseudo endogenous and some synthetic AAS, anti-doping laboratories monitor in a first step, concentrations and concentration ratios of selected EAAS according to the WADA technical document TD2018EAAS in urine samples collected from the athletes [3]. In case of misuse of pseudo endogenous AAS or some synthetic AAS, those steroid profile markers are altered and a confirmative method using gas chromatography combustion isotope-ratio mass-spectrometry (GC-c-IRMS) is

applied. Since it has been shown that ratios of urinary steroids are stable over months and even years in adult humans [4–6] but show interindividual variations, the steroidal module of the Athlete Biological Passport (ABP) was introduced by WADA in 2014 [7]. With this longitudinal monitoring model, it is possible to better detect intraindividual changes, and hence the potential misuse of AAS. However, it was shown that besides various endogenous and exogenous parameters, the intake of selected (non-prohibited) drugs can influence the individual steroid profile and lead to suspicious testing results [8–10]. To better understand how changes in the steroid profile can occur after the intake of specific drugs, it is helpful to understand and further investigate the steroid metabolism and potential points of interference. One enzyme-family which plays a key role in the metabolism of EAAS are aldo-keto-reductases (AKR; Figure 1). In this study we focused on the AKR1C3, which is known to oxidize 17-hydroxy steroids to their corresponding 17-oxo metabolites and vice versa. It was reported that the reduction route is favored in vivo [11]. Furthermore, it was reported by Byrns et al. that the non-steroidal anti-inflammatory drug (NSAID) indomethacin inhibits the AKR1C3 catalyzed reduction in androst-4-ene-3,17-dione in vitro selectively over the closely related AKR1C2 and AKR1C1 [12,13]. No further investigations have been made on the inhibitory effect of indomethacin on 5β-androstanes metabolized by AKR1C3. As NSAIDs are very frequently used drugs (not only) among athletes [14,15], this work aims to further investigate the influence of indomethacin on the steroid metabolism in vitro and wants to show the relevance of indomethacin on the urinary steroid profile in vivo. Hence, the work consists of an in vitro part and an in vivo application trial. The in vitro experiments were analyzed spectro-fluorometrically in real time and by gas chromatography coupled to a quadrupole-time-of-flight mass spectrometer (GC-QToF) as confirmative tests. For the in vivo part, indomethacin was administered to one male and one female volunteer in therapeutic doses over 14 days. Urine samples before, during and after the administration were collected and analyzed. The results first give ideas on the impact of indomethacin intake on steroid profiles in doping control analysis and its potential mechanism of endocrine disruption.

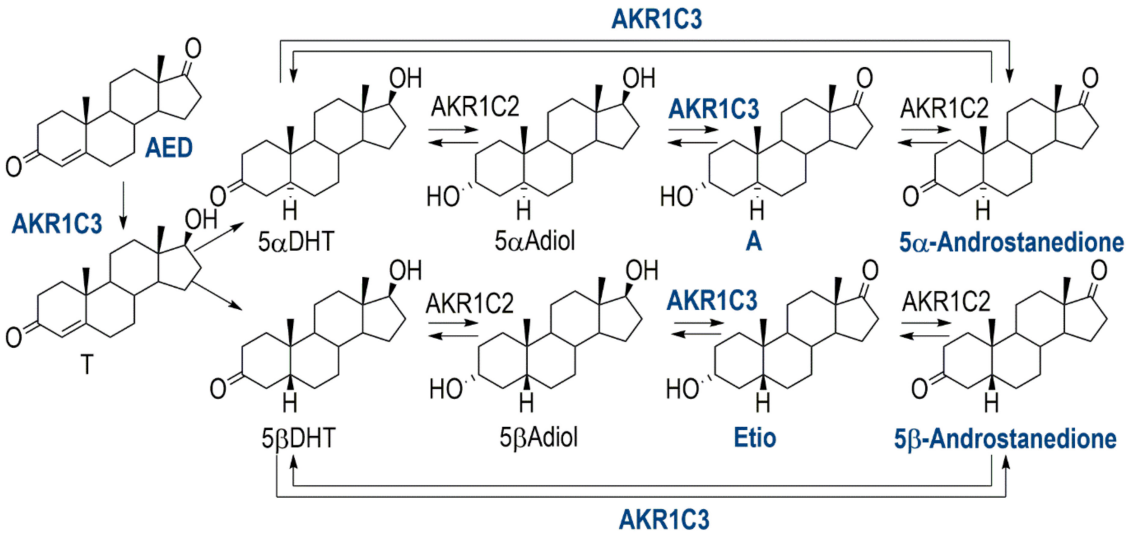

Figure 1. Metabolism of endogenous anabolic androgenic steroids (EAAS); blue and bold: substrates and enzymes used in this publication.

2. Results

2.1. Qualitative Incubation In Vitro

With the applied GC-MS (gas chromatography-mass spectrometry) method, all EAAS of interest were sufficiently separated (no interference between steroids was expected to occur simultaneously during the different incubations). All analyzed EAAS standards are depicted in the upper chromatogram

in Figure 2. For all background incubations (absence of enzyme) no other steroids besides the substrate were detectable. The internal standard (17α-methyltestosterone; substance K in Figure 2) was detected in all samples.

Figure 2 shows chromatograms of samples after enzymatic incubations in solid lines. As no substrate was detected after the incubation of Etio (etiocholanolone, substance D in Figure 2) the sample chromatogram (solid line) was superimposed by the chromatogram of the background-incubation (dotted line). A detailed display of chromatograms of all performed incubations and background samples is available as supplementary data (Supplement S1). Chromatograms are displayed as total ion current chromatograms. Hence, peaks originating from the incubation media are also present. This is assumed to be the case for the two big peaks at 8.79 min and at 9.47 min, as both are also present in background-samples (without enzyme). They are hence neglected in the results presentation and discussion.

In the following paragraph, detailed outcomes of individual steroid incubations with AKR1C3 (aldo-keto reductase 1C3) will be described. After incubation of 5αAD (5α-androstanedione, substance G in Figure 2), small amounts of substrate were detected. In addition, minor amounts of 5αDHT (5α-dihydrotestosterone, substance H in Figure 2) and larger amounts of And (androsterone, substance C in Figure 2) and 5αAdiol (5α-androstane-3α,17β-diol, substance E in Figure 2) were detected. All of these compounds have a 5α-androstane structure in common and are highlighted in orange in Figure 2.

After incubation of 5βAD (5β-androstanedione, substance A in Figure 2) no or small amounts of substrate, but peaks corresponding to 5βAdiol (5β-androstane-3α,17β-diol, substance F in Figure 2) and Etio (substance D in Figure 2), were detected. Furthermore, very minor amounts of 5βDHT (5β-dihydrotestosterone, substance B in Figure 2) were detectable in one of two replicates (not visible in Figure 2). All of these compounds have a 5β-androstane structure in common and are highlighted in green in Figure 2.

After incubation of AED (androst-4-ene-3,17-dione, substance I in Figure 2) with AKR1C3, AED itself and its metabolite T (testosterone, substance J in Figure 2) were detected. Both compounds are highlighted in violet in Figure 2.

After incubation of And (substance C in Figure 2) with AKR1C3, peaks corresponding to And and 5αAdiol (substance E in Figure 2) were detected. Only minor amounts of the substrate were detected (small peak at 8.36 min with framed retention time (RT) in androsterone chromatogram in Figure 2). And and 5αAdiol share a 5α-androstane structure and are hence highlighted in orange in Figure 2.

After incubation of Etio (substance D in Figure 2) the substrate itself was detected in only one of the two replicates (indicated as dotted peak at 8.41 min corresponding to Etio detected in the background sample). The metabolite 5βAdiol (substance F in Figure 2) was present in both replicates. As Etio and 5βAdiol share a 5β-androstane structure they are highlighted in green in Figure 2.

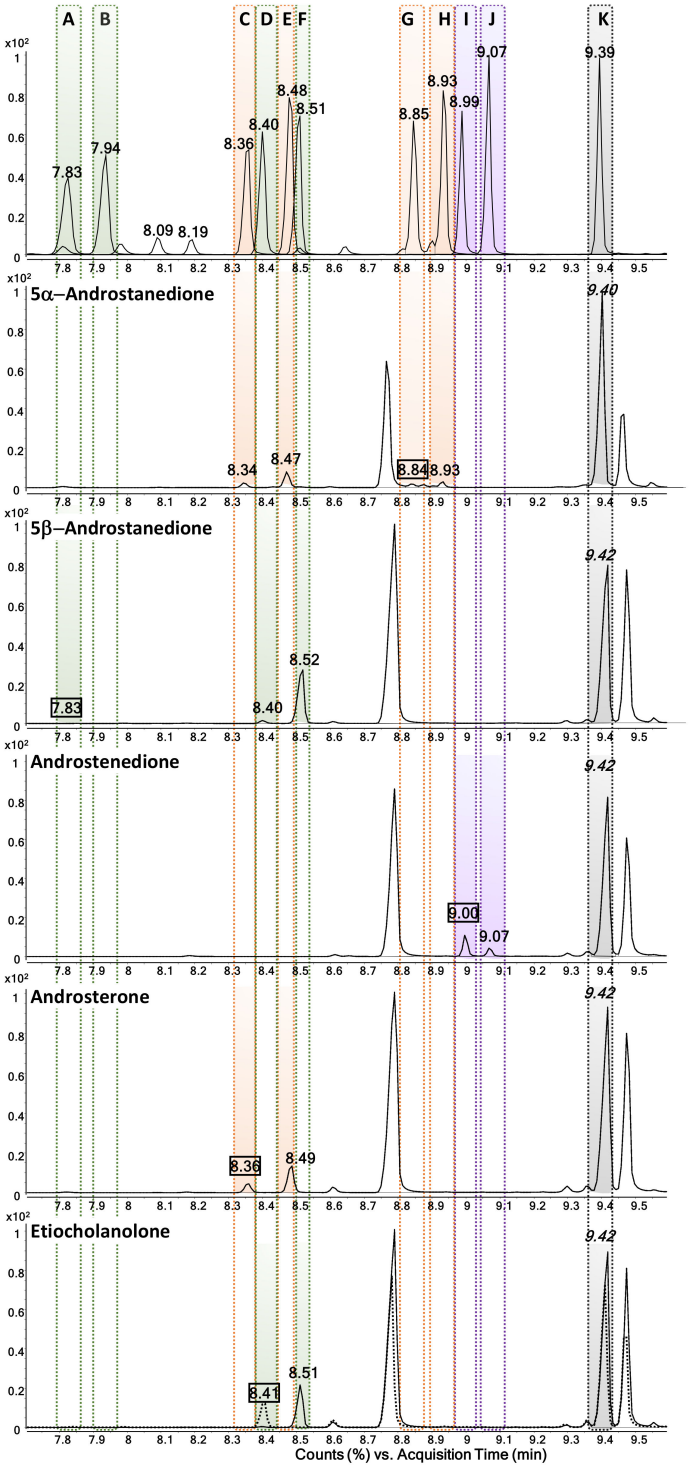

Figure 2. Chromatogram of EAAS standards (upper chromatogram) and steroids detected in overview-incubations (chromatograms below) analyzed on GC-MS with substrates of incubation indicated in upper left corner of each chromatogram and highlighted by framed retention time (RT); colored peaks corresponding to: A: 5βAD (RT: 7.83, 8.09 min, derivatization isomers); B: 5βDHT (RT: 7.94; 8.19 min, derivatization isomers); C: And (RT: 8.36 min); D: Etio (RT: 8.40 min); E: 5αAdiol (RT: 8.48 min); F: 5βAdiol (RT: 8.51 min); G: 5αAD (RT: 8.85 min); H: 5αDHT (RT: 8.93 min); I: AED (RT: 8.99 min); J: T (RT: 9.07 min); K: MeT (RT: 9.39 min; internal standard); orange peaks indicate 5α-androstanes, green peaks indicate 5β-androstanes, violet peaks correspond to T and AED, MeT is colored in black. Peaks not indicated with RT do not correspond to any EAAS standards.

2.2. K_m Determination In Vitro

Experiments to determine the Michaelis-Menten constant (K_m) of AKR1C3 catalyzed Etio metabolism were carried out in 96-well plates to detect real-time changes in fluorescence intensities. The K_m determined with the spectro-fluorometric method was 9.7 µM (standard error of the mean; SE = 1.4 µM; Figure 3B). As previously described, analysis of terminated incubations was additionally carried out using a GC-QToF instrument. The determined K_m with this method was 15.8 µM (SE = 0.9 µM; Figure 3A). Michaelis-Menten curves of both measurements are depicted in Figure 3.

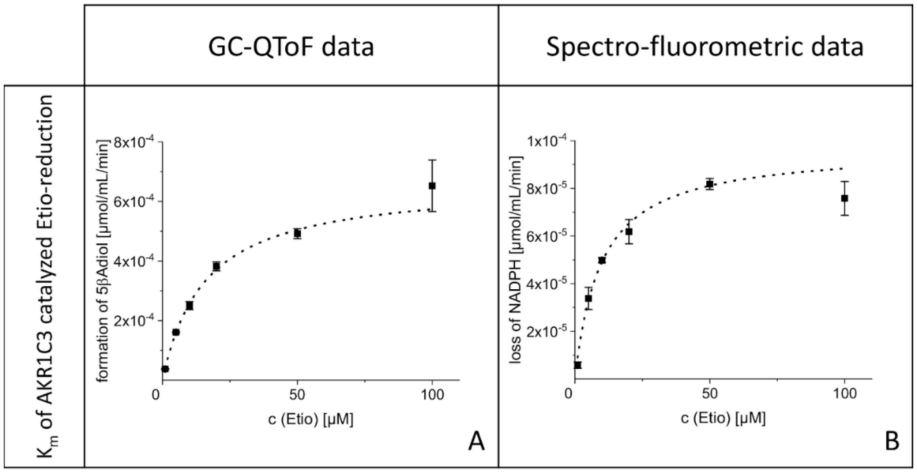

Figure 3. Michaelis-Menten curve of Etio with AKR1C3 generated with data from GC-QToF data (**A**): K_m = 15.8 µM (SE = 0.9 µM) and spectro-fluorometric measurement (**B**): K_m = 9.7 µM (SE = 1.4 µM).

2.3. IC_{50} Determination In Vitro

For the measurements performed with the spectro-fluorometric method, the half maximal inhibitory constant (IC_{50}) could not be calculated. Several background samples (enzyme substituted by phosphate buffered saline (PBS) 0.1 M) showed higher initial velocities than the samples which contained enzyme. Hence, a subtraction of background-velocities from initial velocities of samples would have resulted in negative values and hence negative enzyme activities would have been determined. Therefore, IC_{50} values were determined with the data obtained by GC-QToF analysis.

The IC_{50} determined using the mass-spectrometric method was successful and resulted in a value of 4.8 µM (SE = 1.0 µM). The corresponding curve is depicted in Figure 4.

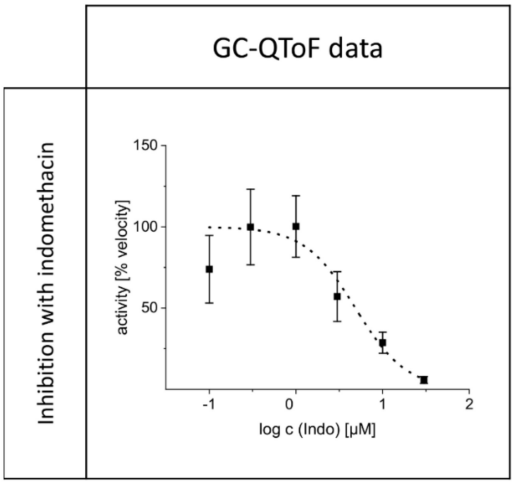

Figure 4. Inhibition curve generated from GC-QToF data: IC_{50} = 4.8 µM (SE = 1.0 µM).

2.4. In Vivo Administration Trial

To illustrate all significant changes in the steroid profile markers of the male and the female volunteers, boxplots of steroid profile markers where significant differences were found between at least two groups are displayed in Figure 5.

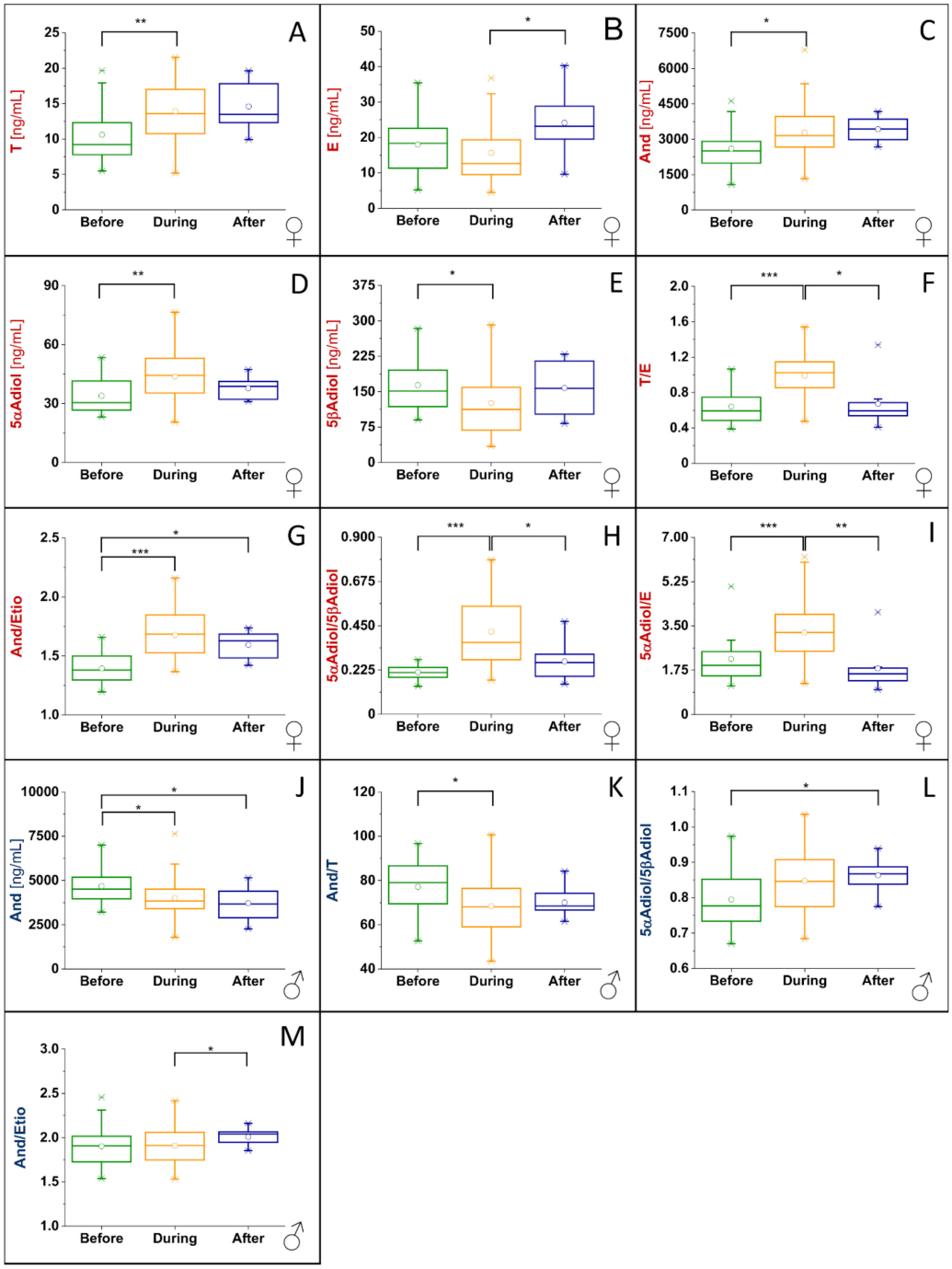

Figure 5. Boxplots of steroid profile markers with significant differences between at least two groups. (**A**–**I**): female volunteer; (**J**–**M**): male volunteer; * significant difference with $p < 0.0167$; ** very significant difference with $p < 0.0033$; *** highly significant difference with $p < 0.00033$.

In the male volunteer, significant changes between at least two groups, i.e., before and during administration, were detected for And (Figure 5J), And/T ratio (Figure 5K), And/Etio ratio (Figure 5M)

and 5αAdiol/5βAdiol ratio (Figure 5L). The concentration of And decreased by 15% (difference in median) during the administration of indomethacin compared to the concentration measured before the administration and decreased by 21% (difference in mean) after the administration compared to before the administration. The And/T ratio decreased by 11% during the administration compared to the ratios before (difference in mean values). The And/Etio ratio increased by 5% (difference in mean) after the administration compared to during administration and the 5αAdiol/5βAdiol ratio increased by 9% (difference in mean) after administration compared to before administration. In the female volunteer, significant differences were detectable for all examined steroid profile markers except for Etio concentration and And/T ratio. Changes solely between the groups before and during administration of indomethacin were detectable for T (Figure 5A), And (Figure 5C), 5αAdiol (Figure 5D) and 5βAdiol (Figure 5E). Changes solely between the groups during and post administration were detectable for E (epitestosterone, Figure 5B). Changes between the groups before/during and during/after were detectable for T/E (Figure 5F), 5αAdiol/5βAdiol (Figure 5H) and 5αAdiol/E (Figure 5I) ratios. Changes between the groups before/during and before/after were significant for And/Etio ratio (Figure 5G).

Furthermore, concentrations and concentration ratios of steroid profile markers, which showed significant changes between at least two groups, were plotted over the whole sampling time. The first sampling point was at t = 0 h. All curves are displayed in Figure 6. From this visualization it is observable that in particular, the concentration of E and 5βAdiol and the T/E, 5αAdiol/E and 5αAdiol/5βAdiol ratios in the female volunteer change a lot over time (Figure 6b,e,f,h and i). Additionally, for the And/Etio ratio in the female volunteer (Figure 6g) and for the 5αAdiol/5βAdiol ratio in the male volunteer (Figure 6l) an increase during the administration of indomethacin is clearly visible.

Indomethacin and its two evaluated metabolites were detected over the whole time of administration. Thereby, the highest concentration of indomethacin was determined in the samples collected after the administration of the drug (13 h). The two following samples before the next intake (18 h and 22 h) were much lower than the detected peak-concentration. Samples collected after the administration period (day 15 and later) contained, if at all, only trace amounts of indomethacin or its examined metabolites (Figure 7).

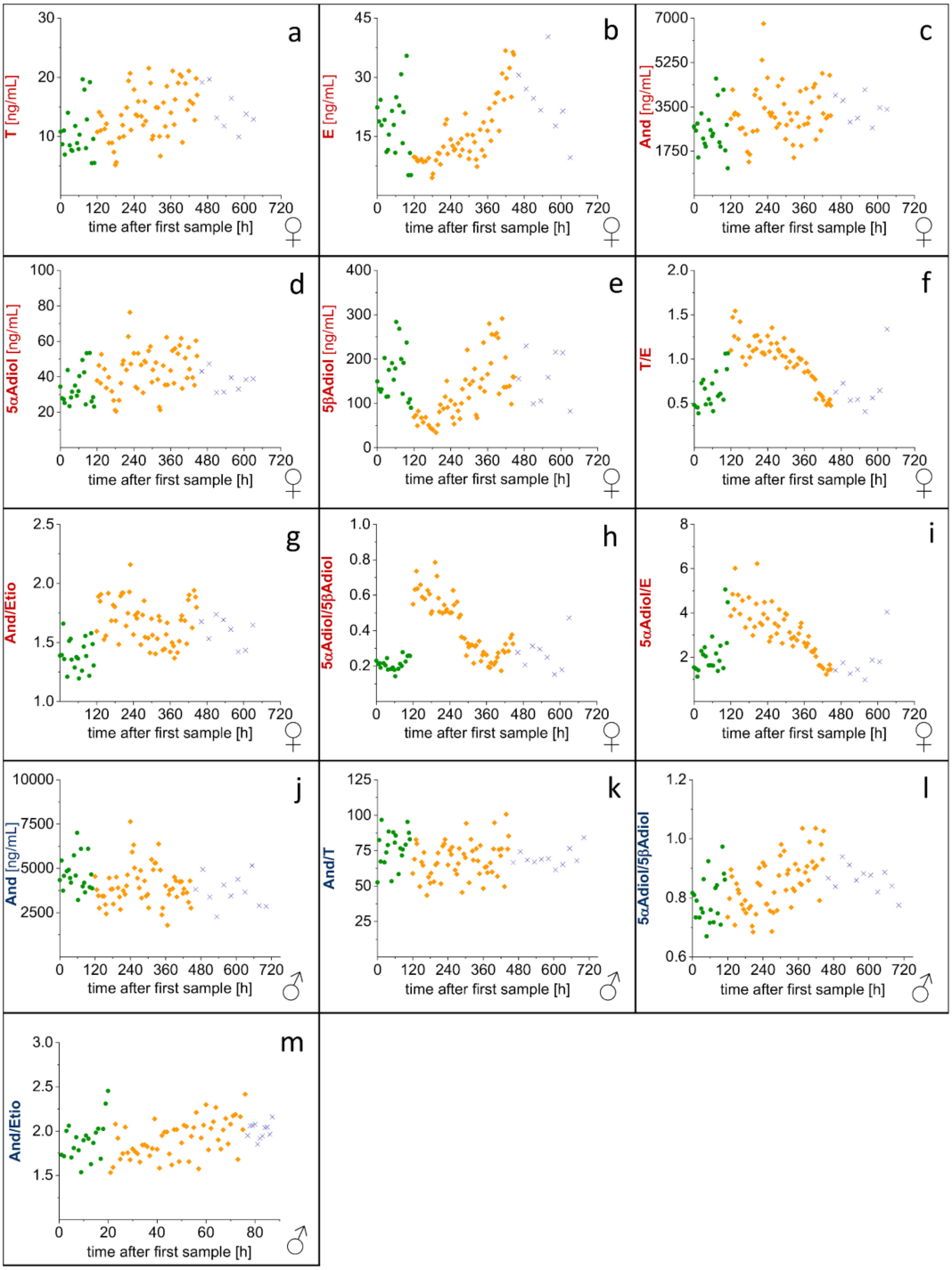

Figure 6. Time course of steroid profile markers which changed significantly over time; (**a**–**i**): female volunteer; (**j**–**m**): male volunteer.

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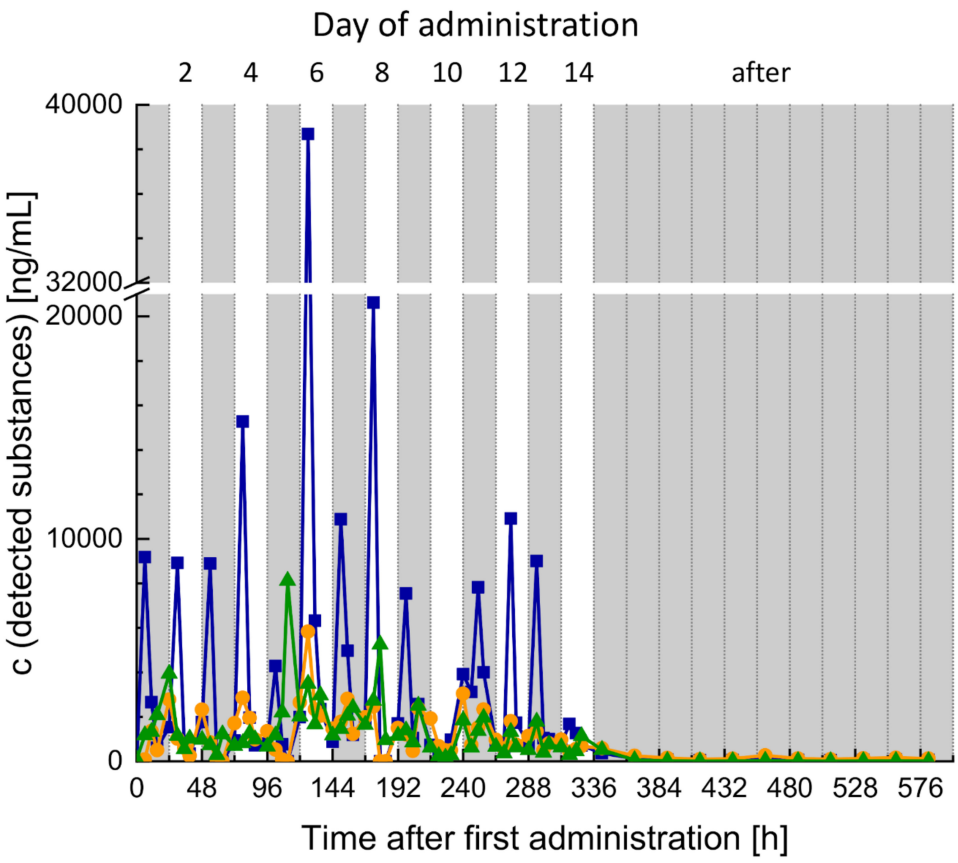

Figure 7. Measured concentrations of indomethacin (blue squares) and its metabolites O-desmethoxyindomethacin (orange circles) and *N*-deschlorobenzoylindomethacin (green triangles) in urine samples collected from the male volunteer. Sampling points for one day were: 7 h, 13 h, 18 h and 22 h.

3. Discussion

3.1. In Vitro Overview Incubation

Overview incubations were performed to examine the suitability of different endogenous steroids as substrate for the subsequent inhibition experiments. Important requirements were:

- The substrate is an endogenous steroid, which plays a role in the steroid module of the Athlete Biological Passport of the World Anti-Doping Agency;
- No side reaction is taking place besides the formation of the desired product;
- The initial velocity is fast enough to be monitored spectro-fluorometrically.

From those requirements it was assumed that a substrate which shows nearly complete formation of product, with no formation of side products after 5.5 h would be an adequate candidate for the following experiments. The requirement of no side reactions guarantees that a "one-to-one reaction" is taking place. Therefore, NADPH (β-Nicotinamide-adenine-dinucleotide-phosphate) consumption measured spectro-fluorometrically directly correlates with product formation measured on the GC-QToF, and the data from spectro-fluorometric measurements and chromatographic confirmation are comparable. The nearly complete formation of the product after a (rather long) incubation period of 5.5 h indicates a higher reaction velocity which would result in a higher sensitivity of the spectro-fluorometric analysis.

For all enzymatic incubations, the formation of product(s) was observed. Hence, a negative effect of methanol or acetonitrile (used as solvents for steroid substrates) on the outcome of the qualitative experiments was not expected.

Five endogenous steroids were chosen for preliminary experiments. 5α- and 5β-androstanedione formed three products each, which was not expected, since all other examined EAAS only showed the formation of one product. Following the pattern of all other observed products, we assumed that also 5α- and 5β-androstanedione would form their 17-hydroxy-metabolite, 5α- or 5β-dihydrotestosterone, respectively. However, we only detected small amounts, of the latter and of the substrates, if any. Instead, we observed the formation of And or Etio and 5αAdiol or 5βAdiol, respectively. Those findings are in accordance with the literature, where AKR1C3 is described to act as 3α-hydroxysteroid dehydrogenase (3αHSD) as well as 17βHSD [16]. Further investigations would be interesting to evaluate the complex kinetics and the metabolite formation sequence to gain further insights into enzyme characteristics.

In the analyzed samples of AED, And and Etio, only one product was detected. Whereas AED and And formed their corresponding product T and 5αAdiol in relatively small amounts in both replicates, Etio was converted nearly completely into its product 5βAdiol in one of two replicates (Figure 2). Due to the complete metabolization and the absence of any side reactions, Etio was chosen as the substrate for the subsequent in vitro kinetic experiments.

3.2. Kinetic Measurements In Vitro

To prevent the potential interfering effects of acetonitrile or methanol (used as solvent of the steroid substrate for qualitative incubations) with the enzyme, Etio was dissolved in DMSO (dimethyl sulfoxide) for all the kinetic measurements. Constant volumes of DMSO and ethanol were added to all incubations (with and without indomethacin and to samples without enzyme) to generate comparable results.

To find the optimal substrate concentration for the subsequent inhibition experiments, the K_m value of the AKR1C3-catalyzed reduction in Etio is needed. These values were determined experimentally since, to our knowledge, no kinetic data on the desired reaction are available in the literature. The K_m values from the spectro-fluorometric and mass spectrometric measurements (K_m(fluoro) = 9.7 μM, standard error of the mean; SE = 1.4 μM and K_m(QToF-MS) = 15.8 μM (SE = 0.9 μM) show that the method used to analyze the kinetic experiments has an impact on the determined K_m value. Despite the difference in the K_m values determined with the two methods, the examined reaction has an efficiency comparable to other steroid-reductions catalyzed by AKR1C3 reported in the literature [12,16]. The reactions of steroid substrates are relatively inefficient compared to other substrates converted by AKR1C3, e.g., 9,10-phenanthrenequinone (K_m = 1.5 ± 0.7 μM) [12,17]. This may explain why the spectro-fluorometric method showed good results for the K_m determination, but not for inhibition experiments. Initial velocities in the inhibition experiments were generally lower than those determined in the K_m experiments, resulting in lower sensitivities. The presence of indomethacin may be another explanation for the unsuitability of the spectro-fluorometric method for the inhibition experiment. In background reactions, we observed that large amounts of indomethacin led to quenching of the fluorescence intensity generated by NADPH. Generally, subtracting the background signal from the sample-signal should compensate for this difference. Nevertheless, we observed that in particular, background samples with high amounts of indomethacin resulted in higher initial velocities than the corresponding samples with the enzyme. As the evaluation of those data would result in negative enzyme activities and in activities far above 100%, the applied spectro-fluorometric method is not suitable to determine the IC_{50} value of the inhibition of AKR1C3 catalyzed reduction in Etio. Due to those difficulties in the spectro-fluorometric method, the IC_{50} was determined using mass spectrometry. The determined value of 4.8 μM (SE = 1.0 μM) was transformed into an inhibition constant (K_I) using the "IC_{50}-to-K_I-converter" [18]. Byrns et al. reported a competitive inhibition type of indomethacin on the reduction in androstenedione by AKR1C3 [12]. Assuming that the inhibition of the AKR1C3 catalyzed reduction in Etio follows the same pattern, the calculated K_I would be 2.2 μM. This result is in accordance with findings from the literature, where different substrates were used [12,19,20]. The determined K_I lies on the upper range of the therapeutic

blood-plasma/serum concentration of the drug (0.84–2.8 μM) [21], resulting in the hypothesis for the in vivo experiments that therapeutic doses of indomethacin may lead to relevant inhibition of AKR1C3.

3.3. In Vivo Administration Trial

As AKR1C3 plays a crucial role in the metabolism of EAAS, used as markers in doping control analysis, we assumed that the intake of therapeutic doses of indomethacin may lead to changes in the steroidal profile. Since the aim of this study was getting a first idea on the detectability of changes in the steroid profile after the intake of therapeutic doses of indomethacin over 14 days, only one male and one female volunteer were included in the study. Despite this limited number of volunteers, we are convinced that the presented results are relevant for the anti-doping community and other fields where steroid quantification is of importance. As the knowledge of so-called confounding factors is of paramount importance for the interpretation of steroid profiles [10], this study will be of value for improved interpretation of steroid profiles. To enhance the exploration of factors influencing the steroid profile, we want to share our findings to raise awareness and induce further in-depth research.

Statistical analysis of in vivo data showed significant changes between the groups (before, during and after indomethacin administration) for several EAAS in both volunteers (Figures 5 and 6). While in the male volunteer only four steroid profile markers changed significantly (A, And/T, And/Etio and 5αAdiol/5βAdiol), nine of the 11 markers changed in the female volunteer. This is in accordance with our previous study on the influence of ibuprofen intake on the steroid profile, where only a few steroid profile markers were affected in the male volunteer, while the majority of markers changed significantly in the female volunteer [22].

Due to the experimental setting, both individual and sexual differences may be explanations for this effect. However, sexual differences seem to be reasonable as an explanation. As females have lower concentrations of EAAS than males, their concentrations and concentration ratios monitored in the steroid profile of the Athlete Biological Passport are much more affected by exogenous and endogenous parameters (e.g., menstrual cycle, intake of contraceptives or emergency contraceptives, ethanol) [23,24]. Mullen et al. showed that the epitestosterone concentration changes significantly during the menstrual cycle, and hence the T/E and 5αAdiol/E ratios are significantly affected. Similar observations are true for the female volunteer in this study who did not take any oral contraceptives for at least six months before and during the study. In particular, the concentration of E, the T/E and the 5αAdiol/E ratios changed a lot over the whole collection time (Figure 6). The influence of physiological changes on these parameters may need further investigation. In a publication by Mareck-Engelke et al. [25] also considering the potential circadian rhythm and the menstrual cycle in females, the And/Etio ratio is reported to be the most stable, but also the 5αAdiol/5βAdiol ratios showed a coefficient of variation (CV) <30%. However, both ratios (A/Etio and 5αAdiol/5βAdiol) changed significantly in the female volunteer during indomethacin application. In addition, the concentrations of And and 5αAdiol increased significantly, while the concentration of 5βAdiol decreased significantly during the intake.

In the male volunteer, the picture is not as complex as in the female volunteer. The significant decrease in And/T ratio may be explained by the decrease in And during the intake of indomethacin. The 5αAdiol/5βAdiol ratio increased significantly during the intake of indomethacin, which is in accordance with the observations from the female volunteer.

For both volunteers, several steroid profile markers changed significantly under the influence of indomethacin but no criteria for a suspicious steroid profile during an anti-doping test were met for a sequence of successive samples. Nonetheless, selected concentrations and concentration ratios were significantly influenced by the intake of the drug. Additionally, the concentrations of indomethacin and its metabolites *O*-desmethylindomethacin and *N*-deschlorobenzoylindometacin were determined in the urine samples collected from the male volunteer to obtain some first information on the long-time influence of indomethacin on the steroid profile. It was found that the highest concentrations of indomethacin are detectable in the sample which was collected after the drug intake. The samples collected after this and before the next indomethacin administration contained much

lower concentrations (Figure 7). This is in accordance with the half-life of 3–11 h [21]. Based on the half-life, and since indomethacin and its monitored metabolites are only detectable in urine samples collected close to the intake of the drug, one would assume that no differences in steroid profile markers are observable between samples collected before and after indomethacin intake. Nevertheless, there are a few significant changes in the steroid profile detectable between samples collected before and after the administration of indomethacin (Figure 5G,J,L). Other profile markers are affected during the intake of the drug but show no significant difference between the groups during and after indomethacin intake (Figure 5A,C–E,K). An explanation of these effects may be a prolonged influence of indomethacin on AKR1C3 and thus also the steroidal profile. To further investigate the significance for doping-control analysis and to reassess our hypothesis a study with a higher number of volunteers is already in preparation.

In addition to the impact on results interpretation in anti-doping analysis, the presented results are also relevant in evaluating potential endocrine disrupting substances (EDS). Like several other NSAIDs, indomethacin has anti-inflammatory, anti-pyretic and analgesic effects. It inhibits the cyclooxygenase and hence the formation of prostaglandins (PGs) which are responsible for the manifestation of pain, inflammation and fever. The literature reports that many putative EDSs inhibit the PG synthesis [26]. Additionally, the literature also discusses the role of PG synthesis inhibition by indomethacin in the mechanism of endocrine disruption [27,28]. However, it is accepted that probably not only the inhibition of PG synthesis is involved. Previous studies showed that indomethacin interferes with the testosterone production in various models [26,27,29–32]. Barkay et al. reported that plasma-testosterone levels in oligospermic men decreased significantly after an intake of indomethacin (75 mg over 90 days) [30]. In comparison, Knuth et al. reported an investigation with an administration of a lower dose and shorter duration (25 mg indomethacin per day over 14 days, i.e., similar to our administration). No significant change was detectable in the serum testosterone concentration in young, healthy male volunteers. This is in accordance with our presented findings, where no significant changes in the urinary testosterone concentration were detectable in the male volunteer. In the female volunteer, where steroid profile markers were showed to be more easily affected, significant changes in the urinary testosterone concentrations were detectable during the intake of indomethacin compared to pre-administration. Future investigations could hence investigate whether higher daily doses or prolonged administrations of indomethacin would also affect testosterone levels in the male volunteer. Additionally, studies on the impact of indomethacin on AKRs were published [12,19], and in vitro experiments presented in our study showed the effect of indomethacin on the AKR1C3 catalyzed reduction in Etio. All investigations commonly show that indomethacin inhibits AKR1C3 in vitro. Among other catalyzed reactions, this enzyme plays a key role in the metabolism of androgens, estrogens and progestagens [16]. As AKR1C3 is implicated in some hormone dependent malignancies and endocrine disorders, drugs selectively inhibiting AKR1C3 are investigated to be used as cancer treatment or drugs treating endocrine disorders for patients where this enzyme is overexpressed [33]. Our work demonstrates that the inhibition of AKR1C3 by indomethacin alters selected markers of the urinary steroid profile in healthy volunteers. In the context of AKR1C3 involvement in androgen, estrogen and progestin metabolism, an effect on estrogens may also be hypothesized. Based on our study and on findings from the literature, the risk potential of indomethacin and its role as endocrine disruptor should be reinvestigated.

4. Materials and Methods

4.1. Materials

Androst-4-ene-3,17-dione (AED), 17β-hydroxyandrost-4-en-3-one (testosterone; T), 17α-hydroxyandrost-4-en-3-one (epitestosterone; E), 3α-hydroxy-5α-androstan-17-one (androsterone; And), 5α-androstane-3,17-dione (5α-androstanedione; 5αAD), 17β-hydroxy-5α-androstan-3-one (5α-dihydrotestosterone; 5αDHT), 5α-androstane-3α,17β-diol (5αAdiol), 3α-hydroxy-

5β-androstan-17-one (etiocholanolone; Etio), 5β-androstane-3,17-dione (5β-androstanedione; 5βAD), 17βhydroxy-5β-androstan-3-one (5β-dihydrotestosterone; 5βDHT), 5β-androstane-3α,17β-diol (5βAdiol) and 17α-methyltestosterone (MeT; used as internal standard) were from Steraloids (Newport, RI, USA), Sigma-Aldrich (Milano, Italy) or TCI (Eschborn, Germany). Deuterated standards T-d3, E-d3, And-glucuronide-d4, Etio-d5, 5αAdiol-d3, 5βAdiol-d5 were obtained from the National Measurement Institute (NMI, Lindfield, NSW, Australia). Indomethacin, *O*-desmethylindomethacin and *N*-deschlorobenzoylindomethacin used as reference compounds for the excretion study were all purchased from TRC (Toronto Research Chemicals, North York, ON, Canada). Probenecid as internal standard was from Sigma Aldrich (Milan, Italy). Indomethacin, used as inhibitor for in vitro experiments, was of European Pharmacopoeia (Ph. Eur.) quality and purchased from Sigma-Aldrich (Steinheim, Germany). For the in vitro assay, solutions of different concentrations of the substrate Etio (20 µM, 100 µM, 200 µM, 400 µM, 1 mM, 2 mM) and the inhibitor indomethacin (6 µM, 20 µM, 60 µM, 200 µM, 600 µM, 2 mM) were prepared from stock solution by dilution of the solid substance in DMSO in the case of Etio and in ethanol in the case of indomethacin. Solvents (ethanol, acetonitrile, DMSO, tert-butyl methyl ether (TBME), methanol or ethyl acetate) and reagents (sodium phosphate and sodium hydrogen phosphate, potassium carbonate and sodium hydrogen carbonate, acetic acid and sodium acetate) were of analytical, high performance liquid chromatography (HPLC) or of high performance liquid chromatography-mass spectrometry (HPLC-MS) reagent grade and were purchased from Merck (Darmstadt, Germany), Carl Roth (Karlsruhe, Germany), Honeywell Fluka (Milan, Italy) or Sigma Aldrich (Milan, Italy). NADPH regenerating system solution A and solution B were used for the overview incubations and were purchased from Corning Gentest (Woburn, MA, USA). The cofactor for the enzymatic incubations to determine the kinetic values K_m and IC_{50} was NADPH-tetra-sodium salt (NADPH-Na_4) and was purchased from Carl Roth (Karlsruhe, Germany). The human recombinant enzyme AKR1C3 (expressed in *Escherichia coli* (*E. coli*, catalogue number: NBC1-21051) originated from Novus Biologicals Europe (Abingdon, United Kingdom). Phosphate buffered saline (PBS, 0.5 M, pH 7.4) from Corning Gentest (Woburn MA, USA) was diluted and used for all in vitro experiments. The preparation of β-glucuronidase from *E. coli* was from Roche Diagnostic (Mannheim, Germany). *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) was supplied by Chemische Fabrik Karl Bucher GmbH (Waldstetten, Germany), ammonium iodide (NH_4I), mercaptoethanol and ethanthiol were from Sigma Aldrich (Milano, Italy or Taufkirchen, Germany). Water was obtained from a "MilliQ" (Millipore S.p.A., Milano, Italy) or from "SG LaboStar" (Guenzburg, Germany) water purification system.

4.2. In Vitro Qualitative Incubation

Incubations were performed in duplicate in parallel to a background sample where the volume of enzyme was substituted by PBS 0.1 M. NADPH regenerating system (consisting of 1.3 mM $NADP^+$, 3.3 mM glucose-6-phosphate and 3.3 mM magnesium chloride and 0.4 U/mL glucose-6-phosphate dehydrogenase in final assay), AKR1C3 (0.64 µM in final assay) and PBS 0.1 M were added to a 1.5 mL reaction tube, centrifuged briefly and prewarmed at 37 °C for 5 min. AED, And, 5αAD, 5βAD or Etio (dissolved in methanol or acetonitrile; concentration in stock solution 1 mg/mL) were added as substrates (final concentration in assay: steroid ~50 µM, solvent 1.5% (*v*/*v*)). After brief centrifugation, incubation was carried out for 5.5 h at 37 °C. Incubation was terminated by the addition of 200 µL cold acetonitrile and samples were extracted immediately or stored at −20 °C until extraction.

4.3. In Vitro Kinetic Assay

All incubations were performed in triplicate. The incubations were carried out in 96-well plates (black, flat black bottom, Sarstedt, Nümbrecht, Germany) and analyzed spectro-fluorometrically in real-time. After incubation, the entire assay-volume was transferred to a 0.5 mL reaction tube and 200 µL of cold acetonitrile were added to denature the enzyme and thereby end the reaction. Samples were stored at −20 °C until confirmative GC-QToF analysis was performed. For each experiment

NADPH solution (in PBS 0.1 M) was prepared freshly. Ethanol or indomethacin dissolved in ethanol, NADPH (12 µM in final assay) in PBS 0.1 M, AKR 1C3 (1.03 µM in final assay) and PBS 0.1 M were added to the appropriate location on the well-plate. The mixture was prewarmed for 3 min at 37 °C under agitation. After the addition of Etio, the plate was centrifuged briefly, and incubation was performed for 1000 s in the plate reader. Concentrations of Etio for Michaelis-Menten-constant (K_m)-determination were 1 µM, 5 µM, 10 µM, 20 µM, 50 µM or 100 µM in the final assay. In experiments to evaluate the half maximal inhibitory concentration (IC_{50}), Etio was used in a concentration slightly below the determined K_m (7 µM in final assay) throughout. Indomethacin (dissolved in ethanol) was added, resulting in concentrations of 0.1 µM, 0.3 µM, 1 µM, 3 µM, 10 µM, 30 µM or 100 µM in final assay.

4.4. Administration Study

Two healthy volunteers, one male and one female (additional information on volunteers in Table 1), were treated with indomethacin (Indoxen 25 mg; Recordati S.p.A.; Milan, Italy) for 14 days. Urine samples were collected 4 times per day for five days prior to the treatment and the whole treatment phase (sampling points: 07:00 h; 13:00 h, 18:00 h and 22:00 h) and one time per day after the treatment phase (sampling point 13:00 h over 11 days for male volunteer and 8 days for female volunteer). Urine samples were anonymized and stored in sterile plastic tubes at −20 °C until analysis. The study was approved by the "Comitato Etico Lazio 1" with the reference number: 1553/CE Lazio 1. All volunteers gave informed consent.

Table 1. Additional information on male and female volunteer included in this study.

	Male Volunteer	Female Volunteer
Age	29	30
Weight	63 kg	60 kg
Hight	172 cm	178 cm
BMI	21.3 kg/m^2	18.9 kg/m^2
Dietary habits	Normal diet (no restriction)	Normal diet (no restriction)
Training	Three times/week (swim and run)	No
Oral contraceptive	Ø	No

4.5. Sample Preparation before Chromatographic Analysis

In case of in vitro samples (overview incubations and kinetic experiments), MeT (in methanol; 10 µL of 1 g/L-solution for overview study or 10 µL of 0.1 g/L-solution for kinetic study) was added. Samples were centrifuged (at 9660× *g*) to sediment the denatured enzyme. Supernatant was transferred to fresh glass-tubes and 1 mL PBS 0.1 M and 5 mL TBME (in case of kinetic experiments) or 2 mL ethyl acetate (in case of the overview incubations) were added.

For all urine samples, specific gravity was determined, to normalize steroid and NSAID concentrations according to the WADA technical document TD2018EAAS, using a digital refractometer RM40 (Mettler Toledo, Novate Milanese, Italy) [3]. Steroids (free and glucuronide fraction) were extracted using an already established protocol for steroid profiling of endogenous steroids, which is used routinely in the Italian anti-doping laboratory, Rome [34–36]. In brief, 2 mL of urine, 30 µL of β-glucuronidase, 750 µL of 0.8 M PBS and 50 µL of internal standard (T-d3: 100 ng/mL, E-d3: 25 ng/mL, And-d4 glucuronide: 200 ng/mL, Etio-d5: 200 ng/mL, 5αAdiol-d3: 50 ng/mL, 5βAdiol-d5: 50 ng/mL, MeT: 250 ng/mL) were mixed and heated for 1 h at 55 °C. After hydrolysis, 500 µL of carbonate/bicarbonate buffer (20% (*w*/*v*), pH 9) and 5 mL TBME were added.

For the extraction of indomethacin and its metabolites, 1 mL of urine, 50 µL probenecid (1 µg/mL) as internal standard, 30 µL of β-glucuronidase and 500 µL PBS 0.8 M were added and hydrolysis was achieved after 1 h of incubation at 55 °C. Before extraction, 500 µL sodium acetate buffer (pH 5) and 5 mL TBME were added.

For in vitro and urine samples, liquid-liquid extraction was performed during agitation on an automated shaker. Samples were centrifuged briefly (relative centrifugal force was 1008× *g*) to separate the phases. The organic layer was transferred to new glass tubes after 5 min freezing in an ethanol bath (−25 °C) or overnight at −20 °C in the freezer. The solvent was evaporated under nitrogen at elevated temperatures and derivatization was performed at 75 °C for 30 min with 50 µL MSTFA/mercaptoethanol/NH_4I (1000:6:4 *v*/*v*/*w*; in case of in vitro kinetic samples and urine samples) or MSTFA/ethanthiol/NH_4I (1000:3:2 *v*/*v*/*w*; for overview incubations).

4.6. Fluorometric Analysis

Spectro-fluorometric measurements to determine the kinetic values of the enzymatic reaction were carried out on a Safire II microplate reader (Tecan, Männedorf, Switzerland) in top-read mode. Excitation wavelength was 340 nm, emission wavelength was 450 nm with both bandwidths set to 5 nm. For all experiments, gain was 120 and Z-position was 10,300 µm. Time between move and flash was 10 ms, integration time 40 µs and 0 µs lag time. In the beginning of all experiments performed on the plate reader, a calibration with 8 different concentrations of NADPH in PBS 0.1 M was performed. For each concentration, 200 µL of NADPH solution were pipetted to 10 individual wells and each well was measured with the same method used for kinetic experiments. The resulting calibration curve was tested for linearity (Mandel-test, p = 99%), homogeneity of variances following DIN 38 402 A51 (p = 99%, including Grubbs outlier-test) and limit of quantification (LOQ) was determined following DIN 32 645 (p = 95%, confidence level: 95%).

4.7. GC-MS Analysis

Samples originating from overview incubations were analyzed on an Agilent 7890A GC-System coupled to an Agilent 5975C MSD (Agilent Technologies, Santa Clara, CA, USA). Injection volume was 2 µL with split injection in a ratio of 1:10. Injection temperature was set to 300 °C. Chromatographic separation was achieved on an Agilent HP1 column (length: 17 m; diameter: 0.2 mm; film-thickness: 0.11 µm) with helium as carrier gas and a flow-rate of 1 mL/min. Oven program started at 193 °C with 3 °C/min heating rate to 215 °C followed by a ramp with 40 °C/min to 310 °C and hold for 1 min. Ionization was electron ionization (EI) and analysis was performed in scan mode with acquisition from *m*/*z* 40 to *m*/*z* 750.

4.8. GC-QToF Analysis

Instrumental analysis of kinetic in vitro samples was performed on an Agilent GC-QToF 7890B/7200 (Agilent Technologies, Milano, Italy), equipped with an Agilent HP1 column (length: 17 m; diameter: 0.2 mm; film-thickness: 0.11 µm) with helium as carrier gas and a flow of 0.8 mL/min. Injection was performed in split mode with a 1:10 ratio at 280 °C. The oven program had the following heating rates: 188 °C hold for 2.5 min, 3 °C/min to 211 °C and hold for 2 min, 10 °C/min to 238 °C, 40 °C/min to 320 °C and hold for 3.2 min. The coupled QToF was operated in full scan with an EI source and ionization energy of 70 eV. Ions were detected from *m*/*z* 50 to *m*/*z* 750.

Calibration was performed with 9 different concentrations of 5βAdiol with fixed concentrations of MeT. Each calibration level was measured twice. Calibration was tested for linearity applying the Mandel Test, tested for outliers (no residuals >3 standard deviations) and weighted with a factor 1/x. A signal to noise ratio of 10 to 1 was regarded as LOQ. Samples with signals below the LOQ were regarded as not containing any analyte.

4.9. GC-MS/MS Analysis

For urinary steroid profile analyses, a previously described method was used [34–36]. It is validated and currently used in routine analysis for the detection and quantification of pseudo-endogenous steroids in the Italian anti-doping laboratory in Rome and accredited under ISO17025. In brief, analyses were performed on an Agilent GC-MS/MS 7890 A/7000 (Agilent Technologies, Milano, Italy) equipped

with an Agilent HP1 column (length: 17 m; diameter: 0.2 mm; film-thickness: 0.11 µm). The carrier gas was helium with a flow rate of 1 mL/min, and the injection and transfer line temperature were set to 280 °C and injection was performed in split mode with a ratio of 1:20. The oven program was as follows: 180 °C hold for 4.5 min, 3 °C/min to 230 °C, 20 °C/min to 290 °C and 30 °C/min to 320 °C. Ionization was achieved by EI and ionization energy was 70 eV. Analyses were performed in multiple-reaction monitoring mode (MRM mode) with transitions used in the reference method. Quantitation of the urinary steroids was based on the peak area ratio of the analyte to the corresponding internal standard. Calibration and quality control samples were prepared in synthetic urine according to previously published methods [37].

For quantification of indomethacin and its metabolites, a similar method was used. Aberrantly, the oven program was 85 °C initial temperature hold for 1 min, 15 °C/min to 320 °C. Transitions for analyzed compounds are listed in Table 2. Selectivity, linearity, repeatability, recovery and limit of detection (LOD) were tested for the evaluation of method suitability (data not shown).

Table 2. Transitions for GC-MS/MS analysis of indomethacin and its metabolites *O*-desmethylindomethacin and *N*-deschlorobenzoylindomethacin and the internal standard probenecid.

Analyte	Precursor Ion [*m*/*z*]	Product Ion [*m*/*z*]	Collision Energy [eV]
Indomethacin (mono-TMS)	429	139; 246; 312	30; 30; 30
O-desmethylindomethacin (bis-TMS)	487; 304	139; 216; 232	30; 30; 30
N-deschlorobenzoylindomethacin (bis-TMS)	363; 348; 246	246; 320; 174	30; 10; 30
Probenecid (mono-TMS)	178; 342	104; 268	20; 20

4.10. Data Analysis of Kinetic Values

To determine the Michalis-Menten constant, initial velocities of the enzymatic reactions were plotted against the concentration of Etio. Nonlinear regression was applied to the data using OriginPro, Version 2019 (OriginLab Corporation, Northhampton, MA, USA). For spectro-fluorometric measurements, the loss in fluorescence intensity over time curves were transformed in loss of NADPH-concentration over time using a calibration curve. By applying linear regression to those concentration-time curves, the initial velocity was determined. Initial velocities for the individual samples were corrected with the background velocities. For mass spectrometric data the determined concentration of formed product (5βAdiol) over time of incubation was regarded as initial velocity. As no product was present in background incubations, no correction of this value was performed. GC-QToF data were analyzed using Agilent MassHunter Workstation Quantitative Analysis for TOF (Version 10.1; Agilent Technologies, Santa Clara, CA, USA).

To determine the IC_{50} values of the inhibition experiments, activity of the enzyme was plotted against logarithmic concentrations of the inhibitor indomethacin in the assay. The activity of the enzyme was calculated as a quotient of the initial velocities of the sample and the initial velocities of the positive control (no inhibitor added). Nonlinear regression was performed using OriginPro, Version 2019.

4.11. Data Analysis of Steroid Profiling and Indomethacin Detection in Urine Samples

Measured concentrations of EAAS, indomethacin and its metabolites were normalized for the specific gravity of the sample according to the WADA technical document TD2018EAAS [3]. Samples with a specific gravity at or below 1.001 were excluded from the analysis. For samples in which selected analytes had concentrations below the limit of quantification (LOQ), the analyte in question and its related ratios were excluded from analysis.

For the steroid-profiling samples of each volunteer were divided into three groups: before treatment with indomethacin (n = 20) during the administration (n = 56 for male volunteer; n = 55 for female volunteer due to exclusion of one sample where specific gravity was 1.001 and n = 54 for female volunteer for E, T/E, 5αAdiol/E because concentration of E was below LOQ in this sample) and after

indomethacin intake (n = 11 for male volunteer and n = 8 for female volunteer). The data were regarded as independent from each other. All measured concentrations belonging to one group (per volunteer, per substance, and phase during treatment) were tested for normal distribution (Shapiro Wilk test, $\alpha = 0.05$). In case of normal distribution the homogeneity of variances between the groups was also tested (F-Test, $\alpha = 0.05$). If the hypothesis of normal distribution was not rejected for both groups examined, a two-sided parametric significance test for independent samples was applied. In case of homogeneity of variances between groups, a two-sided t-test for independent groups was applied. In case of rejection of homogeneity of variances between groups, Welch-Test was used. When for at least one of the two examined groups the null-hypothesis of normal distribution had to be rejected, the less powerful non-parametric Mann-Whitney U-Test was applied. For all significance tests (parametric and non-parametric) the significance level was $\alpha = 0.0167$ to deal with multiplicity problems of statistical tests [38].

Normalized concentrations of indomethacin and its metabolites were plotted against the time after first dose of indomethacin was administered.

5. Conclusions

The presented work shows the complexity of steroid metabolism in vitro and in vivo. It furthermore reveals the advantages and drawbacks of different analysis methods for in vitro enzymatic assays. A combination of in vitro and in vivo experiments was performed showing the interference of the non-steroidal anti-inflammatory drug indomethacin with androgen metabolism and the effect of indomethacin on the urinary steroid profile. The study provides results which can help to interpret steroid profiles in anti-doping analysis. The presented results may also be relevant for other fields of forensic or clinical toxicology and for the investigation of endocrine disrupting substances (EDS). Future investigations on long-term effects or the influence of higher doses of indomethacin may extend our findings.

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"Influence of indomethacin on steroid metabolism: Endocrine disruption and confounding effects in urinary steroid profiling of anti-doping analyses"

Supplement S1: Chromatograms of in vitro qualitative incubations

Anna Stoll [1], Michele Iannone [2], Giuseppina De Gregorio [2], Francesco Molaioni [2], Xavier de la Torre [2], Francesco Botrè [2,3] and Maria Kristina Parr [1,*]

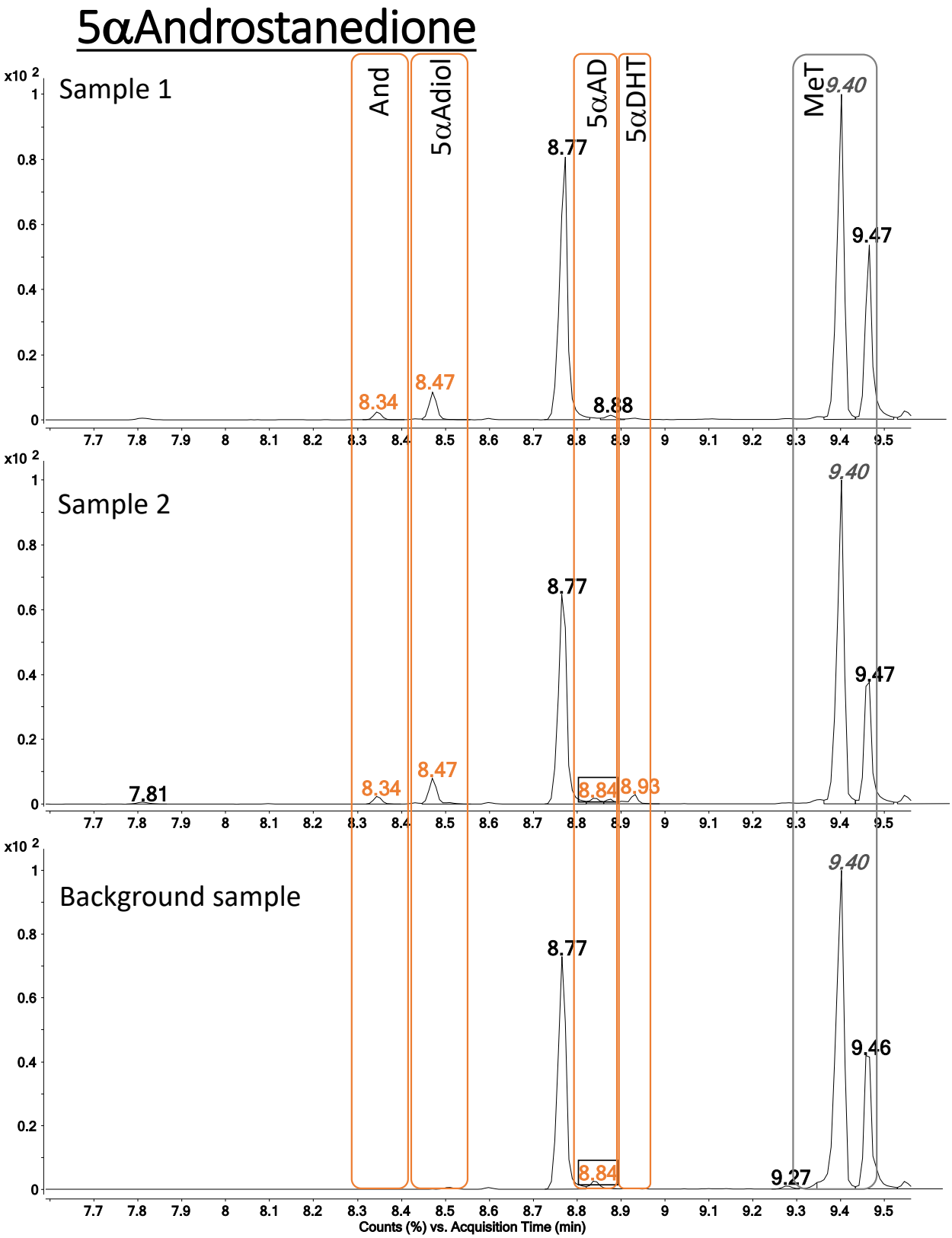
[1] Freie Universität Berlin, Institute of Pharmacy (Pharmaceutical and Medical Chemistry), 14195 Berlin, Germany; anna.stoll@fu-berlin.de (A.S.); maria.parr@fu-berlin.de (M.K.P.)
[2] Laboratorio Antidoping Federazione Medico Sportiva Italiana, 00197 Rome, Italy; micheleiannone14@gmail.com (M.I.); degregorio.giuseppina@gmail.com (G.D.G.); molaioni@gmail.com (F.M.); xavier.delatorre@gmail.com (X.T.)
[3] ISSUL – Institute del sciences du sport, Université de Lausanne, Synathlon – Quartier Centre, 1015 Lausanne, Switzerland; Francesco.Botre@unil.ch (F.B.)
* Correspondence: maria.parr@fu-berlin.de; Tel.: +49-30-838-51471

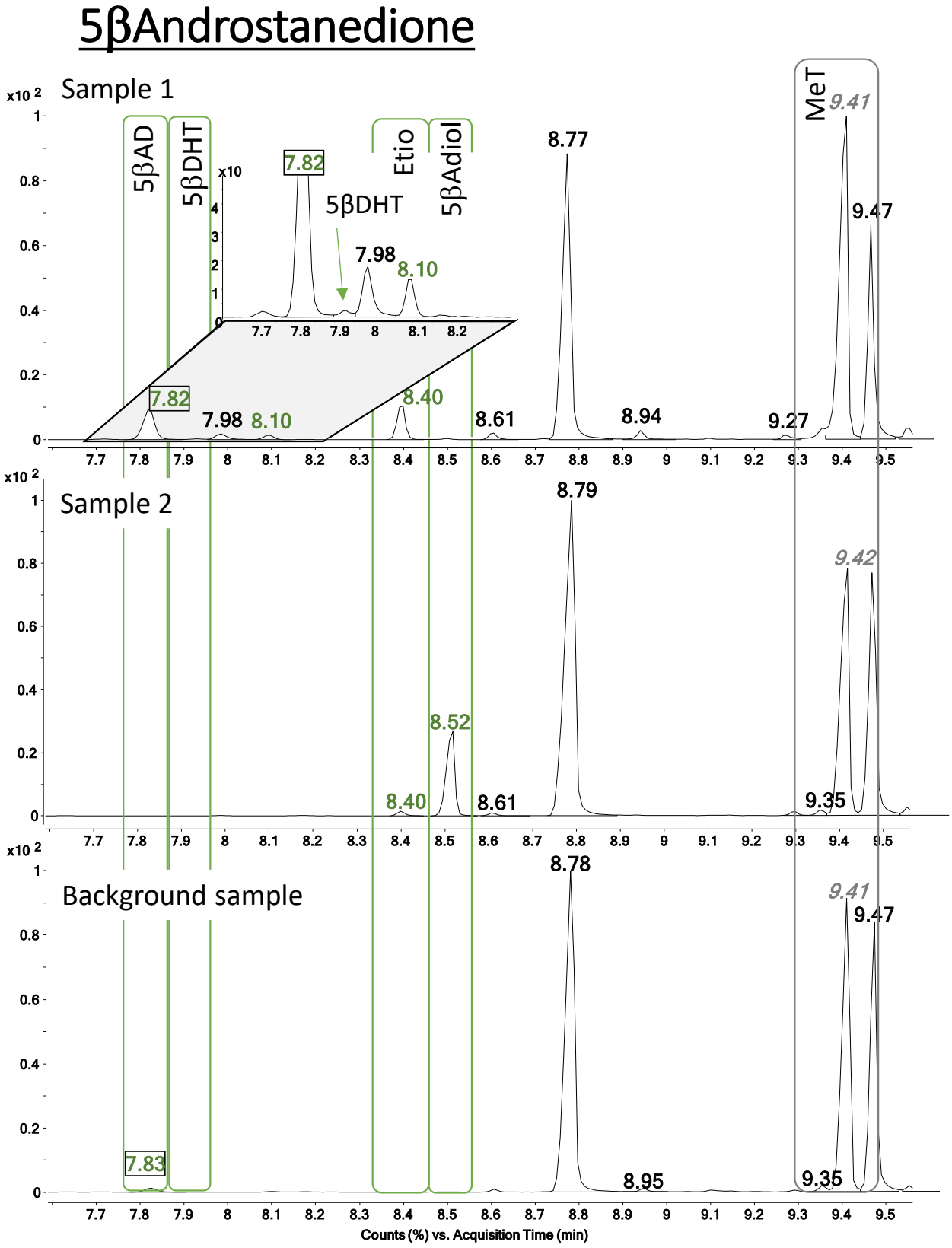
The following document displays chromatograms obtained after incubation of the endogenous anabolic androgenic steroids (EAAS) 5α-androstanedione, 5β-androstanedione, androstenedione, androsterone and etiocholanolone with isolated recombinant human aldo-keto reductase (AKR) 1C3.

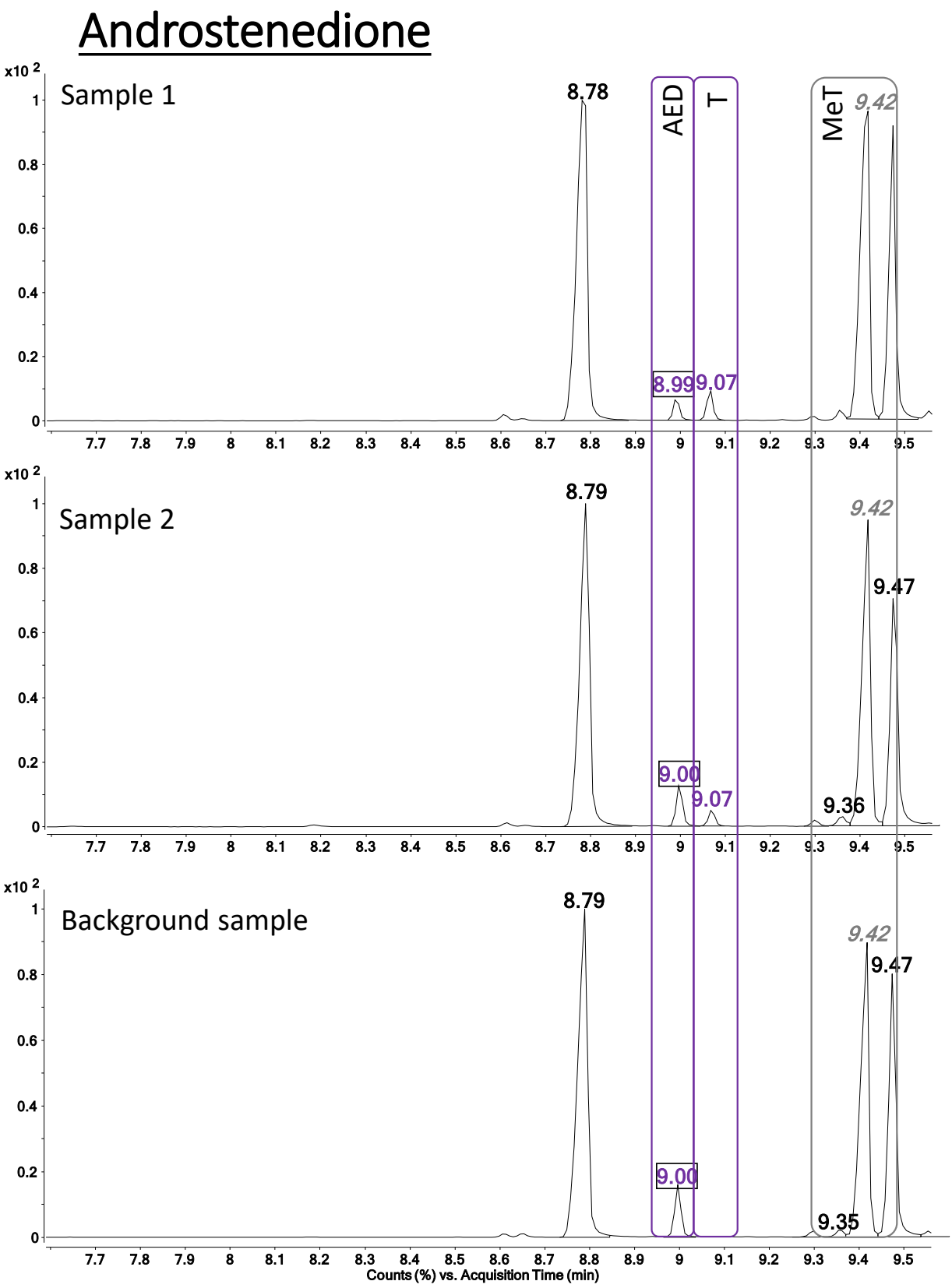
Similarely to the main document all 5α-androstanes are highlighted in organge, all 5β-androstanes are highlighted in green, testosterone and androstenedione are highlighted in violett and the internal standard methyltestosterone (MeT) is highlighted in grey.

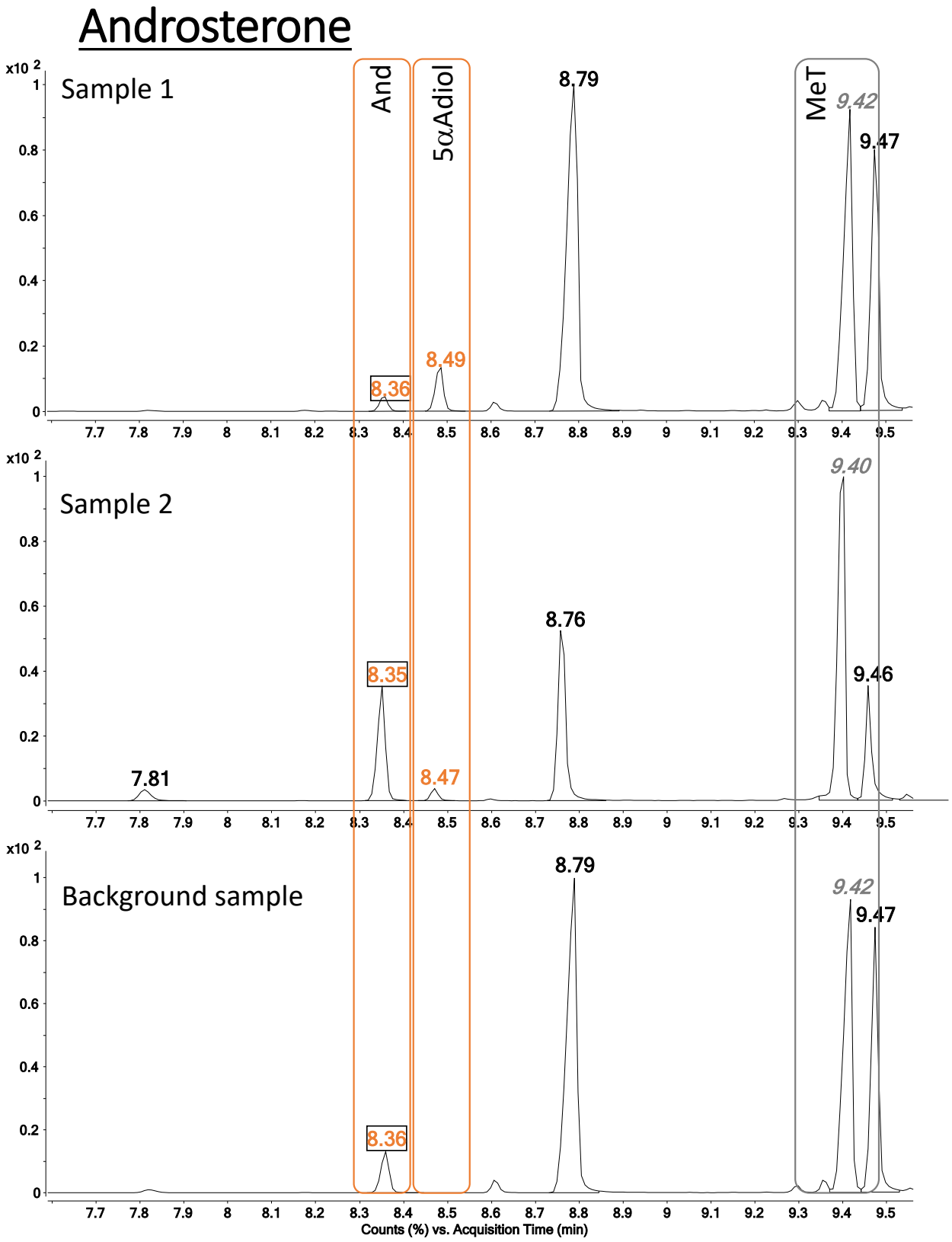
To simplify the graphics, substrates are highlighted by framed retention times (RT) and all detected EAAS are labeled with abbreviations: And: androsterone, 5αAdiol: 5α-androstanediol, 5αAD: 5α-androstanedione, 5αDHT: 5α-dihydrotestosterone, Etio: etiocholanolone, 5βAdiol: 5β-androstanediol, 5βAD: 5β-androstanedione, 5βDHT: 5β-dihydrotestosterone, T: testosterone, AED: androstenedione.

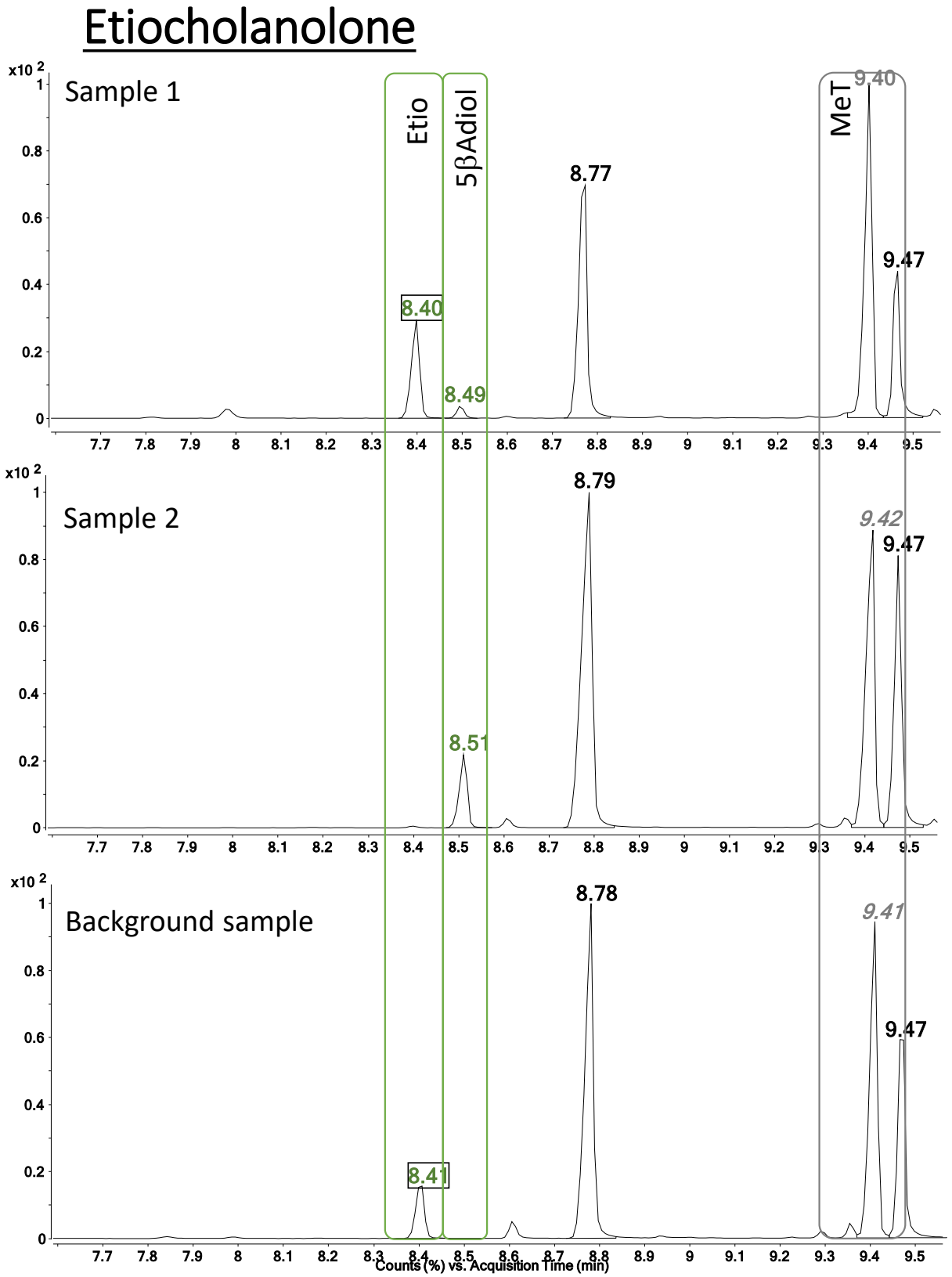
Comment: As the peak corresponding to 5βDHT in sample 1 (incubation of 5βAD with AKR1C3) is very small, an enlarged segment of the chomatogram is provided.


5αAndrostanedione
Sample 1
Sample 2
Background sample
And
5αAdiol
5αAD
5αDHT
MeT
x10 2
8.77
9.40
9.47
8.34
8.47
8.88
7.81
8.84
8.93
9.46
9.27
Counts (%) vs. Acquisition Time (min)


5βAndrostanedione
Sample 1
5βAD
5βDHT
Etio
5βAdiol
MeT
7.82
5βDHT
7.98
8.10
8.40
8.61
8.77
8.94
9.27
9.41
9.47
Sample 2
8.79
8.52
8.40
8.61
9.42
9.35
Background sample
7.83
8.78
8.95
9.41
9.47
9.35
x10 2
x10
Counts (%) vs. Acquisition Time (min)


Androstenedione
Sample 1
8.78
AED
T
MeT
9.42
8.99
9.07
Sample 2
8.79
9.42
9.47
9.00
9.07
9.36
Background sample
8.79
9.42
9.47
9.00
9.35
x10 2
Counts (%) vs. Acquisition Time (min)


Androsterone
Sample 1
And
5αAdiol
MeT
8.79
9.42
9.47
8.49
8.36
Sample 2
9.40
8.76
8.35
9.46
7.81
8.47
Background sample
8.79
9.42
9.47
8.36
x10 2
Counts (%) vs. Acquisition Time (min)


Etiocholanolone
Sample 1
Etio
5βAdiol
MeT
8.40
8.49
8.77
9.40
9.47
Sample 2
8.51
8.79
9.42
9.47
Background sample
8.41
8.78
9.41
9.47
x10 2
Counts (%) vs. Acquisition Time (min)

4 DECLARATION OF OWN CONTRIBUTION

In the following, the author´s contribution to the individual publications, which are included in this cumulative work, are disclosed:

Manuscript I:

- Conception, design and execution of metabolite purification together with co-authors
- Metabolite characterization together with co-authors
- Revision of manuscript in cooperation with co-authors

Manuscript II:

- Conception and design of the experiments in cooperation with co-authors
- Synthesis of compounds used as substrates for subsequent biotransformation
- Execution and evaluation of biotransformation experiments with co-authors
- Analysis and evaluation of data in cooperation with co-authors
- Cooperation for manuscript preparation and revision

Manuscript III:

- Design of *in vitro* protocols
- Conception and execution of *in vitro* experiments
- Analysis and evaluation of results from *in vitro* experiments
- Evaluation of *in vivo* data in cooperation with co-authors
- Preparation of manuscript and revision in cooperation with co-authors

Manuscript IV:

- Design of *in vitro* protocols
- Conception and execution of *in vitro* experiments
- Analysis and evaluation of results from *in vitro* experiments
- Evaluation of *in vivo* data in cooperation with co-authors
- Preparation of manuscript and revision in cooperation with co-authors

5 Discussion and Outlook

The results presented in this work are of interest in scientific areas performing steroid analysis or in fields where the knowledge of steroid metabolism and its influenceability is of important relevance. This includes but is not limited to forensic toxicology, clinical and endocrinological analysis and trace analysis (e.g. in wastewater or dietary supplements). The central topic of this work, however, is anti-doping research, which is intended to exemplify the relevance of the results presented. Some major challenges in the detection of AAS misuse in sport will be illustrated based on a publication by Geyer *et al.* [80]. The impact of the presented work on the mentioned challenges will be discussed in a first step. Subsequently, broader applications will be assessed to address the two major aims of this work. The first aim is to illustrate the relevance of studies on the steroid metabolism and its influenceability for a broader community. The second aim is the provision of helpful insights to enhance currently used methods to detect the misuse of anabolic androgenic steroids and hence to improve the detection of prohibited performance enhancing substances in sports doping. The fundamental knowledge on the general testing procedure for the detection of AAS administration used in anti-doping analysis is favourable to identify challenges in the testing process. Chapter 2.3 gives a deeper insight in the whole process. However, to facilitate the understanding of this discussion the basic steps will be repeated.

The basis for the detection of AAS misused as doping substances is steroid profiling. Therefore, concentrations and concentration ratios of EAAS are determined in urine samples collected from the athletes. Results are reported to the steroidal module of the athlete biological passport. Intake of pseudo endogenous AAS and long-term administration of synthetic AAS leads to steroid profile alterations which allows the detection of the prohibited intake of these substances. Hence, by evaluating alterations of specific endogenous steroids used as markers, a broad number of known, as well as unknown analytes are detectable in an indirect manner [80]. In addition to the prohibited intake of AAS, altered steroid profiles may be caused by other endogenous or exogenous factors, as already mentioned in Chapter 2.3.4. Therefore, anti-doping laboratories monitor the presence of known confounding factors, meaning conditions influencing markers monitored in the steroid profile, to interpret testing results more accurately. Also non-prohibited drugs have been reported to leading to steroid profile alterations mainly by interfering with the steroid metabolism [91, 94, 104, 107]. If changes in the steroid profile are interpreted in a wrong manner, they may adulterate testing results in doping control and hence cause false decisions. The awareness of substances causing steroid profile alterations helps to prevent incorrect decisions and save cost- and time-consuming confirmative analysis in doping control by tracing back atypical profiles to the presence of specific confounding factors (e.g. intake of drugs).

The use of the ABP and hence the calculation of individual limits for each athlete and each marker of the steroidal profile made it possible to detach analytical results from individual

endogenous factors influencing the steroid profile (e.g. sex, age and genetic factors). Nonetheless, research on confounding factors that do not have an endogenous origin (e.g. medication or stress) is crucial for interpretation of results, as they are not adequately recorded by the ABP and therefore possibly cause divergent analysis results. One example may be a potential scenario from anti-doping analysis: the occasional application of drugs influencing the steroid metabolism. The infrequent intake of such drugs may cause testing results outside of the individual limits calculated by the adaptive model of the ABP and hence cause a suspicious or atypical testing result. Another scenario from anti-doping analysis may be the repeated or regular intake of non-prohibited drugs interfering with the steroid profile. This potentially causes steroid profile alterations which are still in the individual limits calculated by the adaptive model but may cause higher variations and hence lead to broader individual limits as it would be the case without the intake of these drugs. A potential intake of pseudo endogenous AAS, especially when administered in micro doses only, may hence be missed. Consequently, investigations on factors interfering with the endogenous steroid profile are of paramount relevance.

As already mentioned in Chapter 2.1.2, the influence on metabolic reactions of endogenous compounds is one key characteristic of endocrine disrupting substances. Investigations on substances influencing the metabolism of EAAS may hence contribute to a renewed risk-assessment of drugs. Based on the same mechanism, these substances interfering with certain metabolic steps can be used in the development of novel drugs. Thereby the endocrine disrupting effect of these substances is used to suppress the production of specific endogenous steroids. With 5α-reductase inhibitors and aromatase inhibitors two classes of substances currently used as therapeutic drugs are mentioned exemplarily. Furthermore, the knowledge of confounding factors leading to divergent analytical results is of relevance in all fields where steroid detection and quantification is performed.

Manuscript III and **Manuscript IV** are investigating effects of ibuprofen and indomethacin on selected enzymatic reactions involved in the steroid metabolism *in vitro* and on selected endogenous steroid concentrations and ratios, used as markers in the steroidal profile of the ABP, *in vivo*. NSAIDs are very frequently used not only in athletes [15, 27, 109-111]. Hence, the presented results may be of important relevance for the evaluation of confounding factors in anti-doping analysis as well as in other fields where steroid metabolism and the detection of intermediates in the steroid metabolism plays a role. To further illustrate the relevance for anti-doping analysis one example for the previously described scenario of chronical intake of potentially confounding substances may be an athlete regularly administering NSAIDs. The consequences for the health and the spirit of sport are discussed in the recently broadcasted German documentary "Geheimsache Doping - Hau rein die Pille" [108]. The results from this thesis present that, besides the risk for health and the violation of the spirit of sport discussed in the documentary, also steroid profile alterations may result from the intake of ibuprofen and indomethacin. Especially the

prolonged administration of higher doses may lead to androgen disbalances. Women in particular may be affected, as their androgen levels are naturally much lower than those of men. As mentioned earlier, NSAIDs are currently neither included in the WADA Prohibited List nor in the monitoring list and not even considered as confounding factors, yet. The results of Manuscript III and Manuscript IV hence provide a valuable basis for further investigations on the potential of ibuprofen and indomethacin to enhance performance in sport.

In a broader context the presented work demonstrates that the inhibition of enzymes involved in steroid metabolism is of relevance in the mechanism of steroid profile alteration, which may cause endocrine disruption. Endocrine disrupting effects had been described previously for NSAIDs (Chapter 2.1.2). The presented findings may hence be of relevance in reinvestigating the risk potential of NSAID intake. In this context the evaluation of potential interfering effects of NSAIDs on the metabolism of endogenous corticoids or on the pharmacokinetic of simultaneously taken glucocorticoid drugs would be interesting. As the application of NSAIDs and glucocorticoids is a very common therapeutical combination of drugs, the evaluation of potentially interfering effects may be of great value.

As mentioned earlier, the ability of substances to interact with the endogenous metabolism can be used to develop new drugs. Several publications show promising *in vitro* results on the use of NSAIDs and related compounds as possible new drug candidates in the treatment of cancer [112, 114, 115]. Findings from this thesis may help to get one step closer to this goal.

Finally, the two manuscripts illustrate, that results from *in vitro* experiments help to better plan and perform *in vivo* application trials (also discussed in Chapter 2.2). *In vitro* results can be used to estimate a suitable dose for subsequent application trials. Furthermore, expected changes in the *in vivo* metabolism may be postulated based on observations from *in vitro* experiments. This facilitates the subsequent analysis, as potentially expected analytes are already foreseen. Additionally, it was demonstrated that it is of paramount importance to include male and female volunteers in studies evaluating the metabolism of steroids. In both manuscripts it was observed, that detected changes in steroid profiles were not completely similar in the male and the female volunteer. As discussed previously and reported by literature as well, the steroid profile of females is more challenging to interpret and hence needs special attention and in-depth investigations. The knowledge gained from these two manuscripts may build a basis to improve interpretation of analytical results in future steroid analysis.

Besides the above-mentioned steroid profiling, additional methods are necessary for the identification of synthetic steroids and so called “designer steroids”. Usually screening methods are performed, monitoring the presence of drugs themselves and/or known (long-term) metabolites. Especially long-term metabolites are of interest for the detection, since substances which are no longer administered (e.g. stop of intake before a competition) may hence be detected as well. It is furthermore known, that the effect of AAS on the performance is prolonged [80]. A prolonged

detection of the substances is hence reasonable and desirable. For the new discovery of long-term metabolites, it is of paramount importance to perform metabolic studies. As mentioned earlier in this chapter and also in Chapter 2.2 *in vitro* studies should build the basis for subsequent *in vivo* trials. Results obtained from *in vitro* experiments can provide valuable information for the planning and execution of *in vivo* studies. Generally, two different approaches for the detection of new metabolites are discussed subsequently. First, procedures to detect metabolites of already known synthetic steroids are explained. Second, procedures to detect metabolites of unknown and potentially novel designer steroids are described.

In the first case, involving known synthetic steroids, metabolic studies with the compound of interest can directly be performed. *In vitro* experiments can be used to evaluate enzymatic contribution and give an idea on which metabolites may be expected *in vivo*. As the detection of metabolites in humans is the ultimate interest, only *in vivo* application trials or retro perspective studies can provide definite information on formed metabolites. For retro perspective studies samples which have been detected to contain the substance of interest with already existing methods in the past may be re-evaluated. This includes but is not limited to patient samples (for approved substances used as pharmaceuticals), samples tested positive for a specific doping compound or samples of previous application trials with the same compound but another research interest. One problem of retro perspective evaluations is that specific side-conditions possibly remain mostly unknown. This may include the time between the intake of the drug and the sample collection, the administered amount of drug or endogenous and exogenous factors potentially influencing the metabolism. In addition, for samples tested positive for a compound of interest, detected metabolites may also derive from another (potentially novel) compound and not from the one the sample was tested positive for. Hence, only tentative outcomes can be torn from this kind of study. To determine a definite detection window, which is important to know for long-term metabolites, a controlled *in vivo* trial is inevitable. Chapter 2.2.1 describes potential setups and some aspects to consider when applying non approved drugs, which is often the case for doping substances. Usually, samples are collected over a sufficiently long time after the intake of the drug. All samples are then analysed, potential new metabolites may be uncovered and their detection window determined.

Once a new long-term metabolite has been identified using *in vitro* and *in vivo* methods described above, the next challenge is the generation of reference material. Reference compounds are of paramount importance in all fields of analytical chemistry. Similarly to other areas, in anti-doping analysis reference substances are used to unambiguously identify an analytical compound and sometimes also quantify the compound of interest. Usually, quantification in anti-doping analysis is only required for so called threshold substances which means substances prohibited at amounts above a specific predefined concentration in the examined sample, which also includes

endogenous steroids. One important aspect for identification of analytes is that the reference material had been characterized adequately prior to utilization [60].

Manuscript I describes the combined chemical and biochemical synthesis and subsequent characterization using high-resolution mass spectrometry (HRMS) of 20OH-NorDHCMT (**21**), a long-term metabolite of the synthetic steroid Oral Turinabol (**22**). The use of biotechnological methods made it possible to relatively easy introduce a hydroxy group to the chemically synthesized precursor in a stereoselective way. The work shows that metabolic processes can help to simplify organic synthesis of reference compounds especially when stereoselectivity is of importance. The substances generated may then be used as reference compounds for the unambiguous identification and quantification in routine analysis.

As mentioned earlier, the second procedure for metabolite detection may be of relevance especially for potentially novel and hence unknown designer steroids. As the exact structure of these newly designed substances is mostly unknown, neither application trials nor retro perspective studies for detection of metabolites are applicable. Metabolic studies may thus help to transfer knowledge from related substances and therefor help to predict expected metabolites of designer steroids. The investigation on enzymes involved in the metabolite formation of already known steroids is therefore of paramount importance.

Manuscript II comprehensively evaluates structural requirements of CYP21A2 substrates. This enzyme had been reported by Parr *et al.* to play a role in the formation of long-term metabolites in a series of 17α-methyl steroids. However not all examined compounds showed the formation of 17α-methyl-17β-hydroxymethyl metabolites which may serve as potential long-term metabolites [33]. Manuscript II lines out strict requirements and accepted structural properties for the conversion of 17,17-dimethyl steroids to corresponding 17α-methyl-17β-hydroxymethyl metabolites by CYP21A2 using whole cell biotransformations with *S.pombe* strains recombinantly expressing human enzymes (described in detail in Chapter 2.2.2). The presented findings help to estimate whether CYP21A2 is of relevance in the metabolite formation of other (unknown designer) steroids. To do so, a potential procedure may be the analysis of mass spectra of known metabolites for characteristic fragments, as presented in the manuscript. Suspicious samples from doping control analysis may then be screened for analytes containing these characteristic fragments and potential elemental compositions obtained from HRMS data. This may build the basis to detect and postulate a structure for newly discovered designer steroids. Based on these structure proposals, promising compounds can be synthesized and compared with the unknown analyte for final confirmation. Similar approaches are conceivable in other fields where unknown analysis is of interest.

In conclusion, the results presented in this work contribute to the improvement of methods used for the detection of steroids not only in anti-doping analysis. This work shows that this can be achieved in different ways. First, by investigating factors which influence the endogenous

steroid metabolism and hence lead to altered steroid profiles. Second, by generating reference material which helps to unambiguously identify and quantify metabolites. And third, by investigating metabolic steps of known compounds and transferring this knowledge to other and potentially unknown compounds. Furthermore, the potential of metabolic studies to investigate the endocrine disrupting ability of drugs was demonstrated. The impact of this knowledge for further risk-assessment and drug development is discussed.

6 SUMMARY

This thesis addresses the steroid metabolism and its role in the detection of steroids. The results were achieved by performing metabolic studies in combination with different analytical techniques. Three main outcomes were achieved. First, the generation of reference material for a long-term metabolite of the synthetic steroid Oral-Turinabol (OT) was presented. The obtained results enhance unambiguous detection and quantification of this metabolite and hence help to uncover the prohibited OT administration in sports doping. Second, structural requirements and tolerated functionalities for substrates of the enzyme Cytochrome P450 subtype 21A2 (CYP21A2) were evaluated. These insights help to extrapolate known metabolic reactions to new and potentially unknown compounds. Third, investigations on steroid profile changes caused by the intake of non-steroidal anti-inflammatory drugs were described. These findings provide knowledge on substances influencing steroid metabolism, which is of high relevance for accurate steroid determination and hence the correct interpretation of analytical results.

All results were obtained using mainly three models to study steroid metabolism and its influenceability. First, incubations with *Schizosaccharomyces pombe* strains recombinantly expressing human CYPs were used to generate reference material in small amounts and to evaluate metabolic reactions. Second, incubations with isolated recombinantly expressed human enzymes were used to examine metabolic reactions and their influenceability by other drugs. Third, *in vivo* drug administration was used to study steroid metabolism in humans and to investigate whether the intake of non-steroidal anti-inflammatory drugs leads to changes in steroid concentrations and concentration ratios. To subsequently evaluate the samples generated by the methods described above different analytical techniques were utilized. High-resolution mass spectrometry (HRMS) was used for quantification and tentative structure identification. Liquid chromatography and gas chromatography were both combined with HRMS. Additionally, gas chromatography coupled to single or triple quadrupole mass spectrometry was employed to identify and accurately quantify compounds where reference material was already available. Finally, fluorometric measurements carried out in real time were used to determine kinetic characteristics of enzymatic reactions.

The results presented in this thesis build a solid base for the refinement of steroid detection. It was shown that combinations of *in vitro* and *in vivo* metabolic studies and investigations on metabolic reactions together with adequate analytical techniques are of high relevance for the improvement of steroid detection methods. Furthermore, the results are of relevance to enhance methods for generation of reference compounds, to identify potentially new drugs and to re-evaluate the risk potential of drugs. Further areas that can benefit from the results of this work include clinical and toxicological analysis, analysis of environmental and biological samples and endocrinology.

Finally, the results are of high importance for anti-doping analysis. This field shall be mentioned separately as the results presented particularly contribute to tackle challenges relevant in this research area.

ZUSAMMENFASSUNG

Diese Arbeit befasst sich mit dem Steroidmetabolismus und dessen Rolle in der Steroidbestimmung. Die Ergebnisse wurden mit Hilfe von Stoffwechselstudien in Kombination mit unterschiedlichen analytischen Techniken erzielt. Drei Hauptergebnisse können formuliert werden. Ein erstes Ergebnis ist die Beschreibung der Erzeugung von Referenzmaterial eines Langzeitmetaboliten von Oral-Turinabol (OT). Die dabei gewonnenen Erkenntnisse vereinfachen den eindeutigen Nachweis und die Quantifizierung dieses Metaboliten und tragen somit dazu bei, die verbotene Einnahme von OT als Doping im Sport zu enthüllen. Weiterhin wurden strukturelle Voraussetzungen und tolerierte Funktionalitäten von Substraten des Enzyms Cytochrom P450 Subtyp 21A2 (CYP21A2) untersucht. Die daraus resultierenden Erkenntnisse ermöglichen die Extrapolation bekannter Stoffwechselreaktionen auf neue und möglicherweise unbekannte Verbindungen. Als drittes Hauptergebnis wurden Untersuchungen beschrieben, die sich mit Steroidprofilveränderungen befassen, die durch die Einnahme von Nichtsteroidalen Antirheumatika hervorgerufen werden. Dabei wurden Erkenntnisse gewonnen, die einen besseren Überblick über Substanzen, welche den Steroidstoffwechsel beeinflussen, ermöglichen. Dieses Wissen ist von großer Bedeutung für den eindeutigen Steroidnachweis und somit die korrekte Interpretation von Analysenergebnissen.

Alle Ergebnisse wurden größtenteils durch die Verwendung von drei Modellen zur Untersuchung des Steroidstoffwechsels und seiner Beeinflussbarkeit generiert. Als erstes Modell wurden *Schizosaccharomyces pombe* Stämme genutzt, welche rekombinant humane CYP Enzyme exprimieren. Einerseits wurden sie verwendet, um Referenzsubstanzen in kleineren Mengen zu erzeugen, andererseits um Stoffwechselreaktionen zu bewerten. Als zweites Modell wurden Inkubationen mit isolierten rekombinanten humanen Enzymen durchgeführt, um Stoffwechselreaktionen und deren Beeinflussbarkeit durch andere Arzneistoffe zu untersuchen. Als drittes Modell wurden *in vivo* Arzneimittelanwendungen genutzt, um den Steroidstoffwechsel im Menschen zu untersuchen sowie die Auswirkungen der Einnahme von Nichtsteroidalen Antirheumatika auf Steroidkonzentrationen und -konzentrationsverhältnisse zu beleuchten.

Um die durch die oben genannten Methoden erzeugten Proben anschließend zu analysieren, wurden verschiedene analytische Techniken verwendet. Hochauflösende Massenspektrometrie (HRMS) wurde zur Quantifizierung und vorläufigen Strukturaufklärung genutzt. Dabei wurden Flüssigchromatographie und Gaschromatographie gekoppelt mit HRMS angewendet. Zusätzlich wurde eine Kombination aus Gaschromatographie gekoppelt mit Single- oder Triple-Quadrupol Massenspektrometrie verwendet um Verbindungen, für welche Referenzmaterialien bereits verfügbar waren, nachzuweisen und exakt zu quantifizieren. Abschließend wurden fluorometrische Messungen in Echtzeit durchgeführt, um kinetische Kenndaten von Enzymreaktionen zu bestimmen.

Die im Rahmen dieser Arbeit vorgestellten Erkenntnisse bilden eine zuverlässige Grundlage für die Verbesserung des Steroidnachweises. Es wurde gezeigt, dass eine Kombination aus *in vitro* und *in vivo* Stoffwechselstudien und Untersuchungen zu Stoffwechselreaktion in Verbindung mit geeigneten analytischen Techniken von hohem Stellenwert für die Verbesserung von Steroidnachweismethoden ist. Außerdem sind die Erkenntnisse von Bedeutung, um Methoden zur Erzeugung von Referenzmaterialien zu verbessern, potenzielle neue Arzneistoffe zu erkennen und das Risikopotenzial von Arzneistoffen neu zu evaluieren. Weitere Gebiete, die von den Erkenntnissen dieser Arbeit profitieren können, sind unter Anderem, die klinische und toxikologische Analytik, die Umweltanalytik, die Untersuchung von biologischen Proben und die Endokrinologie.

Weiterhin sind die Ergebnisse von hoher Bedeutung für die Anti-Doping Analytik. Dieses Wissenschaftsfeld soll gesondert genannt werden, da die vorgestellten Ergebnisse dazu beitragen, Herausforderungen, die in diesem Gebiet von besonderer Relevanz sind, zu überwinden.

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8 List of Publications

8.1 Publications in scientific peer reviewed journals

Stoll A, Iannone M, De Gregorio G, Molaioni F, de la Torre X, Botrè F, Parr MK. Influence of Indomethacin on Steroid Metabolism: Endocrine Disruption and Confounding Effects in Urinary Steroid Profiling of Anti-Doping Analyses. *Metabolites* 10 (2020) article number 463;
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Stoll A, Iannone M, De Gregorio G, Molaioni F, de la Torre X, Botrè F, Parr MK. Influence of Pain Killers on the Urinary Anabolic Steroid Profile. *Journal of Analytical Toxicology* 44 (2020) 871-879;
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Stoll A, Loke S, Joseph JF, Machalz D, de la Torre X, Botrè F, Wolber G, Bureik, M, Parr MK. Fine-mapping of the substrate specificity of human steroid 21-hydroxylase (CYP21A2). *Journal of Steroid Biochemistry and Molecular Biology* 194 (2019) 105446;
https://doi.org/10.1016/j.jsbmb.2019.105446

Liu J, Chen L, Joseph JF, Naß A, Stoll A, de la Torre X, Botrè F, Wolber G, Parr MK, Bureik M. Combined chemical and biotechnological production of 20βOH-NorDHCMT, a long-term metabolite of Oral-Turinabol (DHCMT). *Journal of Inorganic Biochemistry* 183 (2018) 165–171;
https://doi.org/10.1016/j.jinorgbio.2018.02.020

8.2 Oral presentations in national and international conferences

The speaker is emphasized by underlining.

Stoll A, Iannone M, de la Torre X, Botrè F, Parr MK. No Pain No Gain – Influence of Pain Killers on the Anabolic Steroid Profile. The 57th Annual Meeting of the International Association of Forensic Toxicologists, Book of Abstracts (2019) oral presentation SP6

Parr MK, La Piana G, Stoll A, Joseph JF, Loke S, Schloerer N, de la Torre X, Botrè F. Tracing back drug misuse – proper metabolite identification requires synthesis. The 57th Annual Meeting of the International Association of Forensic Toxicologists, Book of Abstracts (2019) SP1

Stoll A, Joseph JF, de la Torre X, Liu J, Bureik M, Botrè F, Parr MK. Recombinant Fission Yeast – A Versatile Tool for Metabolite Studies and Synthesis. DPhG Conference 2018 "Shaping future pharmaceutical research", Book of Abstracts (2018) oral presentation V6

Parr MK, Liu J, Joseph JF, Gorczyca D, Stoll A, Machalz D, Wolber G, de la Torre X, Botrè F, Bureik M. Old friends, new relatives – the science behind recent revelation of doping abuse at Beijing and London Olympics. In: Recent Developments in Pharmaceutical Analysis RDPA 2017, Book of Abstracts (2017) 45

Joseph JF, Stoll A, de la Torre X, Botrè F, Parr MK. Metabolic hydroxylation reactions of androstenedione and derivatives. DPhG Conference 2016 "Shaping future pharmaceutical research", Berlin (GER), Book of Abstracts (2016) V3

8.3 Poster presentations in national and international conferences

Stoll A, de la Torre X, Botrè F, Parr MK. Steroids and Pain Killers – Influences of NSAIDs on Human Steroid Metabolism. 20th International Congress on *In Vitro* Toxicology - ESTIV, Book of Abstracts (2018) Poster P177

Stoll A, de la Torre X, Botrè F, Parr MK. In-Vitro Investigation on the Effect of NSAIDs on Human Steroid Metabolism. Annual Meeting of the German Pharmaceutical Society – DPhG, Book of Abstracts (2018) Poster P179

Parr MK, Stoll A, Gorczyca D, Krzemiński M, Loke S, Joseph JF, de la Torre X, Botrè F. Towards the synthesis of long-term metabolite of Oral-Turinabol for revelation of doping abuse at Beijing and London Olympics. 24th Conference on Isoprenoids Book of Abstracts, The Polish Chemical Society (2018) 104

Loke S, Stoll A, Parr MK. Controlled administration trial of Oral-Turinabol - metabolite confirmation and elimination profiles with special respect to long-term metabolites. DPhG Conference 2018 "Shaping future pharmaceutical research ", Book of Abstracts (2018) Poster P28

Joseph JF, Stoll A, de la Torre X, Botrè F, Parr MK. Minor metabolic pathways of xenobiotic steroid metabolism. 3rd Congress on Steroid Research (2015) P053

Parr MK, Stoll A, Botrè F, de la Torre X. Structure reactivity relationship of A-ring hydroxylation of steroids by aryl hydrocarbon hydroxylases - from estrogens to androgens. 3rd Congress on Steroid Research (2015) P041

9 INDEPENDENCE DECLARATION

I hereby affirm that I have completed the presented cumulative dissertation independently and without unauthorized assistance. No aids other than these listed in the text were used in the writing of the dissertation.

A doctoral procedure has never been completed at any other university or applied to another department.

Anna Stoll

10 APPENDIX

10.1 List of figures

10.2 List of tables