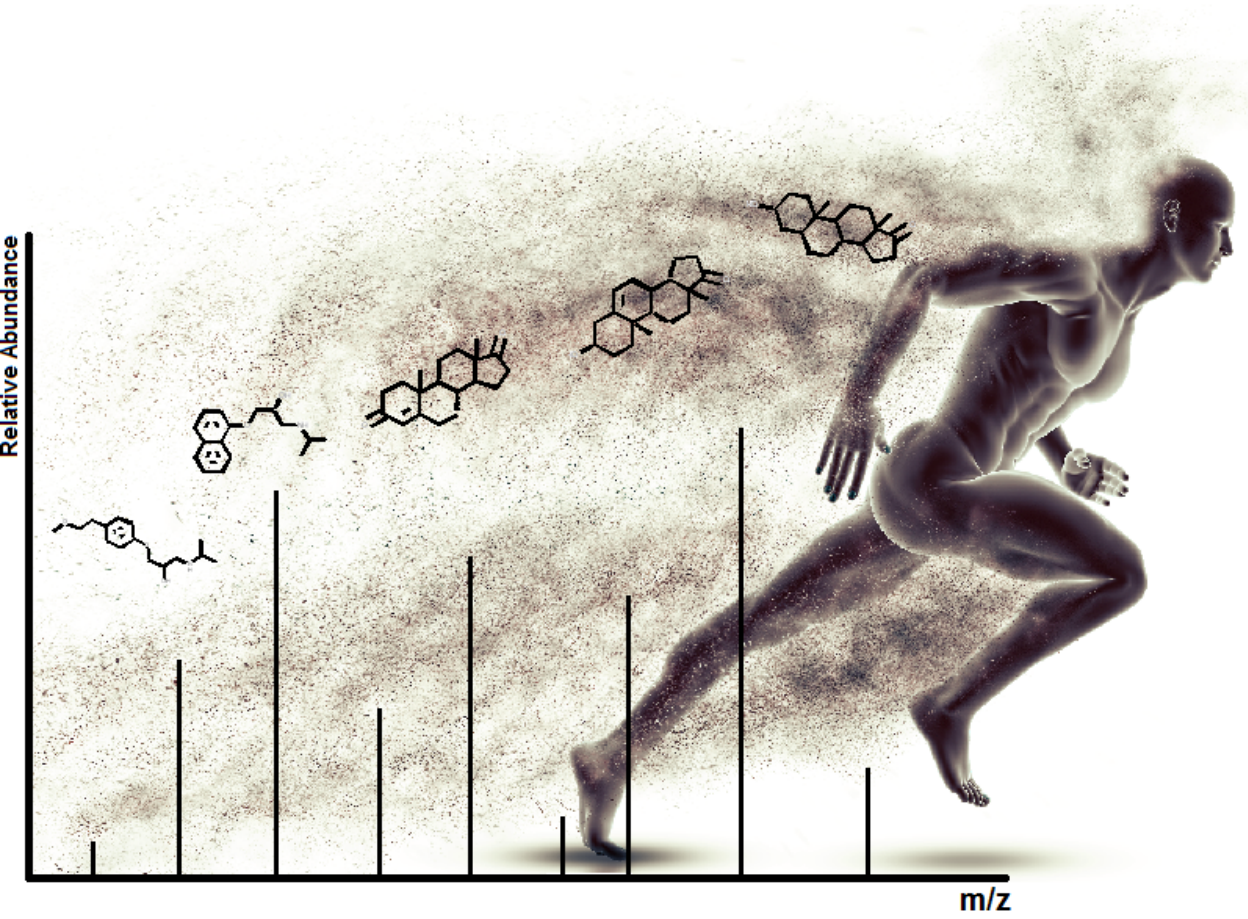
Stockholm University

Analytical Methods For Sports Drugs: Challenges and Approaches

Hatem Elmongy



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Hatem Elmongy

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Abstract

Drugs used to enhance human performance in sport competitions are prohibited by the world anti-doping association (WADA). Biological samples from athletes are continuously tested for adverse analytical findings regarding the identity and/or quantity of the banned substances. The current thesis deals with the development of new analytical methods to determine the concentrations of certain drugs used by athletes and even by regular users for therapeutic purposes. The developed methods aim to analyze the contents of these drugs in the biological matrices; plasma, serum and saliva to provide a successful approach towards either doping detection or therapeutic monitoring. β-adrenergic blockers such as propranolol and metoprolol are used in sports to relief stress and as therapeutic agents in the treatment of hypertension. Both drugs are in chiral forms and available only as racemic mixtures. The different pharmacology of each enantiomer necessitates the monitoring of each enantiomer by stereoselective analytical technique such as chiral liquid chromatography for separation and mass spectrometry for selective detection. The Endogenous anabolic androgenic steroids (EAAS) on the other hand are only notoriously used in sports to increase muscle mass and strength. A method utilizing high-resolution mass spectrometry (HRMS) coupled to ultra-high performance liquid chromatography (UHPLC) was developed for the simultaneous determination of EAAS and their conjugated metabolites to provide a better insight into the steroidal module of the athlete biological passport (ABP). Moreover, the steroidal profile was assessed in serum using the proposed method after the administration of Growth hormone injection as an approach toward the implementation of a new endocrinological module based on steroids biomarkers to hormone doping. Biological samples contain many components that may interfere with the analytical measurements. Therefore, sample preparation methods were developed using solid phase extraction (SPE) and miniaturized techniques such as microextraction by packed sorbents (MEPS) for the purification and pre-concentration of analytes prior to LC/MS analysis.

Keywords: *Sports Drugs, Doping in Sports, Steroids, LC-MS/MS, Chiral analysis, high-resolution mass spectrometry, Sample preparation, Biological samples, solid phase extraction.*

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Cover image: Mass spectrometric chart, drugs chemical structures and running man. The running male figure created by kjpargeterimages.co.uk and used with permission.

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To My Beloved Family

Populärvetenskaplig Sammanfattning

Substanser som används i syfte att öka idrottsutövares styrka och uthållighet under tävling är förbjudna av World Anti-Doping Association (WADA). Idrotsutövares urin och blod testas kontinuerligt för att bedöma om dom är positiva eller negativa genom att analysera för förbjudna substanser. Den här avhandlingen handlar om utveckling av nya metoder för att bestämma koncentrationerna och identitet för endogena substanser, förbjudna när dom är exogena och andra droger som används både i syfte att fuska eller terapeutiskt.

Avsikten med de utvecklade metoderna har varit att analysera innehållet av dessa substanser i biologiska matriser; plasma, serum och saliv för att finna metoder för antingen detektion av doping eller terapeutiska läkemedel. β-blockerare som tex. propranolol and metoprolol används som doping inom visa idrotter för att minimera stresskänslor och terapeutiskt för att behandla högt blodtryck (hypertoni). Båda dessa läkemedelsubstanser är kirala men är endast kommersiellt tillgängliga i sina respektive racemiska former. Enantiomerernas olika Farmakologi nödvändiggör behovet av stereoselektiva analytiska metoder för att bestämma innehållet av respektive enantiomer. Metoden som används i denna avhandling baseras på kiral kromatografi och detektion med masspektrometri.

Kroppsegna steroider så som testosteron används inom idrott för att öka muskelmassa och sålunda styrka och uthållighet. En metod för bestämning av kroppsegna konjugerade (sulfat och glukuronid) och fria steroider baserad på vätskekromatografi och högupplöst masspektrometri har utvecklats för serumprover. Metoden kan ses som en ett första steg till ett endokrint biologiskt pass för idrottsutövare. Idag existerar två ben i det

biologiska passet, ett baserat på kroppsegna steroider i urin och ett hematologiskt baserat på ett antal blodparametrar i helblod. Metoden har applicerats på serumprover från en klinisk studie där tillväxthormon har administrerats. Biologiska prover är mycket komplexa och provberedning kan behövas, i studierna i denna avhandling utvecklades metoder med fast fas-extraktion, (SPE) och microextraktion (MEPS) för rening och koncentrering av prover före analys med kromatografi och masspektrometri.

الملخص باللغة العربية

تحظر الوكالة العالمية لمكافحة المنشطات الأدوية المستخدمة لتحسين الأداء البشري في المسابقات الرياضية. ويتم اختبار العينات البيولوجية من الرياضيين بشكل مستمر لمعرفة النتائج التحليلية المتعلقة بهوية أو كمية المواد المحظورة.

تتناول الأطروحة الحالية تطوير طرائق تحليلية جديدة لتحديد تركيزات بعض الأدوية التي يستخدمها الرياضيون وحتى المستخدمون المنتظمون لأغراض علاجية. تهدف الطرق المطورة إلى تحليل محتويات هذه الأدوية في السوائل البيولوجية للانسان كما في البلازما والسيرم واللعاب لتوفير نهج ناجح تجاه الكشف عن المنشطات أو التقييم العلاجي. تستخدم مضادات المستقبلات بيتا الأدرينالية مثل البروبرانولول والميتوبرولول في الألعاب الرياضية لتخفيف التوتر كما أنها تستخدم كأدوية علاجية في علاج ارتفاع ضغط الدم.

كلا الدوائين مركبات كيرالية ومتاحين بشكل تجاري كخليط من المتناظرين الضوئيين. وبسبب الاختلاف في الفاعلية الدوائية للمتناظرين فان تعيين كل منهما ضروري باستخدام طرائق تحليلية انتقائية للايزومرات كاستخدام كروماتوجرافيا السوائل ذات
.الكفاءة العالية الكيرالية و مطياف الكتلة للرصد الانتقائي
ومن ناحية اخري فان الستيرويدات الداخلية المنشطة تستخدم فقط بشكل غير قانوني في المسابقات الرياضية من بعض الرياضيين لزيادة الكتلة العضلية والقوة البدنية
تم تطوير طريقة تحليلية تعتمد علي كروماتوجرافيا السائل ذات الكفاءة الفائقة المرتبطة بمقياس الكتلة عالي الفصل لتعيين تركيزات الستيرويدات المنشطة والمركبات الايضية لتقديم رؤية افضل للنموذج الستيرويدي الداخل في تكوين الملف البيولوجي للرياضي. الي جانب ان المحتوي الستيرويدي في سيرم الانسان تم تعيينه باستخدام الطريقة المطورة وذلك بعد استخدام هرمون النمو من قبل أشخاص اصحاء كتطبيق.
ويهدف هذا التطبيق للطريقة المقترحة الي وضع نموذج جديد قائم علي المحتوي الهرموني للاشخاص باستخدام الستيرويدات كدوال بيولوجي
السوائل البيولوجية تحتوي علي العديد من المكونات التي تتداخل مع القياسات التحليلية. ولذلك، تم تطوير طرائق لتحضير العينات تعتمد علي الاستخلاص ذا الطور الصلب و

الاساليب المصغرة كالاستخلاص المصغر باستخدام المواد الماصة المعباءة لتنقية المركبات وتركيزها قبل التحليل باستخدام الكروماتوجرافيا ومقياس الكتلة.

List of Publications

I. **Online post-column solvent assisted and direct solvent-assisted electrospray ionization for chiral analysis of propranolol enantiomers in plasma samples.** Hatem Elmongy, Hytham Ahmed, Abdel-Aziz Wahbi, Hirsh Koyi, Mohamed Abdel-Rehim, Journal of Chromatography A (2015) 1418, 110-118.

The author was responsible for the planning and ideas, development of the analytical method, the sample preparation procedure, the experiments and data interpretation, as well as writing the paper

II. **Determination of metoprolol enantiomers in human plasma and saliva samples utilizing microextraction by packed sorbent and liquid chromatography–tandem mass spectrometry.** Hatem Elmongy, Hytham Ahmed, Abdel-Aziz Wahbi, Ahmed Amini, Anders Colmsjö, Mohamed Abdel-Rehim, Biomedical Chromatography (2016) 30 (8), 1309-1317.

The author was responsible for the development of the analytical method, the sample preparation procedure, the experiments and data interpretation, as well as writing the paper

III. **Development and validation of a UHPLC-HRMS method for the simultaneous determination of endogenous anabolic androgenic steroids in human serum.** Hatem Elmongy, Michèle Masquelier, Magnus Ericsson. (2019) (Manuscript)

The author was responsible for the development of the analytical method, the sample preparation procedure, the experiments and data interpretation, as well as writing the manuscript

IV. **Studies of hematological ABP parameters and putative GH biomarkers in relation to 2 weeks recGH administration.** Tobias Sieckmann, Hatem Elmongy, Magnus Ericsson, Hasanuzzaman Bhuiyan, Mikael Lehtihet, Lena Ekström. (2019) (Manuscript)

The author was responsible for sample preparation and analysis of samples using UHPLC-HRMS and partially in data evaluation.

List of Publications not included in the Thesis

I. **Nanomaterials as sorbents for sample preparation in bioanalysis: A review.** Mazaher Ahmadi, Hatem Elmongy, Tayyebeh Madrakian, Mohamed Abdel-Rehim, Analytica Chemica Acta, (2017) 958, 1-21.

II. **Saliva as an alternative specimen to plasma for drug bioanalysis: A review.** Hatem Elmongy, Mohamed Abdel-Rehim. TrAC Trends in Analytical Chemistry (2016) 83, 70-79.

III. **Enantioselective HPLC-DAD method for the determination of etodolac enantiomers in tablets, human plasma and application to comparative pharmacokinetic study of both enantiomers.** Ismail I Hewala, Marwa S Moneeb, Hatem A Elmongy, Abdel-Aziz M Wahbi, Talanta, (2014) 130, 506-517.

List of Abbreviations

17-OHP	17 α-hydroxyprogesterone
A	Androsterone
AAS	Anabolic Androgenic Steroids
ABP	Athlete Blood Passport
A-dione	Androstenedione
A-G	Androsterone Glucuronide
A-S	Androsterone Sulphate
AGC	Automatic Gain Control
APCI	Atmospheric pressure Chemical Ionization
APPI	Atmospheric Pressure Photoionization
BIN	Barrel Insert in a Neddle
C/IRMS	Combustion isotopic ratio mass spectrometry
CID	Collision induced Dissociation
CRM	Charged Residue Module
CSPs	Chiral Stationary Phases
CYP	Cytochrome P
DHEA	dehydroepiandrosterone
DHEA-G	Dehydroepiandrosterone Glucuronide
DHEA-S	Dehydroepiandrosterone Sulphate
DHT	5α-dihydrotestosterone
DHTG	Dihydrotestosterone Glucuronide
DHTS	Dihydrotestosterone Sulphate
E	Epitestosterone
E-G	Epitestosterone Glucuronide
E-S	Epitestosterone Sulphate
EAAS	Endogenous Anabolic Androgenic Steroids
ESAs	Erythropoiesis-Stimulating Agents
ESI	Electrospray Ionization
Etio	Etiocholanolone
Etio-G	Etiocholanolone Glucuronide
Etio-S	Etiocholanolone Sulphate
ExAAS	Exogenous Anabolic Androgenic Steroids
GC/MS	Gas Chromatography/ Mass Spectrometry
GH	Growth Hormone
GPC	Gel Permeation Chromatography
HGB	hemoglobin
HILIC	Hydrophilic interaction liquid chromatography
HRMS	High-resolution mass spectrometry
HSDs	Hydroxyl Steroid dehydrogenases
HQC	High concentration quality control

IAAF	International Athletic Federation
IEM	Ion Evaporation Model
IGF-1	Insulin-like growth factor 1
IL-ISTD	Isotope-labelled internal standard
IOC	International Olympic Committee
LC/MS	Liquid Chromatography / Mass Spectrometry
LLE	Liquid-Liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
LQC	Low concentration quality control
MEPS	Microextraction by Packed Sorbent
MIPs	Molecular imprinted Polymers
MRM	Multiple Reaction Monitoring
MRPL	Minimum Required Performance Level
MQC	Medium Concentration Quality Control
NPLC	Normal-phase Liquid Chromatography
OPSAI	Online post-Column Solvent Assisted Ionization
P-III-NP	Procollagen III amino-terminal propeptide
PS-DVB	Polystyrene-Divinylbenzene copolymer
RAM	Restricted Access Material
recGH	Recombinant Growth Hormone
RET %	reticulocytes percentage
SAESI	Solvent Assisted Electrospray Ionization
SCX	Strong Cation Exchange
SLE	Solid-liquid Extraction
SPE	Solid Phase Extraction
SRM	Selected Reaction Monitoring
T	Testosterone
TG	Testosterone Glucuronide
TS	Testosterone Sulphate
UGT2B17	Diphospho glucuronosyltransferase 2b17
WADA	World Anti-Doping Association

Table of Contents

1. Introduction

1.1. Doping in sports

Doping was first mentioned in 1889 as a mixed remedy of opium that was used to "dope" horses. Zulu warriors used a spirit prepared from the residues of grapes as a stimulant and called it "Dope". Later the word "Dope" was extended in meaning to include other substances with stimulating properties [1]. In the modern sense, doping in sports means the administration or use of doping agents or doping methods by athletes which appear on the list of banned substances by the anti-doping agency.

Stimulants were the early doping agents including among others the notorious cocaine, caffeine and strychnine. The use of stimulants in competitions was increased after the introduction of the strong acting synthetic phenylethylamine derivatives such as amphetamine and methamphetamine [2].

Anabolic agents or steroids were used in sports first as recovery aids after extreme stress and exhaustion. They were developed from the male sex hormone "testosterone". Testosterone was successfully isolated in a pure crystalline form in 1935 [3]. With the structure elucidation and chemical synthesis, a Nobel Prize was awarded to A. Butenandt and L. Ruzicka in 1939. Soon after, synthesis of numerous derivatives was involved in pharmaceutical industry in parallel to the natural hormone.

The international Olympic committee (IOC) have addressed doping problems since IOC sessions in Warsaw and Cairo in 1937/1938. A medical commission was established at the IOC session in Athens in 1961. The first doping tests at the Olympics took place during the winter games in Grenoble and summer games in Mexico in 1968, where the first

disqualification based on positive results occurred. The International Athletic Federation (IAAF) was the first to ban the use of stimulating substances in sport, but this remained inefficient until testing possibilities were available.

The IOC proposed the idea of an international Anti-doping Agency in 1998. First discussed at a World Conference in Lausanne in February 1999, the International Olympic Committee, the Council of Europe and the Monitoring Group to its Anti-Doping Convention, as well as several representatives of Governments, played an active role in supporting the foundation of the World Anti-Doping Agency, WADA, in December 1999.

Ever since, WADA has implemented the world anti-doping code and regulatory documents that include the prohibited list of substances [4]. The code is intended to protect clean athletes and to ensure fair play in competitions with special attention to detection, deterrence and prevention of doping [5]. The compounds and methods are classified in the list to ten categories for substances (S0 to S9) and three categories for methods (M1 to M3) and P1 which includes β-blockers that are prohibited in specific sports. The different classes of the substances and methods with examples are illustrated in table 1. The compounds are further categorized to non-threshold substances, that their detection in the tested samples indicates an adverse analytical finding (AAF) such as β-blockers [6]. WADA has established the minimum required performance levels (MRPL) to harmonize the analytical performance of the methods applied to the detection of non-threshold substances in all laboratories. The threshold substances indicate AAF only upon exceeding certain limit (e.g. Salbutamol, Morphine, and Ephedrine) [7].

Doping testing is routinely applied to urine and blood (whole blood, serum, and plasma). Urine is non-invasive and can be collected in large volumes unlike blood. Thus, the majority of anti-doping routine tests is still carried out on urine samples. However, urine exhibits some limitations that can markedly challenge the routine analytical methods such as enzymatic polymorphism [8-10], microbial contamination, and concomitant use of masking agents and/or diuretics. Serum on the other hand lacks such challenging features and can provide an interesting alternative especially with the application of selective means of detection such as mass spectrometry.

1.2. Minor level Substances

Substances such as stimulants, narcotics, diuretics, β-agonists and β-blockers are easily ionizable with limited metabolism that facilitate their detection and quantitation. Moreover, their minimum required detection levels are relatively high in the range of a few tens to hundreds of ng/mL (minor levels) [11]. Continuous improvement of analytical methods needed for fast, sensitive and selective determination of such drugs is crucial in anti-doping laboratories.

β-blockers (β-adrenergic antagonists) such as propranolol and metoprolol are used by athletes in sports that required improved psychomotor coordination. The peripheral blockade of β_2-adrenergic receptors can alleviate symptoms associated with anxiety and stress such as tremors [12]. The selective extraction of such drugs from the complex biological matrices is routinely achieved prior to the analytical step.

Table 1. The prohibited substances and methods according to WADA prohibited list 2019.

Category	Sub-category	Examples	Prohibition
S0 Non-approved substances		Drugs under pre-clinical or clinical development or discontinued, designer drugs, substances approved only for veterinary use.	
S1 Anabolic Agents	**1. Anabolic androgenic steroids**		In and out of competition
	a) Exogenous	1-Androstenediol, 1-Testtosterone, Bolasterone, Clausterone, Clostebol, Danazole,	
	b) Endogenous	5-Androstenedione, Epitestosterone, DHEA, 5α-dihydrotestosterone, Testosterone.	
	2. Other anabolic agents	Clenbuterol, selective androgen receptor modulators (SARMs, e.g. andarine), tibolone, zeranol and zilpaterol.	
S2 Peptide hormones, growth factors, related substances, and mimetics	**1. Erythropoietins (EPO) and Agents Affecting Erythropoiesis**		In and out of competition
	1.1 Erythropoietin-Receptor Agonists	Darbepoetins (dEPO), Erythropoietins (EPO)	
	1.2 Hypoxia-inducible factor (HIF) activating agents	Argon, Cobalt, Daprodustat, Molidustat, Xenon.	
	1.3 GATA inhibitors	K-11706	
	1.4 TGF-beta (TGF-β) inhibitors	Luspatercept, Sotatercept	
	1.5 Innate repair receptor agonists	Asialo EPO, Carbamylated EPO (CEPO)	
	2. Peptide Hormones and their Releasing Factors		
	2.1 Chorionic Gonadotrophin (CG) and Luteinizing hormone (LH) and their releasing factors in males	Buserelin, deslorelin, gonadorelin, goserelin, leuprorelin, nafarelin and triptorelin;	
	2.2 Corticotrophins and their releasing factors	Corticorelin	
	2.3 Growth Hormone (GH), its fragments and releasing factors	Growth Hormone fragments, e.g. AOD-9604 and hGH 176-191, Growth Hormone Releasing Hormone (GHRH) and its analogues.	
	3. Growth Factors and Growth Factor Modulators	Fibroblast Growth Factors (FGFs), Hepatocyte Growth Factor (HGF), Insulin-like Growth Factor-1 (IGF-1) and its analogues.	
S3 Beta-2 Agonists	All selective and non-selective beta-2 agonists	Fenoterol, Formoterol, Higenamine, Indacaterol, Olodaterol, Procaterol, Reproterol, Salbutamol, Salmeterol.	In and out of competition
S4 Hormone and	**1. Aromatase inhibitors**	2-Androstenol, 2-Androstenone, 3-Androstenol, 3-Androstenone.	In and out of competition

	Metabolic Modulator			
		2. Selective estrogen receptor modulators (SERMs)	Raloxifene, Tamoxifen, Toremifene.	
		3. Other anti-estrogenic substances	Clomifene, Cyclofenil, Fulvestrant.	
		4. Agents preventing activin receptor IIB activation	Activin A-neutralizing antibodies; Activin receptor IIB competitors such as: Decoy activin receptors (e.g. ACE-031).	
		5. Metabolic modulators		
		5.1 Activators of the AMP-activated protein kinase (AMPK)	AICAR, SR9009, and Peroxisome Proliferator Activated Receptor δ (PPARδ) agonists.	
		5.2 Insulins and insulin-mimetics		
		5.3 Meldonium		
		5.4 Trimetazidine.		
S5	Diuretics and Masking Agents		Desmopressin, probenecid, plasma expanders, e.g. intravenous administration of albumin, dextran, hydroxyethyl starch and mannitol	In and out of competition
S6	Stimulants	**a) Non-Specified Stimulants**	Adrafinil, Amfetamine, Cocaine, Phentermine, Mephentermine; Mesocarb.	In competition
		b) Specified Stimulants	Ephedrine, Epinephrine, Sibutramine, Strychnine, Methylphenidate.	
S7	Narcotics		Buprenorphine, Dextromoramide, Diamorphine (heroin), Fentanyl and its derivatives.	In competition
S8	Cannabinoids	**- Natural**	Cannabis, hashish and marijuana,	In competition
		- Synthetic	Δ9-tetrahydrocannabinol (THC) and other cannabimimetics.	
S9	Glucocorticoids		Betamethasone, Budesonide, Cortisone, Deflazacort, Dexamethasone, Fluticasone	
P1	Beta-Blockers (β-blockers)		Propranolol, metoprolol, Atenolol, Acebutolol, Timolol, Carvedilol, Oxeprenolol.	
M1	Manipulation of blood and blood components		Administration of RBCs, haemoglobin-based blood substitutes	
M2	Chemical and Physical Manipulation		Tampering samples, IV infusions	
M3	Gene and Cell Doping		Gene editing agents, polymers of nucleic acids	

On the other hand, a simple dilute and shoot procedure is often used as a screening assay for such compounds to provide a non-selective approach to their detection in test samples [11]. Most of β-blockers are chiral in nature which means two isomeric forms exist that are usually administered in a 1:1 racemic mixture.

1.2.1. Chirality in drug analysis

Chiral drugs are compounds that contain at least one chiral center and are widely used in the treatment of human diseases. Chiral drugs constitute over half of the commercially available therapeutic agents and they are mostly administered as racemates [13]. Racemates are mixtures containing equal proportions of (R)- and (S)-enantiomers, yet in most cases each enantiomer exhibits a different pharmacological action [14, 15]. The different pharmacological behavior of enantiomers is due to the different three-dimensional configurations that implement the selective drug-receptor interaction in the body [16]. However, the individual drug enantiomers present identical physicochemical properties in an achiral environment, which constitute a challenging aspect during analytical determinations using conventional separation methods [17].

Analysis of the stereoisomers of chiral pharmaceuticals is necessary to determine the enantiomeric purity and hence the drug potency. Due to the different pharmacological action of each enantiomer, the determination of each enantiomer in the mixture is crucial using chiral chromatography. On the other hand, isolation of the pure enantiomer can be done using preparative chiral chromatography in case of production of enantiopure drugs [18].

The Separation of chiral isomers can be carried out using HPLC or GC through direct and indirect methods. Indirect methods are based on

the use of chiral additive to the mobile phase. Each enantiomer covalently reacts with the chiral additive resulting in adducts that can be separated on an achiral stationary phase. It involves the formation of diasteriomeric complexes with the chiral selector of the stationary phase which vary in their stability and partitioning with the mobile phase leading to the differential elution. The direct methods include the separation of the isomers on a chiral stationary phase.

1.3. Trace level substances

1.3.1. Steroids in sports

Anabolic steroids affiliated with the male sex hormone testosterone are the most frequently detected doping substances in sports reported by WADA. Due to the anabolic effect exhibited by increased muscle growth, boosting strength, and accelerated recovery, anabolic agents are detected as doping agents in almost all sports [19-21]. The WADA list of prohibited substances classify anabolic androgenic agents (AAS) as exogenous anabolic androgenic steroids (ExAAS) including for instance stanozolol, oxandrolone, metandienone, etc., and endogenous anabolic androgenic steroids (EAAS) such as testosterone (T), epitestosterone (E), 5α-dihydrotestosterone (DHT), dehydroepiandrosterone (DHEA), androstenedione (A-dione), etiocholanolone (Etio), androsterone (A) . [22] (Table 1). Usually low but frequent doses of AAS are favored in case of endurance athletes while larger doses mainly via injections are more enhancing in case of strength sports. Moreover, testosterone can also increase the muscles ability to replenish its glycogen reserve besides its role in muscle regeneration after physical exercise [23, 24]. Synthetic AAS can be found by some athletes as a powerful compensating agents to sustain the testosterone levels and to increase the capacity for more intense training sessions.

The synthesis of steroids, i.e. steroidogenesis from the precursor cholesterol, has been extensively explained in literature [25] including the role of the metabolizing enzymes; the cytochrome P450 (CYP) isozymes [26]. These enzymes catalyze the reactions of hydroxylation and cleavage, the hydroxyl steroid dehydrogenases (HSDs) which catalyzes the reduction or oxidation reactions of steroids (Figure 1) [26, 27]. Other enzymes such as glucuronidases and sulfatases catalyze the conjugation of steroids prior to excretion.

AAS are required to be monitored in trace levels and due to their multiple isomeric and metabolic forms, selective and sensitive analytical determinations are crucial to address analytical findings accurately. Therefore, a sample preparation procedure is often a pre-requisite for purification and pre-concentration of analytes of interests.

Since most analytical instrumentation cannot distinguish between the administered and natural T, (T/E) ratio was adopted to be the first widely used indirect marker of doping with anabolic steroids to detect T administration with an authorized upper limit of 6.0 [19]. E was shown to not increase after T intake which results in an increase in the T/E ratio [28]. T and E are almost totally excreted in urine in conjugated form. Therefore, T and E levels has been assessed in urine samples after deconjugating the glucuronide moiety by enzymatic hydrolysis (b-glucuronidase) and derivatization (trimethylsilylation) prior to gas chromatography and mass spectrometric detection (GC/MS) [29, 30]. The major enzyme responsible for T glucuronidation is uridine diphospho-glucuronosyltransferase 2B17 (UGT2B17) [31]. It was shown that the gene deletion polymorphism of UGT2B17 [32] highly affecting the rate of urinary T excretion [31]. The polymorphism of UGT2B17 gene deletion is observed between individuals with different ethnicity who show either

homozygous deletion allele (del/del) or heterozygous (del/ins) which significantly affects T levels. The T/E ratio in healthy volunteers [33] and in AAS abusers [34] was found to be highly dependent on the UGT2B17 deletion genotype [35] in healthy volunteers [36] and in sport samples [37].

The variation of T/E ratio with different genotypes of UGT2B17 made it urgent to identify other strategies, independent of this polymorphism, which could be used universally for detection of T doping. This eventually led to the adoption of the steroidal module in the Athlete Biological Passport. A subsequent confirmation analysis by gas chromatography combustion isotope ratio mass spectrometry (GC/C/IRMS) was established in the technical documents of WADA (TD2014IRMS) [38] to determine the carbon isotope composition of targeted androgens. It is known that synthetic sources of T has different 13C/12C isotopic ratio from the natural hormone produced by means of cholesterol metabolism [39] which can make the discrimination possible. However, doping manufacturers are continuously improving the ratios during the synthesis by monitoring 13C content to counteract the IRMS strategy for detection.

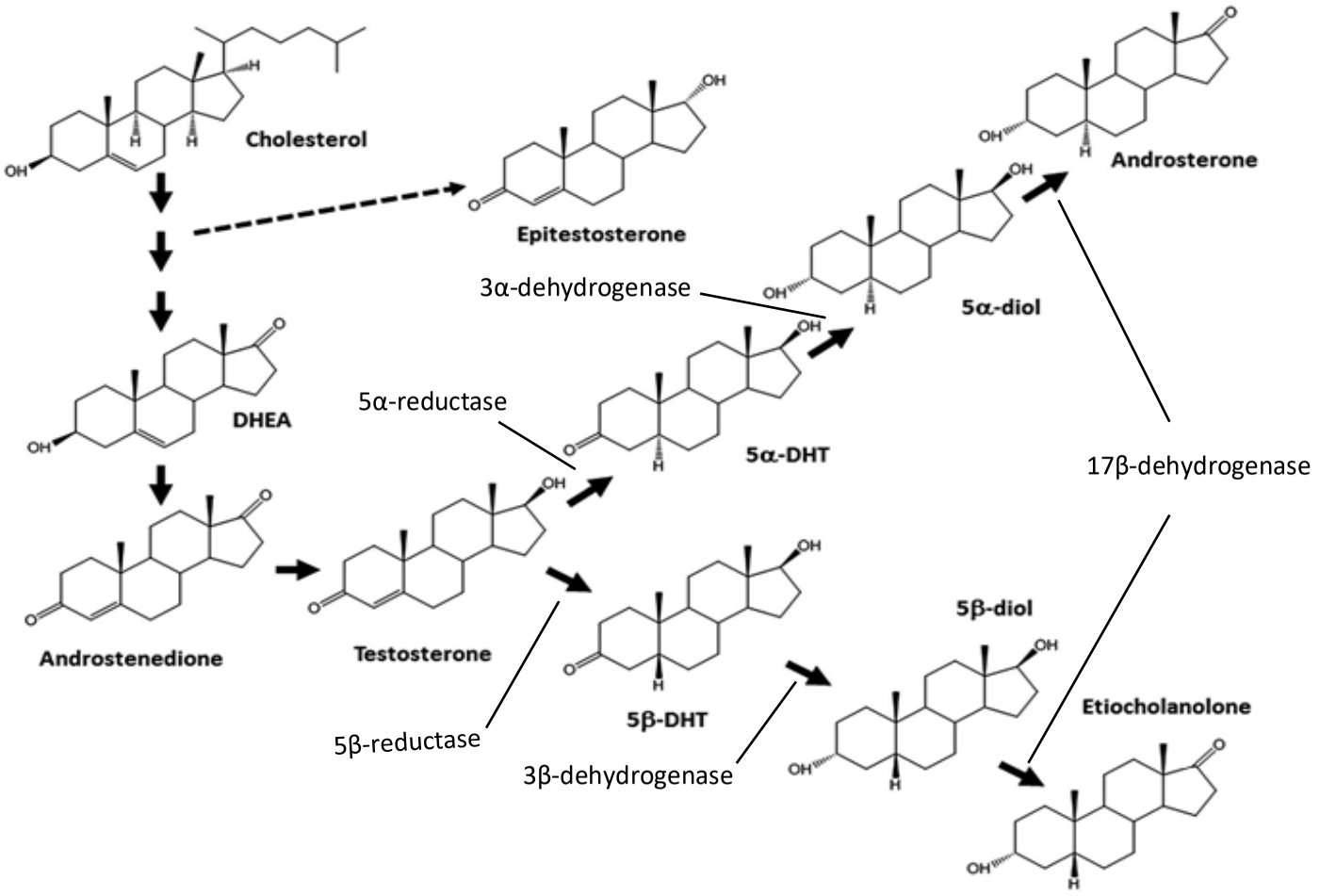


Figure 1. Target analytes of the endogenous anabolic androgenic steroids and their metabolic pathways.

1.3.2. Athlete biological passport and steroidal module

The scientific community has proposed the term "athlete biological passport" first in the early 2000s when monitoring biomarkers of blood doping to define an individual's hematological profile. WADA has further developed and validated the concept in conjunction with several stakeholders and medical experts that resulted in formal operating guideline and mandatory standards known as the Athlete Biological Passport (ABP) [40]. The passport was first published in 2009, including exclusively the hematological module. In 2014, the steroidal module was included in order to monitor the athlete's steroid variables. ABP constitutes a framework to promote harmonization in ABP programs and facilitate the exchange and mutual recognition of data which enhances the operation efficiencies of Anti-Doping Activities.

Currently ABP includes two distinct modules: the hematological and the steroidal modules. The hematological module of the ABP aims to detect any form of blood doping implemented by the use of prohibited substances/methods for the enhancement of oxygen transport or delivery, including the use of Erythropoiesis-Stimulating Agents (ESAs) and any form of blood transfusion or manipulation [40-42]. The steroidal module of the ABP, which aims to detect intake of either exogenous or endogenous anabolic agents [43], shall be emphasized in the current thesis.

The steroidal module provides the information on markers of steroid doping. The module aims to detect the exogenous intake of EAAS and different pro-hormones to T. The ABP steroidal module listed by WADA consider the following markers; T, E, A, Etio, 5α-androstane-3α,17β-diol (5αAdiol) and 5β-androstane-3α,17β-diol (5βAdiol) [44].

Ratios instead of individual concentrations of steroids were used

as they provide more stable and sensitive results to report doping [45]. Moreover, the use of biomarker ratios rather than individual concentration minimizes the fluctuations of steroid concentrations due to inter-subject variations.

1.3.3. Endogenous steroids and biomarkers of doping.

Among different analytical doping tests, detection of doping with endogenous steroids remains one of the most challenging tasks for anti-doping laboratories, as routine doping control cannot distinguish between exogenous intake and endogenous steroids. In that matter, the biomarker ratio of endogenous steroids can provide a helpful tool in reporting any AAF. The ABP is based on using the intra-individual standard values instead of using population-based cut-off ratios, due to genetic polymorphism that may result in alternative values of the ratios as previously discussed with T/E ratio. Samples from athletes are longitudinally monitored [43, 46] including in addition to T and E, other steroid metabolites such as A, Etio, 5αAdiol, and 5βAdiol as well as their ratios (A/T), (A/Etio), (5αAdiol/5βAdiol), and (5αAdiol/E) [40, 45]. Moreover, serum circulating conjugated metabolites can also provide promising markers which have been included in the current study.

1.3.3.1. T/E ratio

It is the most sensitive ratio used for the detection of the exogenous administration of T and the most common parameter in the steroidal profile. As previously described, the administration of T results in an increase of endogenous T while E remains unchanged. As a result, the ratio T/E increases with doping. The cut off ratio was lowered to 4 from 6 in 2004. The ratio was shown to be ineffective due to genetically backed high T/E in some people resulting in false positive results while

others with naturally lower T/E could not reach the cut off value even after T injection resulting in false negative results [47, 48].

1.3.3.2. A/T ratio

This ratio shows lower values (< 20) upon doping which is different than other ratios that show higher values after intake. The reciprocal of the ratio was used earlier but was changed to A/T instead to improve the numerical representation of values to have lower decimals.

1.3.3.3. 5αAdiol/5βAdiol ratio

5αAdiol and 5βAdiol are phase I metabolites of 5α-DHT and 5β-DHT, respectively (Figure 1). 5α-Adiol is an androgenic agent with an activity second to 5α-DHT itself, while 5β-Adiol is devoid of any androgenic activity [49]. The administration of T significantly elevates the levels of diols which markedly depends on the route of administration and the administered steroid. The ratio is most sensitive to the administration of transdermal T due to the high abundance of α-reductase enzyme in the skin [50, 51]. 5α-DHT being a precursor to 5αAdiol leads to an increase in the diols ratio [52]. In general, a ratio higher than 2.4 is considered AAF [44].

1.3.3.4. A/Etio ratio

The ratio describes the products of phase I metabolism of 5-Adiols which is as sensitive as 5αAdiol/5βAdiol to detect the application of DHT and transdermal T.

1.3.3.5. 5αAdiol/E ratio

This is the last ratio that was added to the steroidal module after being reported as the most sensitive for the detection of doping with T gel [51]. It is helpful in the detection of all transdermal T and DHT

preparations.

The passport approach with the longitudinal monitoring over time is generated by a Bayesian Adaptive Model [53]. Based on intra-individual monitoring, it was observed that the detection window of oral T in urine was between 2 and 12 h. The transdermal T administration could never be revealed by $T/E > 4$ due to the slow-kinetic release of the topical application, while longitudinal monitoring in urine revealed its detection window mostly between 8 and 24 h [54].

The urinary longitudinal monitoring has indeed improved the capability of detecting steroids misuse [55]. However, urine is still a vulnerable medium due to confounding factors in the urine matrix, both endogenous (e.g., enzyme induction and inhibition) and exogenous (medications, bacterial contamination, ethanol, etc.) [56]. Therefore, monitoring of blood/serum provides an alternative approach that is currently being studied and continuously improved for detection of doping. Several studies for doping control have already reported biomarkers of interest in blood after oral administration of esterified T by means of immunoassay, radioimmunoassay, or liquid chromatography/mass spectrometry LC-MS/MS [57-59].

1.4. Analytical strategies in doping

All the compounds prohibited by WADA either in or out of competition require careful analytical procedures in order to achieve a proper monitoring of the samples. These compounds possess very diverse physicochemical properties (e.g., polarity, molecular weight, and acido-basic properties) which makes the analytical task very challenging. Besides, several methods in parallel are required to cover all the different categories of substances and to ensure the quality of the analytical results.

Generally, the analytical methods target these compounds as well as their major phase I and phase II metabolites as monitoring of the latter lays down the possibility of discovering more efficient markers of doping. In urine, the excretion pattern of each prohibited substance must be carefully examined to ensure the proper selection of the target compounds for screening purposes, favoring major metabolites or those with long-term urinary excretion profiles [60]. In blood, monitoring all prohibited compounds including major metabolites is crucial.

In practice, the presence and/or absence of a doping agent in tested samples is determined in routine testing through a common workflow including an initial test procedure (screening) followed by a confirmation procedure, if applicable. A schematic representation of the workflow in doping control laboratories is described in Figure 2. The screening step is carried out by fast and selective analytical methods that require good sensitivity, mostly by mass spectrometry, to limit the risk of false-negative and false-positive results. In the case of a suspicious result, the confirmation procedure is applied to the suspected samples that targets the potentially incriminating substance(s), including possible metabolite(s). Considering the chemical diversity and the wide range of physicochemical properties of the prohibited substances, anti-doping laboratories should use multiple analytical techniques, including immunological, biochemical, and chromatography–mass spectrometry methods [11].

All WADA accredited doping control laboratories are working according to the latter protocol which is necessary to keep their accreditation which is periodically monitored and examined. There are only 31 WADA accredited doping control laboratories around the world. The doping control laboratory in Stockholm, Sweden represents one of

only two labs in Scandinavia and 17 other WADA accredited labs in Europe [61].

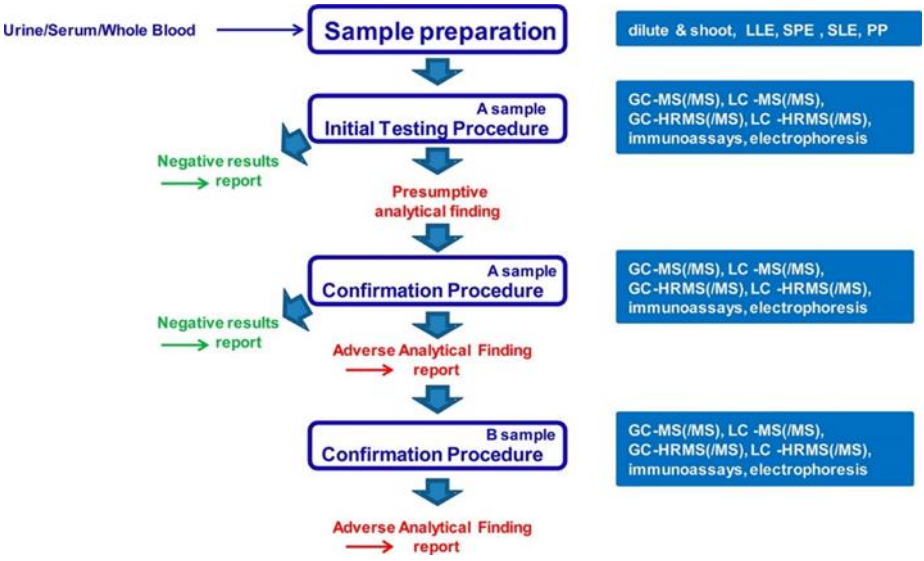


Figure 2. Typical workflow of doping control analysis [11].

1.4.1. Sample preparation

Sample preparation is a critical step due to the vast variations in the analytes' physicochemical properties and the complexity of the matrices containing salts, lipids, and proteins. Sample preparation procedures must ensure reliable sensitivity and selectivity to the analytical method by avoiding the contamination and clogging of the chromatographic column, and possible ion suppression using mass spectrometric detection. There are many types of sample preparation techniques used in doping control ranging from fast and simple dilute and shoot and protein precipitation to multistep sample preparation techniques (e.g. solid-phase extraction (SPE), liquid–liquid extraction (LLE), or supported-liquid extraction (SLE)). All these techniques can be used to provide acceptable recovery for most of the analytes when being used

during the screening step [62].

The use of selective and sensitive mass spectrometric detection facilitates the selection of suitable sample preparation procedures as screening of a wide range of compounds does not require highly selective extraction method. On the other hand, both urine or blood samples introduce matrix effects that can abolish the method selectivity and sensitivity especially in fast and simple procedures as in dilute-and-shoot procedure. The handling of matrix effects is usually done by the use of an isotope-labeled internal standard (IL-ISTD) which aids obtaining accurate and reproducible results, especially for the quantitative determination of threshold substances [63].

1.4.1.1. Liquid-Liquid extraction (LLE)

Substances at trace levels in the biological fluids (e.g. AAS) require a pre-concentration step. An LLE procedure was used formerly as the main pre-concentration technique in doping control when LC-MS or gas chromatography/ mass spectrometry (GC-MS) were less commonly used in the past. It achieves the analyte extraction by its differential partitioning between two immiscible solvents. Although being a simple and cost-efficient technique it was not suitable for polar compounds and it required large volumes of sample and solvent. The LLE procedure is usually required in two parallel extractions, at basic and acidic pH, respectively, for simultaneous extraction of acidic and basic substances during the screening stage [11].

1.4.1.2. Solid-Liquid extraction (SLE)

The extraction is carried out as the biological sample is adsorbed on a cleaned diatomaceous earth stationary phase with high surface area loaded in a cartridge or a well plate. The elution of the analytes occurs

when an immiscible solvent is applied to the cartridge. SLE, in other way, can be considered a sort of simplified and automated LLE. Moreover, the sample preparation is faster than LLE because problems associated with phase separation is of no concern, like emulsion formation. In addition, a high-throughput sample preparation platform is available for SLE techniques by using 96-well plates. SLE shows higher recovery values than LLE [62]. However, there is not sufficient demonstration about the applicability of SLE to the multiclass screening of the prohibited substances including both the acidic and basic compounds. Consequently, SLE can serve a valuable role in confirmatory procedures of certain substances, particularly low polarity compounds such as steroids and glucocorticoids [11].

1.4.1.3. SPE

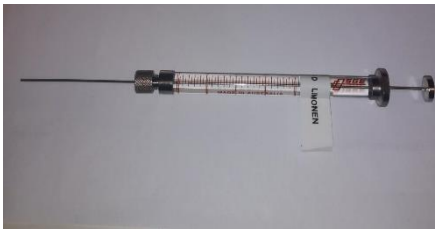
SPE is one of the main routine sample preparation protocols in doping analysis due to its suitability to many substances including anabolic agents, β2-agonists, hormone antagonists and modulators, diuretics, stimulants, narcotics, glucocorticoids, and β-blockers. It is superior to LLE and SLE techniques regarding solvent consumption and high-throughput analysis (96- or 384-well plates format), with simultaneous clean up and pre-concentration. In addition, SPE provides the ability to utilize a wide range of sorbents in the normal phase (extraction of polar analytes from non-polar organic solvents), reversed phase (extraction of hydrophobic analytes or polar organic analytes from aqueous matrices), ion exchange (extraction of charged analytes from aqueous or non-polar organic samples), and mixed mode, which allows the extraction of any compound due to several different interaction mechanisms. Polymeric C18 sorbents provides a good extractability for many prohibited substances in the screening process. Mixed-mode

cartridges have proved to be a promising technique as it improves the analyte retention due to its dual mechanism by C18 sorbent bonded with ion exchange groups. This enhances the applicability of such sorbent in screening purposes when large number of compounds are included [64, 65]. Polymeric sorbents that include polar and non-polar groups provide very applicable sample preparation procedures in doping analysis due to the compatibility with the different physicochemical properties of the prohibited substances.

1.4.1.4. Microextraction by packed sorbent (MEPS)

Microextraction by packed sorbent (MEPS) is a miniaturized SPE that is constituted of a milliliter to microliter packed bed volumes of sorbent [66], that can be connected online to GC and/or LC without any further modifications [67, 68]. This technique has been applied successfully to the extraction of a wide range of analytes from different biological matrices, such as urine, plasma, saliva and blood [66, 69, 70].

In MEPS, a syringe (100–250 µL) is packed with approximately 1–2 mg of sorbent as a plug, between the barrel and the needle as a cartridge or as a barrel insert in a needle (BINs) operated by eVol device produced by Trajan scientific and medical (Figure 3) [71]. Different modes of separation sorbents can be applied to the MEPS approach. It can be used for reversed phases, normal phases, mixed mode, and ion exchange sorbents [69, 70, 72]. MEPS can include reversed phase sorbents (C18, C8 and C2), normal phase (silica), restricted access material (RAM), HILIC (hydrophilic interaction liquid chromatography), carbon, polystyrene-divinylbenzene copolymer (PS-DVB), molecular imprinted polymers (MIPs), strong cation exchange (SCX) and mixed mode (C8/SCX) chemistries [70].



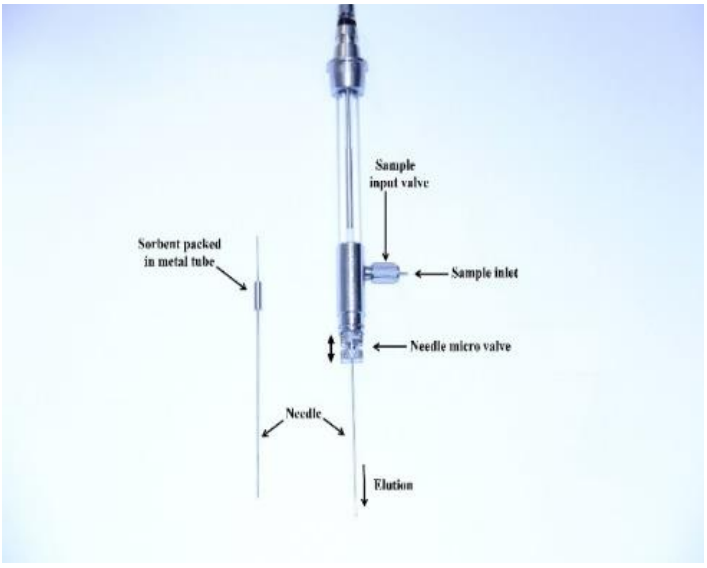


Figure 3. Different types of MEPS syringes used in our studies including eVol semi-automated dispensing device.

MEPS provides a suitable tool for sample purification and pre-concentration especially when only microliters (10 µL) up to 1000 µL of sample is available which is common in case of biological or environmental samples [73]. Furthermore, the packed sorbent can be used more than 100 times [70, 73], even when using plasma or urine samples, whereas the conventional SPE column is dispensable and used only once. The elution of analytes from sorbent beds can be carried out using small volumes of an organic solvent, such as methanol or other mobile phases, which applies a greener sample preparation approach while ensuring a high yield of analytes. MEPS can provide a very promising alternative to conventional SPE due to the fast and ease of use, the possibility of being fully automated for online procedures, the reduction of organic solvent and sample volumes used.

1.4.1.5. Alternative Samples

There is a growing trend towards the use of alternative samples to blood, plasma and urine to detect drugs for clinical and forensic applications [74]. These alternative samples can be hair [75], sweat [76], breath [77] and saliva [78]. Saliva, unlike blood and urine, provides a quick and non-invasive sampling. While collecting blood samples require experienced personnel, saliva sampling does not require professional expertise. In sports competitions, sample collection has to be supervised. Unlike urine samples, saliva can be collected under supervision without any privacy violation due to lack of direct observation of private functions [79]. However, the collection of saliva samples is usually opposed by the lack of sufficient fluid due to either physiological factors or even the drug use itself [80, 81]. Substances and/or techniques that stimulate the production of saliva can also alter drug concentration. In addition, only a limited number of drugs were clinically monitored in saliva as the

correlation between saliva and plasma concentrations were not attained for many substances [82]. Plasma or serum samples can reflect the actual circulating concentration of the analytes while urine permits the measurement of the accumulated concentration of analytes and metabolites [79]. Saliva, on the other hand, contains only the free (protein unbound) fraction of drugs [82]. Most drugs are highly bound to blood proteins, but it is only the free fraction that is pharmacologically active [83]. Therefore, the drug concentration in saliva is a better representation of the therapeutically active fraction of drugs than the drug concentration in plasma [82, 84]. This reflects the importance of saliva as a sample for the therapeutic monitoring of drugs as well as a diagnostic medium for the measurement of endogenous markers [85-92].

1.4.2. Chromatographic analysis

GC–MS, despite being used routinely for comprehensive screening methods in doping analysis, is usually time-consuming in regards of sample pretreatment. It is often based on hydrolysis and derivatization procedures prior to the analytical step. On the other hand, LC–MS methods have proven to be successful in the identification and determination of steroids and their metabolites in different biological matrices [93-96]. The introduction of UHPLC with tandem mass spectrometry or high-resolution mass spectrometry has become the technique of choice for steroid analysis. UHPLC has improved the methods speed, sensitivity, reproducibility and specificity with respect to HPLC. It provided high applicability for multi-component mixtures of steroids and their metabolites, especially when it comes to the conjugated metabolites of AAS [97-100]. Thousands of samples can be analyzed per month thanks to the modern multiplex instruments with improved specificity and resolution offered by time-of-flight, quadrupole time-of-

flight or quadrupole orbitrap mass spectrometry.

Nowadays, LC systems can use vast number of stationary phases that suites the purpose of the analysis. Moreover, the technique provides high versatility towards the use of different mobile phases in order to achieve the best separation.

One of the most challenging tasks in chromatographic separations is chiral separations. The chiral stationary phases (CSPs) usually consist of either small chiral molecules or chiral polymers immobilized on solid support such as silica gel. The chiral recognition is attributed to "*Three point attachment theory*" [101] which states that the interaction between the chiral molecule and selector is the binding of three groups (colored) of the tetrahedral carbon atom to a receptor surface at specific sites A, B, and C (Figure 4).

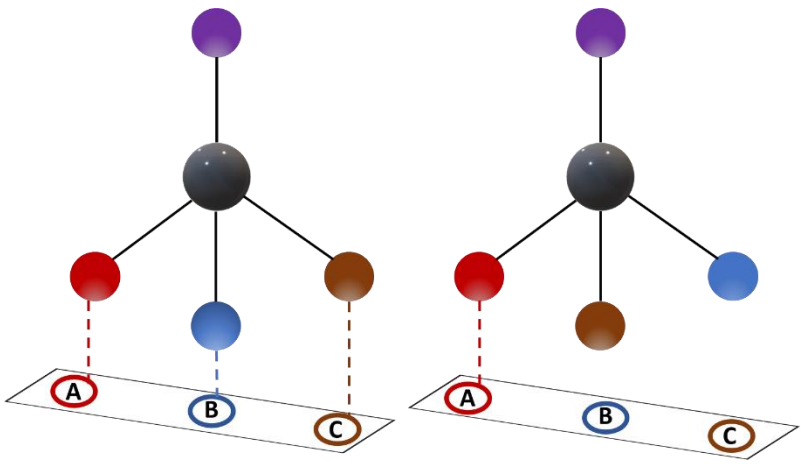


Figure 4. Three-point attachment for chiral interaction with analyte-selector interaction (enantiomer to the left) and no interaction (enantiomer to the right).

The specific configuration of the receptor would make it impossible for the enantiomer to undergo an equivalent binding via the same three-contact points. The most commonly used CSPs are polysaccharides. They provide high affinity towards many analytes due to the unique configuration of the polysaccharide backbone (cellulose or amylose derivative) with attached carbamate derivatives that provide high functionality to link with the enantiomers via H-bonds (Figure 5). Polysaccharide CSPs favors normal phase conditions [102, 103], which usually counteract the analyte ionization at the interface of mass spectrometry. Incorporation of assistant polar would enhance the ionization of the analyte molecules at the ESI-interface, hence increasing the signal intensity.

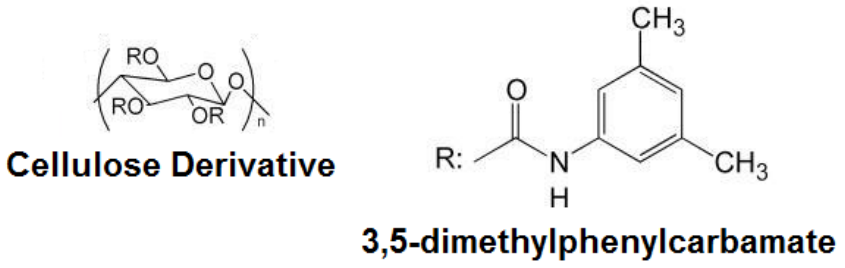


Figure 5. Polysaccharide chiral stationary phase chemical structure showing cellulose backbone for chiral selection and carbamate derivative for polar interaction.

1.4.3. Mass spectrometric detection (in LC-MS)

Basically, the mass spectrometer generates ions from molecules and separates them according to their *mass-to-charge ratio (m/z)* which can be qualitatively or quantitatively detected according to their respective *m/z* and signal intensity. The modern definition of mass spectrometry has

added up that the ionization of a sample is not only achieved by the influence of electrons but it could also be due to photons, neutral energetic atoms, massive cluster ions and others [104]. A typical basic scheme that most mass spectrometers follow is: an ion source, a mass analyzer, and a detector.

At the front end of a mass spectrometer, the ion source is the one part responsible for ion production. The solution of the analyte is injected into the ion source under atmospheric pressure in a stream of heated nitrogen gas (≈ 200 °C) to assist the evaporation of the solvent. The most commonly used platforms for sample ionization are ESI, APCI and APPI.

1.4.3.1. Electrospray Ionization (ESI)

Under the influence of an Electric field, a mist of electrically charged droplets is generated. This mist is consistently exposed to a hot stream of nitrogen which act as an evaporation gas leading to continuous shrinking of the droplets until the formation of completely desolvated ions. The stream of liquid experience the high electric field at the open end of the spray capillary which results in the charge separation in the electrolytic solutions and the formation of *Taylor Cone* [105, 106] into jet of microdroplets that are of same charge. Hence, repelled by coulombic repulsion and directed towards the counter-electrode. The generated desolvated ions are then being focused into the mass analyzer [107].

Two models explain how ions are formed from the charged droplets. The older model, *The charged – residue model (CRM)* [108, 109] assumes that the complete loss of the solvent molecules until a full desolvation of ion contained in a sufficiently small droplet is responsible for the ion introduction in the gas phase. The charges (protons) are then transferred to the molecule, preferentially on the exposed basic sites. The

later model, *the ion evaporation model (IEM)* [110-112] assumes that a direct escape of ions from the surface of highly charged microdroplets results in complete desorption of analyte ions. The ion evaporation can occur after the shrinkage of charged droplets to allow maximum charge density and under the influence of a suitable electric force to provide the energy needed for ion escape.

1.4.3.2. Atmospheric Pressure Chemical Ionization (APCI)

Ions are generated via a needle electrode in a proximity to the sampling orifice. The solution is exposed to a heated cartridge to about 500 °C for evaporation and the formation of ions under atmospheric pressure takes place by the corona discharge [113, 114] i.e. the ions are actively generated from neutrals which provide an advantage over ESI for analyses of low/non-polar compounds [115, 116]. APCI requires higher flow rates than ESI for effective vaporization (200 – 1000 µl/min) [117].

1.4.3.3. Atmospheric Pressure Photonization (APPI)

The analytes introduced to the ionization chamber are first vaporized with the aid of the nebulizing gas then exposed to ultraviolet light from a krypton lamp. The photons emitted from this lamp have a sufficient energy level to ionize molecules before entering the mass spectrometer [118, 119]. The technique is useful for non-polar analytes that are difficult to ionize with the conventional ESI [120].

1.4.4. Mass Analyzers

The mass analyzers used in the current study which are highly applicable in doping analysis are briefly described in the following:

1.4.4.1. Quadrupole Mass Analyzers

A quadrupole mass analyzer is a set of four conducting rods arranged in

parallel and extended along the Z-axis, with a space in the middle. Each of the opposing pairs of rods are electrically connected to each other [104, 121]. The filtration of ions takes place by maintaining a stable trajectory of target ions through the quadrupole until the detector. The ions travel through the quadrupole under the influence of an oscillating electric field. A radiofrequency RF voltage is applied on one pair of opposing rods which can aid as a sort of ion focusing. When a DC offset voltage is applied to the second pair of rods, only ions with a specific *m/z* ratio can maintain their trajectory to the detector while other ions bombard against the rods and will not reach the detector (Figure 6). By continuously varying the applied voltages, the analyst can scan for a wide range of *m/z* values [121].

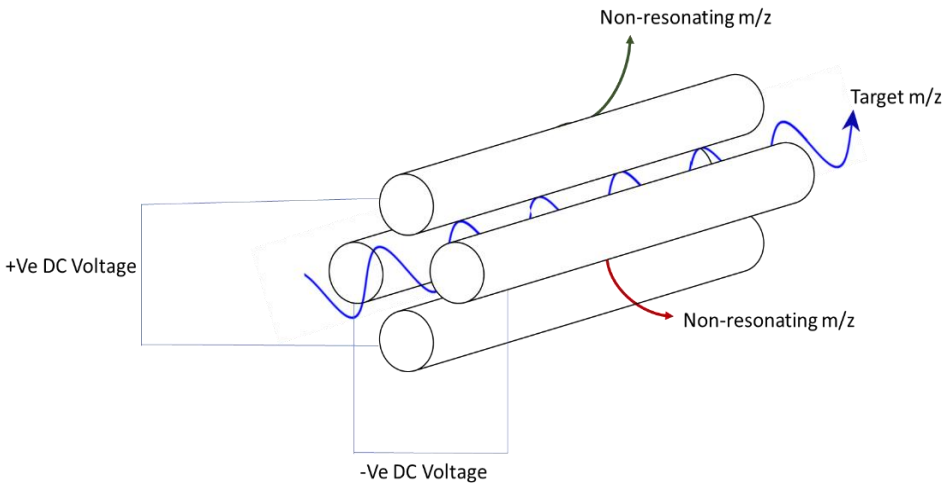


Figure 6. A schematic diagram of a quadrupole mass analyzer showing the trajectory of the resonating charged ions under the orthogonally applied positive (+ve)/ negative (-ve) DC voltage to the quadrupole filter

A single quadrupole mass spectrometer can only detect the ions formed at the ionization source that are intact molecules or possibly fragment ions that are formed by in-source fragmentations. Therefore, a single quadrupole does not provide sufficient structural information with lower specificity if compared to tandem mass spectrometers.

A tandem mass spectrometer, called a triple quadrupole, consists of two quadrupole mass analyzers separated by a collision cell. The precursor ions that travel through the first quadrupole are selected, focused and then fragmented in the collision cell by a process known as collision-induced dissociation (CID) [122, 123]. CID results from the collisions of the analytes with an inert gas, usually nitrogen or argon, to produce fragment ions or the so-called product/daughter ions. The specific product/daughter ions help make the detection more selective as ions of interest are selected by the final quadrupole mass analyzer and then passed to the detector [104]. The precursor ion/product ion pair selection is called the mass transitions. Only analyte ions having that specified mass transition are able to reach the detector, which gives the high specificity of tandem quadrupole mass spectrometric methods. This mode of data acquisition is known as selected-reaction monitoring (SRM) [124]. When multiple transitions are selected, the data acquisition is called multiple-reaction monitoring (MRM) [125, 126].

Triple quadrupole mass spectrometers are highly useful for confirmation methods in doping analysis when extra specificity is required and when co-eluting substances with identical elemental compositions exist [127]. MRM modes can provide accurate identification and quantitation data of target analytes.

1.4.4.2. Orbitrap Mass analyzers

The Orbitrap mass analyzer consists of a cylindrical electrode with a spindle-like central electrode (Figure 7). When voltage is applied between the two electrodes, a linear electric field is generated along the axis. Thus, ions become captured in a rotational oscillation along the axis. The trajectory of ions is the equilibrium between the centrifugal and electrostatic force under the applied voltage between the axial and the cylindrical electrodes [128, 129].

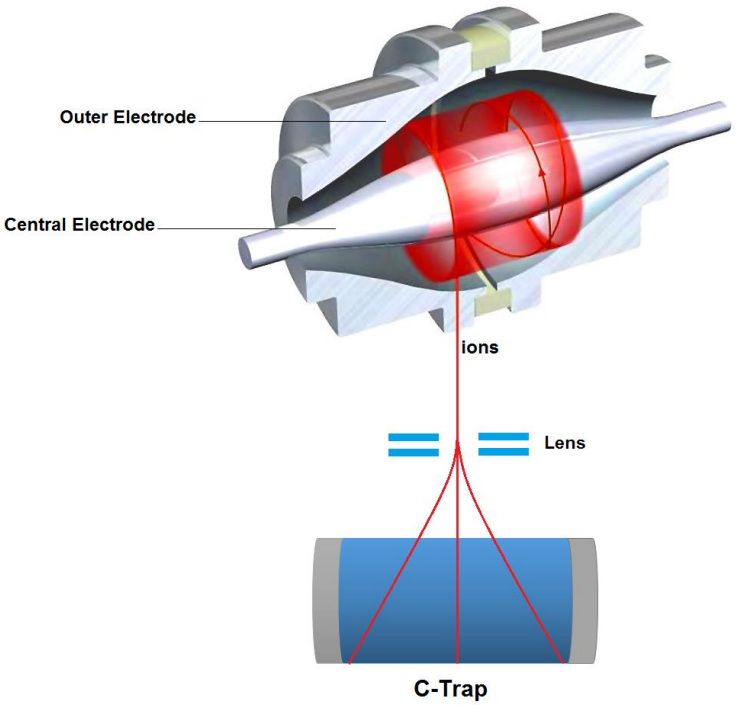


Figure 7. Orbitrap mass analyzer showing the pathway of ions ejected from C-Trap to the radial electric field of the orbitrap

Ions are then ejected into the space between the central and outer electrodes essentially through a deflector electrode which lies in one of the outer electrodes. Under the applied voltage between the central and outer

electrodes, the generated radial electric field bends the ion in a trajectory towards the central electrode. The tangential movement results in an opposing centrifugal force. The axial motions of the ions are completely independent of the motion around the inner electrode and all initial parameters of ions, except their *m/z* [130].

The C-Trap is an external ion storage device that accumulates the ions prior to injection into the orbitrap. A maximum quantity of ions is selected, trapped and analyzed at the same time which is determined by the automatic gain control (AGC) setting. Complex matrices can produce large amount of background ions. The hybrid quadrupole-orbitrap mass spectrometer is designed to reduce the background signals by using a low-resolution quadrupole filter that focuses only precursors of interest to be analyzed by the high-resolution orbitrap [130, 131]. This improves the capability of the orbitrap mass spectrometers for analyzing low molecular weight compounds in various matrices by providing accurate masses of either precursors or product ions in full scan mode.

High-resolution mass spectrometers using orbitrap analyzers play significant roles in doping analysis. As with HPLC retention times and MS full scan data in conjunction with accurate mass measurements can greatly facilitate the identification of prohibited substances components during the screening assay. Nowadays, orbitrap mass spectrometers are highly abundant in doping control laboratories as the high mass accuracy (better than 1 ppm with internal calibration) and resolving power (up to 240,000) makes it an asset to aid both screening and confirmatory assays.

1.5. Challenges with LC-MS analysis

During LC-MS assays, the chromatographic conditions adopted for optimum separations are sometimes not favorable by mass spectrometric detection. For example, chiral separations using the common polysaccharide stationary phases usually favor normal phase conditions as previously discussed. Using highly non-polar solvents such as hexan or heptane as the major component of the mobile phase is important to achieve a proper chiral separation. However, the sensitive detection of such analytes is always thwarted by the lack of sufficient ionization with ESI mode. The need to shift either to APCI detection modes or to post-column addition of makeup solvents are usually utilized [132]. The post column addition of makeup solvents, referred in the current study as Online post-column solvent assisted ionization (OPSAI), is usually easier and cheaper to apply than APCI modes. OPSAI has been used to enhance the ionization of several analytes after chromatographic separation using mass spectrometric detection [132-134].

Another approach to enhance mass spectrometric detection of analytes is the electrospray-based ionization method termed solvent-assisted electrospray ionization (SAESI) [135]. The introduction of the assistant solvents at the tip of the main electrospray needle enhance the ionization of the compounds that are non-electrospray ionization-friendly. The technique can be used in detection of organic reaction intermediates and real-time analysis of polymers and chiral drugs that are separated by gel permeation chromatography (GPC) and normal phase liquid chromatography (NPLC). Furthermore, it can achieve online hydrogen/deuterium (H/D) exchange reaction and even mitigate the signal suppression caused by strong acid modifiers in liquid chromatography

[135]. In addition, the introduction of assistant solvents directly into the ionization chamber of the mass spectrometer only requires simple sprayers that are commercially available or even homemade sprayers which makes SAESI easy to handle.

2. Aims of the thesis

The study presents newly developed analytical methods using LC-MS for analysis of prohibited substances for doping control in sports. The study focuses on the challenging aspects of chiral separations with mass spectrometric detection regarding the low polarity of the mobile phases. In addition, sensitive and selective determination of therapeutic/doping chiral drugs in saliva and plasma is addressed. The study includes the development of analytical methods for the determination of the endogenous anabolic androgenic steroids as an approach to better implementation of the steroidal module of athletes. Moreover, it includes the development of improved sample preparation techniques to reduce matrix effects and increase the method sensitivity.

The specific aims of the thesis are:

- To achieve chiral separation and quantitation of WADA-prohibited β-receptor blocking agents, propranolol and metoprolol, in plasma and saliva to aid monitoring of the drug enantiomers in doping and therapeutics.
- To address the comparative effects of SAESI and OPSAI techniques to improve mass spectrometric detection of chiral drugs using chiral HPLC.
- To develop sample preparation methods based on miniaturized extraction technique (MEPS) for drug extraction and pre-concentration from human plasma and saliva samples.
- To provide new analytical methods for sensitive quantitation of EAAS in human serum using UHPLC-HRMS for doping control in sports.

- To investigate the effect of cross abuse of other substances, such as growth hormone (GH), on the steroidal module in human serum as a future endocrine module of ABP. As an example, the steroidal profile is suggested to provide a potential biomarker for GH doping in athletes.

3. Methods

3.1. Chromatographic separation using HPLC-MS/MS (Paper I & II)

The chromatographic separation was achieved using the chiral column CHIRAL ART Cellulose-SB (150 × 4.6 mm, 5-µm particle size) from YMC Europe GmbH (Dinslaken, Germany) and mobile phase (0.1 % ammonium hydroxide in n-hexane/isopropanol (80:20 %v/v)).

In paper I, the non-polar mobile phase required for the chiral resolution of propranolol enantiomers suppresses the ionization of the target analytes at the ESI-interface. The developed method deals with improving the signal acquisition of the analytes by the introduction of two techniques utilizing assistant polar solvents.

3.1.1. Online post-column solvent assisted ionization (OPSAI) approach

This approach depends on the introduction of makeup solvent into the mainstream of LC effluent before the ion source (Figure 8). The makeup solvents are selected to be miscible with the mobile phase and sufficiently polar to aid analyte ionization. It was mixed with the LC effluent via a three-way T-junction after the chromatographic column and driven by an isocratic pump (Shimadzu, Kyoto, Japan) with a flow rate of 0.2 mL/min. Makeup solvents composed of (A): 0.5% formic acid in isopropanol (B): 0.5% formic acid in isopropanol-water (1:1), and (C): 0.5 % formic acid in water. The proportion of the makeup solvent was carefully optimized to be 20% of the overall flow to avoid excessive post-column dilution of the analyte. The overall flow was tested and optimized to be 1 mL/min. The chiral separation of propranolol enantiomers was

achieved within 12 min. The inner diameter of all the PEEK tubes used for post-column connections was 0.1 mm to minimize the effect of the extra post-column volume due to the makeup solvent on the obtained separation resolution.

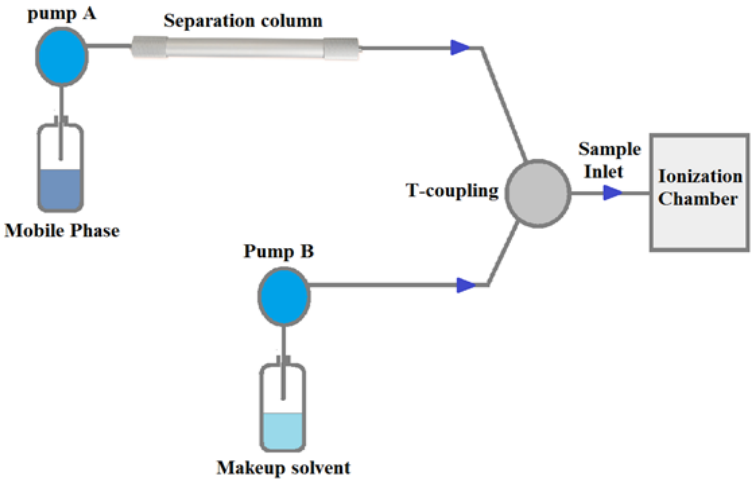


Figure 8. Online post-column solvent assisted ionization (OPSAI) approach

3.1.2. Solvent assisted electrospray ionization (SAESI) approach

The SAESI approach aims to introduce the makeup solvents as a fine spray into the ion source to be mixed with the main electrospray from the LC system. It was performed using a metal probe inclined with a specific angle (45°) for optimum mixing of sprays inside the ionization chamber. The internal diameter of the metal probe was 125 µm and the probe is connected to the nebulizing gas inlet. The makeup solvent was pumped to the ion source via an isocratic pump (Shimadzu, Kyoto, Japan) with a flow rate of 0.2 mL/min and mixed with the nebulizing gas using a T-coupling to produce the spray (Figure 9). Same makeup solvents, A, B and C were used as with SAESI to evaluate the ionization efficiency of the analytes.

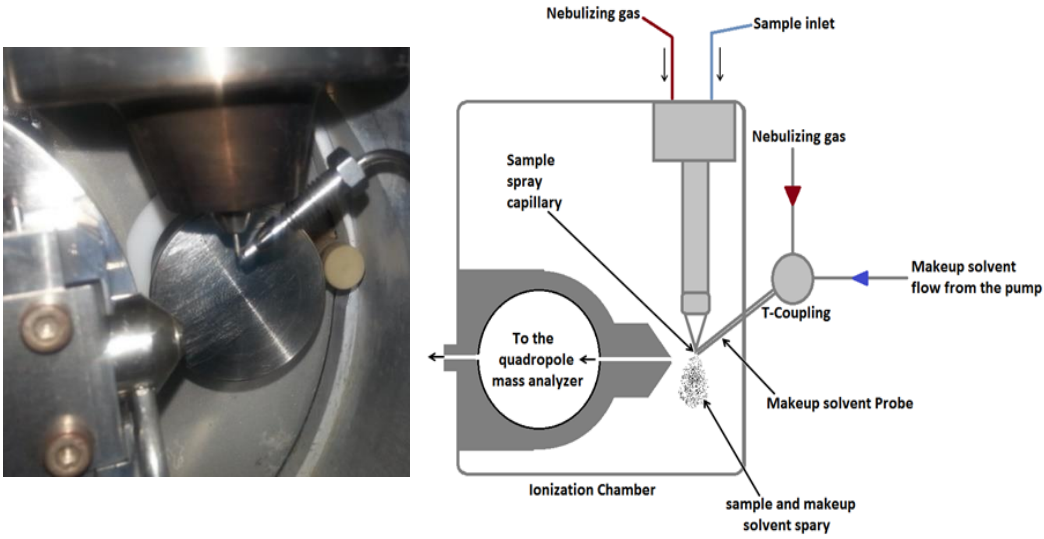


Figure 9. Solvent assisted electrospray ionization (SAESI) approach.

3.2. Sample preparation using MEPS (Paper I & II)

The method was developed using a syringe with a packed-bed of C18-sorbent (3 mg). To monitor the number of aspiration/dispensing cycles, the syringe (250 µL) was used manually to carry out the extraction procedure. Plasma and saliva samples were usually diluted before application to MEPS to reduce the high back-pressure produced upon dispensing solvents. The steps involved in the MEPS procedure are similar to those in the case of conventional SPE. It starts by conditioning the sorbent with organic solvent mainly methanol and activation using water. The samples were spiked with the internal standard and then aspirated by the MEPS syringe. The sample was driven through the sorbent bed inside the syringe several times for maximum analyte recovery (4-6 cycles). The sorbent was then washed to eliminate interfering matrix components. The elution step took place using a suitable solvent (e.g. methanol). The sorbent was reactivated for subsequent extraction simply by washing with methanol/water which showed high efficiency for up to 55 extraction. The

sorbent can be easily replaced either by introduction of a new sorbent material inside the syringe barrel or application of a new BIN into the MEPS syringe operated by an eVOL device. After the development of the method, the extraction procedure could be fully automated by the integration of the MEPS syringe in a CTC autosampler which can perform a high-throughput sample preparation process.

3.3. Chromatographic separation using UHPLC-HRMS (Paper III & IV)

Chromatographic separation was achieved using TriART C18 100 × 2.0 mm with 1.9 µm particles with a precolumn YMC-Triart C18 5 × 2.1 mm (YMC Co. Ltd, Kyoto, Japan). The column is functional at higher pH values (up to 12) which is suitable for the used mobile phase. The mobile phase consisted of: (A) 10 mm ammonium acetate adjusted to pH 9.5 using 20% Ammonium hydroxide and (B) 10 mm ammonium acetate adjusted to pH 9.5 and methanol (10:90 % v/v) at a flow rate of 0.5 mL/min and a column temperature at 50 °C. A high resolution/high accuracy mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) with an HESI-II probe was used for analyte detection operating in full scan mode with a scan range from 300 to 500 *m/z* in the negative mode and from 200 to 400 *m/z* in the positive mode under a resolution value of 70000.

Standard solutions of all analytes were used for setting the calibration of the method as well as the identification of target analytes. System suitability testing was performed for a standard mixture before sample run and daily wash and calibration procedures of the mass spectrometer were carried out for optimum performance.

Analytes in serum samples were extracted using Oasis HLB SPE cartridges, 1 mL, 30 mg, (Waters, Taunton, MA, USA). The elution of the

analytes took place in two steps to increase the yield of the target analytes. First, 1 mL mixture of (acetonitrile/ methanol/ 5% ammonium hydroxide) (50:50:0.1 % v/v) was allowed to elute through the sorbent. Second elution was performed by 1 mL mixture of (acetonitrile/ isopropanol) (40:60 %v/v). The first step was convenient for the elution of the conjugated steroids while the use of the second elution mixture showed more tendency to elute the unconjugated analytes. The eluate was then evaporated to dryness at 60 °C under nitrogen and subsequently reconstituted in 100 µL 40 % methanol.

3.4. Method validation (Paper III)

The method has been validated in accordance with the world anti-doping agency (WADA) guidelines used by accredited laboratories [7].

Calibration of the method was performed using standard solutions of target analytes in serum including a spiked concentration range of 0.1-14 ng/mL and 1-70 ng/mL of the EAAS and conjugated analytes, respectively. The quality control solutions were prepared in three different concentrations to span the calibration range including low (LQC), medium (MQC) and high (HQC) concentration levels. Different isotopic internal standards were used and assigned for quantitative measurements as the most convenient representative of the corresponding analyte. The correlation coefficients for all calibration were $\geq$0.985, with relative standard deviation of each calibrator concentration $\leq$ 20 %.

Accuracy and precision were calculated using QCs and tested for within-day and inter-day results. % trueness, the repeatability of the assay (intra-day precision) and the intermediate precision which implement the inter-day effect were calculated and showed satisfactory results within the limit of 20 % variation. The recovery was tested for all analytes and was

found to vary within the acceptable limit of 70% to the pre-extracted amount. Limit of detection and quantitation (LOD and LOQ) were assessed for all analytes with 3 data points as the lowest threshold for peak detection. Stability was assessed as short-term stability after 24 h of storage at room temperature, the freeze-thaw cycle stability that evaluate the analyte stability after 1-3 freeze cycles and stability on the autoinjector tray held at 5°C for 3 consecutive days with variation limit not exceeding 15% of the determinations.

Healthy volunteers' samples and samples collected before and after GH intake were collected, stored under -80 °C until analysis. All samples were subjected to the developed SPE method before chromatographic separation. The data analysis was performed using TraceFinder for Clinical Research 4.1 software.

4. Results and Discussion

4.1. Paper I

Chiral separation of propranolol enantiomers with a Chiral-SB column was achieved using a mobile phase composed of 80:20 (v/v) n-hexane/isopropanol with ammonium hydroxide 0.1%, v/v. It was found that chiral separation cannot be achieved unless a non-polar solvent, such as hexane or heptane, constitutes the major component of the mobile phase. These solvents interfere with the analyte ionization at the ESI interface and significantly decrease the signal intensities of target analytes. This project aims at improving the poor ionization of chiral analytes at ESI interface of mass spectrometry when using the typical non-polar mobile phase combinations for chiral separation. The developed technique depends on the introduction of a makeup solvent with sufficient polarity to aid in the ionization of the target analytes. The selection of the makeup

solvents is based on their miscibility with the mobile phase to ensure proper mixing as well as sufficient polarity to enable analyte ionization. The use of makeup solvents was investigated by two different approaches.

OPSAI approach

The makeup polar solvents were introduced after LC separation via a T-junction that mixes the main effluent from the LC column and the solvent. The tested mixtures composed of isopropanol and/or water plus formic acid in different proportions to produce solvents (A), (B) and (C). The proportion of the makeup solvent in the overall flow rate was optimized to be 20% to maintain a good balance between the ionization efficiency and the dilution effect. The separation of enantiomers was optimized within a 12 min runtime.

Formic acid improved the MS response of propranolol enantiomers in positive ESI mode. Optimum ionization efficiency and minimum background signal was obtained when formic acid constituted 0.5% of the total makeup solvent. It was found that makeup solvent A (0.5% formic acid in isopropanol) demonstrated the highest S/N ratio for both enantiomers with the highest signal intensity. Decreasing the isopropanol proportions in solvent B to half resulted in a decrease in both S/N and signal intensity of both enantiomers and finally solvent C (0.5% formic acid in water) resulted in the lowest response (Figure 10). Isopropanol is miscible with hexane, which is the major mobile phase component. Hence, distribution of the analyte in the isopropanol portion of the makeup solvent is feasible. This explains the lower signals acquired with solvent B and C due to lower isopropanol proportions.

SAESI approach

In this approach, the makeup solvent is introduced into the ESI-interface using a probe which allow the solvent to be pumped into the ion

source as a fine spray using nitrogen. The solvent sprayer is placed to allow maximum proximity to the capillary tip for optimum mixing of both sprays. The tip of the makeup sprayer was adjusted to be at a 45° angel with respect to the capillary spray.

The detection of propranolol enantiomers using the SAESI approach was best demonstrated when using solvent (C) which gave the highest signal intensities and S/N ratio while the lowest values were observed when using solvent A (Figure 10). Herein, water as makeup solvent produced superior ionization efficiency to isopropanol. Water from the makeup solvent is mixed with the main spray from the ESI capillary and enhances the charge transfer to the analyte molecules due to higher polarity and hence conductivity than isopropanol. Signal intensities using make-up solvents (B) and (C) were higher than those in case of only isopropanol (solvent A).

The comparative study between the OPSAI and SAESI approaches showed that the use of solvent A with OPSAI resulted in better signal intensities than using solvent C with SAESI. Therefore, makeup solvent A was selected for the chiral separation and quantitation of propranolol. We suggest that SAESI can be a promising technique by further investigation of the method parameters such as the distance between the makeup and spray needle, flow rates of the makeup and main electrospray and different compositions of the makeup solvents.

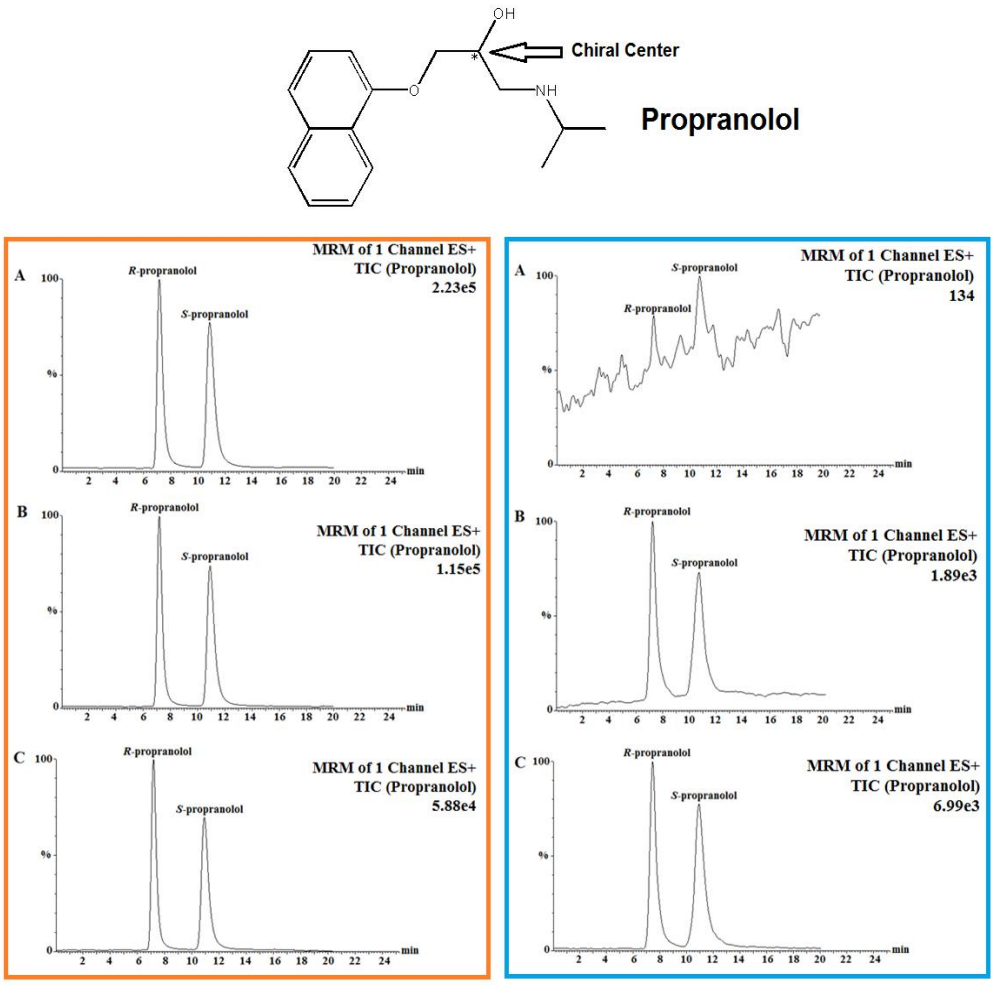


Figure 10. Chiral LC-MS/MS separation of propranolol enantiomers using SAESI and OPSAI approaches to enhance the analyte ionization at ESI interface using makeup solvents A (0.5 % formic acid in isopropanol), B (0.5% formic acid in isopropanol:water 1:1) and C (0.5% formic acid in water).

4.2. Paper II

A method was developed for chiral separation of the metoprolol enantiomers. The method was applied to determine each enantiomer in biological samples including plasma and saliva. The key element for the

successful application of the method was the development of the sample extraction procedure using MEPS. As a miniaturized technique it improved the sample recovery from the test matrix due to efficient extraction and pre-concentration. The MEPS procedure was based on the use of a MEPS syringe operated by a semi-automated eVOL dispenser with C18 sorbent BIN. Pentycaine was used as the internal standard owing to similar elution profile from MEPS as the target analytes and high reproducibility in extraction while lacking interaction with either of the target analytes or the matrix components. The use of C_{18} sorbent BIN showed highest recoveries of the analytes that ranged from 96.11 to 98.61 and 95.4 to 98.99 % in plasma and saliva samples, respectively.

The metoprolol enantiomers were separated using a cellulose SB chiral column and mobile phase composed of 0.1% ammonium hydroxide in n-hexane/isopropanol (80:20, v/v). As a result of low polarity of the mobile phase, a post-column addition of makeup solvent was added to enhance the ionization efficiency of the analytes. The makeup solvent composed of 0.5% formic acid in isopropanol which provided the best results with similar chiral separation conditions addressed in Paper I (Figure 11). The method calibration was tested in both plasma and saliva and for each sample type the method was proved to be linear in the range of 2.5-500 ng/mL with LLOQ of 2.5 ng/mL.

Saliva can provide an alternative sample to plasma to monitor the therapeutic concentrations of administered drugs providing faster, simpler and non-invasive sampling procedure compared to plasma. MEPS is an optimum solution for saliva sample extractions due to the availability of only small volumes per sample and it can also provide a promising sample collection/extraction device.

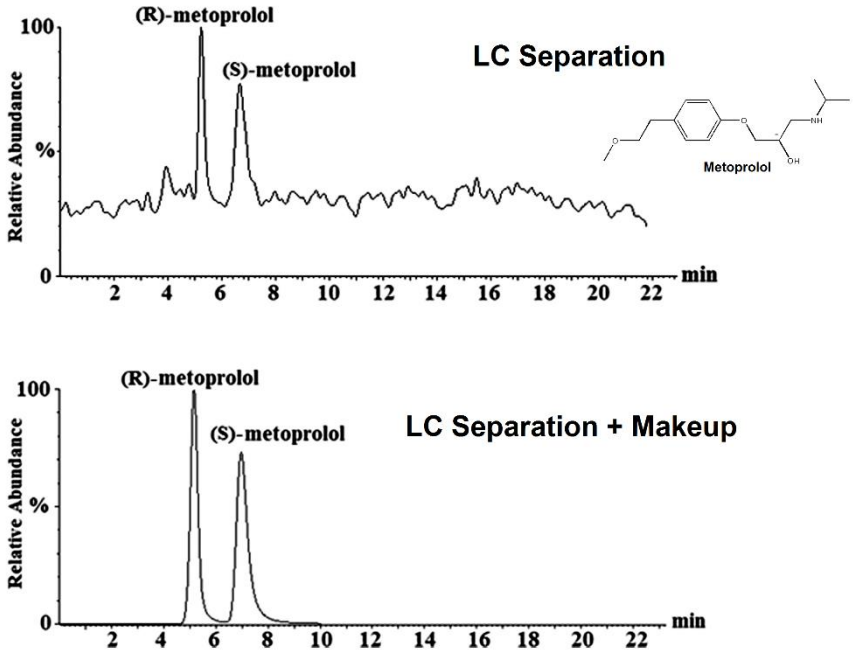


Figure 11. LC/MS separation of metoprolol enantiomers using mobile phase 0.1% ammonium hydroxide in hexane–isopropanol (IPA) (80:20 v /v) with and without makeup solvent addition (0.5% formic acid in isopropanol).

The method was validated in plasma and saliva and provided a sensitive quantitation of metoprolol enantiomers. The method can be applied to doping analysis in plasma and saliva, as well as in therapeutic drug monitoring and clinical and forensic investigations.

4.3. Paper III

The method was developed to chromatographically separate 20 EAAS in human serum samples. Separation is crucial for proper identification of target analytes with similar *m/z* as for the detection of isomeric compounds. Basically, the conjugated EAAS produce the best mass spectrometric response in the negative ESI as $[M-H]^-$ while the unconjugated EAAS produce highest signals in the positive ESI as $[M+H]^+$. Some analytes were detected as different adducts such as $[M+NH_4]^+$ for DHEA and Etio, and $[M+H]^+$ for TS and ES which aided in their identification due to chromatographic overlap with their isomers. The mass spectrometric detection was operating in full scan mode which facilitated the screening of all potential adducts.

Different mobile phase compositions were tested for the optimum separation of targeted analytes. It was found that the pH of the mobile phase is crucial for optimum separation and detection of all analytes. The use of 0.1% formic acid or acetic acid improved the detection signals of EAAS in the positive ESI mode, however the sulphate-conjugated analytes showed very low response. The use of ammonium acetate or formate buffers at pH 4.4-4.6 showed a promising separation and detection of glucuronides and sulphates in the negative ESI mode while unconjugated EAAS showed relatively low and irreproducible signals.

In conclusion, unconjugated EAAS requires acidic mobile phase pH and positive ESI detection while conjugated EAAS, specially sulphates require basic mobile phase pH and favor negative ESI detection. The use of different mobile phase pH is the reason for the lack of common chromatographic/detection conditions for the two groups of analytes. However, when using a higher pH buffer (pH = 9.5) such as ammonium

acetate buffer, the detection signals were improved and more reproducible for all analytes. Some analytes were detected as ammonium adducts $[M+NH_4]^+$ such as DHEA and Etio, which produced higher signal intensities than their precursor ions $[M+H]^+$ and were used for their identification and quantitation. The ammonium adducts detected have resolved the chromatographic overlap of DHEA and Etio with E and A, respectively when detected as $[M+H]^+$ traces. Neutral-loss $[M-H2O+H]^+$ adducts were also observed for DHEA, DHT, A and Etio (Figure 12).

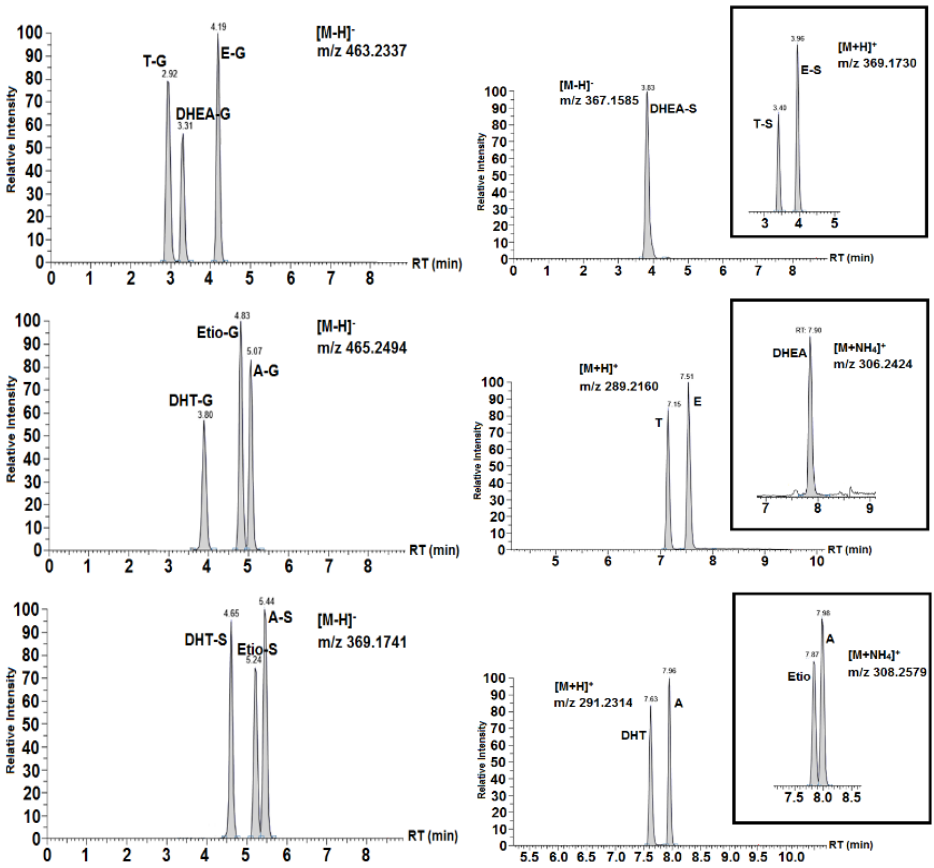


Figure 12. LC-MS chromatograms of EAAS using methanol/ ammonium acetate (90:10 v/v %) at pH 9.5 showing different adducts of target analytes

The use of APCI interface instead of ESI was tested because of the weak ionization of unconjugated EAAS encountered under the developed LC conditions. APCI has wider applications for mass spectrometric detection of poorly ionizable or intermediately to non-polar compounds.

APCI showed slightly improved and more reproducible signal intensities for the unconjugated EAAS in comparison to ESI while lower intensities were observed for the conjugates. However, APCI showed improvements only upon using mobile phase compositions of water/methanol mixtures as the additions of any buffers reduced the signal amplitude of all analytes. This has shown that the buffer component is crucial not only for the detection but also for the chromatographic separation. Hence, APCI could not be pursued due to the lack of proper chromatographic resolution of analytes under the chromatographic conditions used. The effect of applying APCI mode in comparison to ESI is illustrated in Table 2.

All serum samples were subjected to the SPE procedure prior to analysis. Different sorbents were tested to provide the optimum recoveries of target analytes. SPE cartridges with different modes have been compared for the extraction of EAAS including weak anion-exchange polymer Oasis WAX (Waters,, USA), hydrophobic copolymer Chromabond HR-X (X) (Macherey-Nagel,, Germany), strong cation exchanger Chromabond HR-XC (XC) (Macherey-Nagel, Germany) and weak cation exchanger STRATA X-CW (X-CW) (Phenomenex,, USA).

The extraction was evaluated according to the best recoveries obtained using washing and elution solvents suggested by the manufacturer for the elution of target analytes (Table 3). It was found that

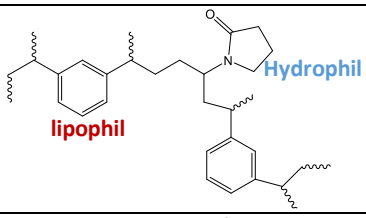
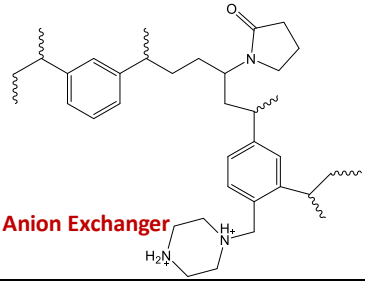
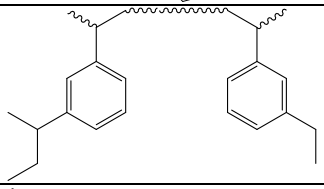
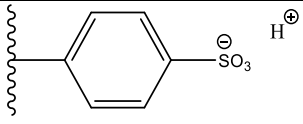
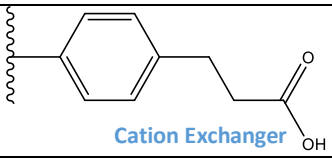
HLB provided the highest recoveries for glucuronides, second to X for sulphates while poor performance observed for the unconjugated (Figure 13). The reason for selecting HLB was the better reproducibility and distinctive improvement in analyte recoveries over other sorbents by modifying elution solvents and steps to the final conditions (see section 3.3.).

Table 2. The performance of APCI and ESI detection mode after LC separation of EAAS.

Compound	Adduct					
	$[M+H]^+$ / $[M+H]^-$		$[M-H2O+H]^+$		$[M+NH_4]^+$	
	ESI	APCI	ESI	APCI	ESI	APCI
Androsterone	+++	+	-	++	++	-
Androsterone Glucuronide	+++	+	-	-	-	-
Androsterone Sulphate	+++	++	-	-	-	-
DHT	++	++++	-	-	+	-
DHT Glucuronide	+++	++	-	-	-	-
DHT Sulphate	+++	++	-	-	-	-
Testosterone	+++	++++	-	-	-	-
Testosterone Glucuronide	+++	+	-	-	-	-
Testosterone Sulphate	+++	+	-	-	-	-
DHEA	+	++	+	+	++	-
DHEA Glucuronide	+++	++	-	-	-	-
DHEA Sulphate	+++	++	-	-	-	-
Epitestosterone	+++	++++	-	-	-	-
Epitestosterone Glucuronide	+++	++	-	-	-	-
Epitestosterone Sulphate	+++	++	-	-	-	-
Etiocholanolone	-	+++	-	++	+	-
Etiocholanolone Glucuronide	+++	+++	-	-	-	-
Etiocholanolone Sulphate	+++	++	-	-	-	-
Androstenedione	+++	+++	-	-	-	-
17α–Hydroxyprogesterone	+++	++++	-	-	-	-

- undetected signal

Table 3. SPE sorbents and solvents for EAAS extraction.

Sorbent	Type	Structure (Active sites)	Washing solvent*	Elution solvent	Properties of the elution solvent
HLB	Hydrophilic-lipophilic balance co-polymer		5 % methanol in water	(2 mL) methanol	Mainly elution of neutrals
WAX	Weak Anion Exchanger (Analytes Pka < 1)		2 % formic acid in Water	1. (1 mL) Methanol 2. (1 mL) 5 % (20 % ammonium hydroxide) in methanol	1. Elution of weaker acids and neutrals. 2. Elution of Strong acids and weak bases.
X	Hydrophobic Polymer		2 % formic acid in water	(2 mL) 5 % (20 % ammonium hydroxide) in methanol	Elution of neutrals and basic compounds
XC	Strong Cation Exchanger (Analytes Pka 2-10)		2 % formic acid in water	1. (1 mL) methanol 2. (1 mL) 5 % (20 % ammonium hydroxide) in methanol	1. Elution of neutrals and acidic compounds 2. Elution of basic compounds
XC-W	Weak Cation Exchanger (Analytes Pka > 10)		10 % methanol in water	(2 mL) 5 % formic acid in methanol	Elution of neutrals and strong bases

* Washing liquids were used as 1 mL X 2

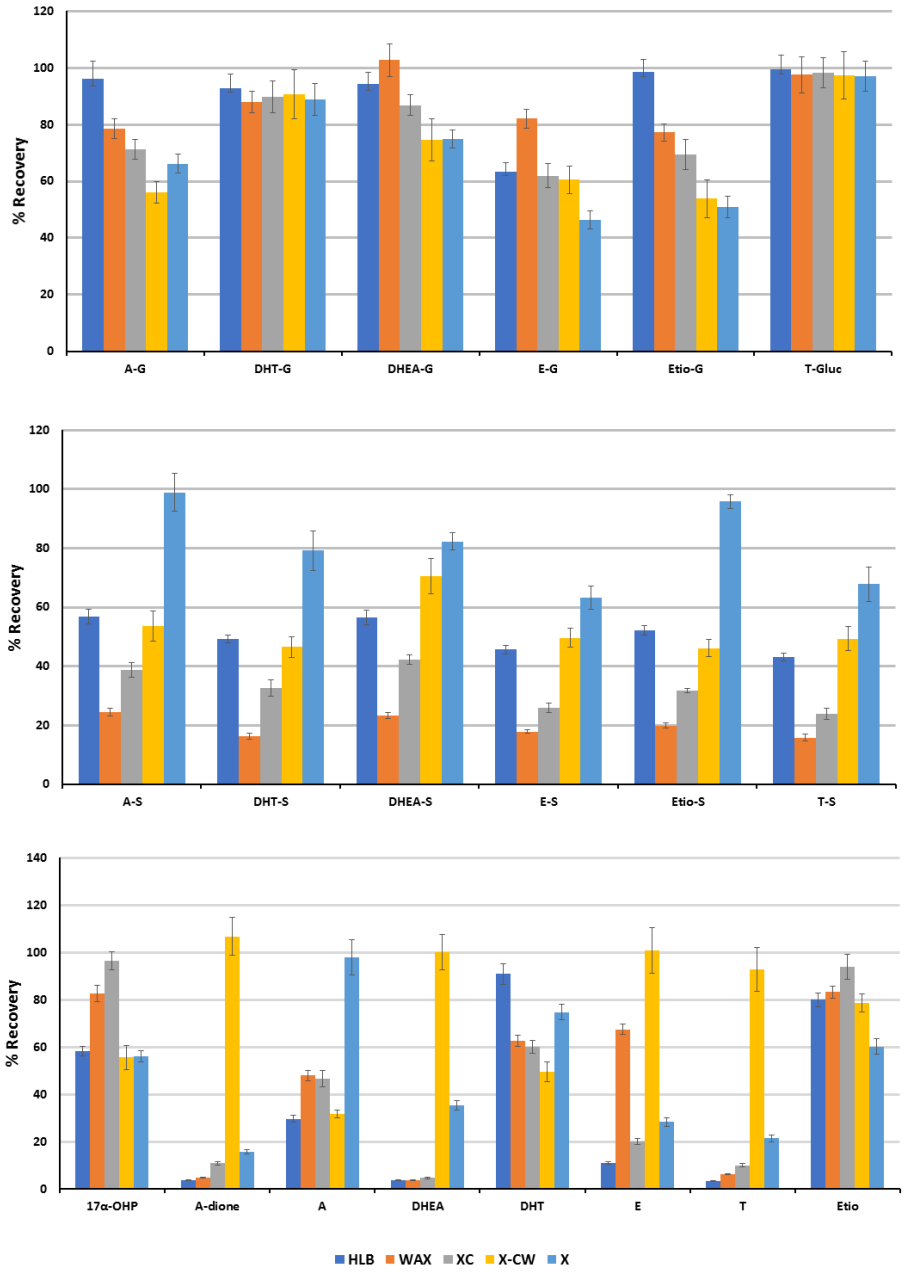


Figure 13. Recovery of EAAS using different-mode SPE sorbents

The method was applied for the determination of targeted EAAS in healthy volunteers to estimate baseline values. Samples collected from five healthy subjects were analyzed using the proposed method and it was found that DHEA-S had the highest concentration of conjugated EAAS. DHEA and A-G were found in highest concentration for unconjugated EAAS and glucuronides conjugates, respectively. For the unconjugated EAAS, Etio was undetected in all samples, whereas the levels of E were very low, and A was only detected in a single Male subject.

The investigated glucuronides were detected in all samples, which makes them promising as potential markers of EAAS and possible control compounds of doping. In addition, DHEA-S, DHT-S and A-S were found in all samples, while E-S and T-S were found in low to undetected levels in the analyzed samples.

The developed method was applied to the samples collected from the subjects in order to provide a preliminary information about the baseline levels. This reflects the applicability of the method to the routine doping analysis, anticipating the detection and quantification levels required in human serum.

4.4. Paper IV

The method from paper III was utilized to analyze the steroidal profile in human serum after the administration of recGH for 2 weeks to nine healthy males (20-45 years) [136]. In addition to the athlete ABP steroidal and hematological modules, IGF-I, P-III-NP, besides the circulatory levels of EAAS, especially DHT were suggested as a future endocrine module [137]. Serum DHT levels have been modified after therapeutic treatment with recGH [138], but there are no studies where DHT has been quantified in relation to supra-physiological doses. The hematological ABP module includes parameters such as reticulocytes percentage (RET %), hemoglobin (HGB), and an OFF-score to see if blood doping or tempering with the blood has occurred [139, 140]. It has been shown that recGH treatment may affect erythropoiesis in GH deficient patients [141]. We suggest that recGH may influence DHT levels and the ABP hematological parameters also in healthy subjects, but that has to our knowledge not been studied. Therefore, we have investigated if the administration of recGH influences the circulatory levels of DHT and the hematological module of ABP.

The current work showed, for the first time, a negative correlation between DHT-S and recGH administration manifested by the decrease of DHT-S concentration in serum after several hours of the last administration (Figure 14). It has been shown that a negative correlation exists in humans between DHT and IGF-1 as the inhibition of DHT synthesis leads to decreased expression of IGF-1 in the prostate and balding scalps [142, 143]. The reason for such correlation is not known, but it has been shown that DHT decreases the production of IGF-1 in dermal papilloma cells in mice by interacting with the androgen receptor [144].

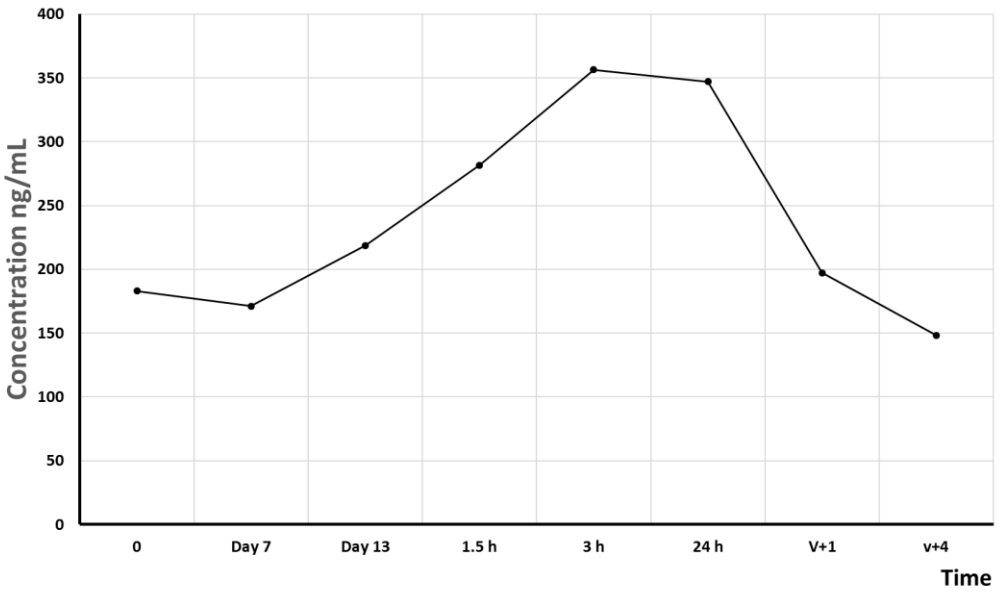


Figure 14. Longitudinal monitoring of DHT-S before and after recGH administration showing median concentrations of nine subjects. 0: represents three baseline values over 3 weeks, Day 7 and Day 13: continuous recGH administration, 1.5h: first time point after discontinuation of recGH adminstration, V+1: week 1, V+4: week 4

DHT-S was found at highest concentrations (range 37.9-892 ng/mL), whereas lower concentrations of unconjugated DHT (range 3.6-87 ng/mL) and DHT-G (range 1.0-10.8 ng/mL) were detected. Unconjugated DHT was only detectable in four of the participants. There were no effect on DHTG levels in relation to recGH administration.

For Glucuronides, A-Glucuronide (A-G) and Etio-Glucuronide (Etio-G) were found in highest concentrations ranging from 10 – 40 ng/mL with no correlation to IGF-1.

Large inter-individual variations were observed for some of the metabolites with A-G and DHEA showing the largest difference (>10.000-

fold difference) between the individuals. On the other hand, the unconjugated steroids were mostly unchanged by recGH administration.

A limitation to our study is the low number of participants. However, the study was designed for individual longitudinal monitoring rather than to identify population based changes. The inclusion of several baseline values also makes it possible to assess the intra-subject variations.

A low intra-subject variation is considered a prerequisite for the longitudinal biomarker besides being abundant in many individuals [136, 145]. Therefore, Pre-recGH administration samples were investigated for baseline values of steroids to monitor inter- and intra-subject varaiations. DHEA-S, DHT-S and T-S showed the lowest variations with CV % values; 10.48, 13.23 and 6.59 % respectively (Table 4). This suggests that these three metabolites are promising biomarkers in future ABP, at least for men.

Table 4. Determination of EAAS by UHPLC-HRMS in serum samples from nine male subjects.

Compound	Inter-individual Variations Fold variation (Range ng/mL)	Intra-individual Variations n = 4 (mean CV %)
Androsterone	6 (9.90-62.81)	25.38
Androsterone Glucuronide	17 (10.70-185.01)	12.10
Androsterone Sulphate	11600 (0.09-1048)	19.09
DHT	5910 (0.01-59.10)	NQ
DHT Glucuronide	970 (0.01-9.70)	16.88
DHT Sulphate	16 (47.20-780)	13.23
Testosterone	3 (0.66-2.20)	10.28
Testosterone Glucuronide	229 (0.01-2.29)	17.01
Testosterone Sulphate	19 (0.47-9.12)	6.59
DHEA	12700 (0.01-127.30)	38.17
DHEA Glucuronide	200 (0.01-2.01)	NQ
DHEA Sulphate	2 (264-625)	10.48
Epitestosterone	ND	NQ
Epitestosterone Glucuronide	350 (0.01-3.51)	11.58
Epitestosterone Sulphate	370 (0.01-3.70)	24.38
Etiocholanolone	5570 (0.01-55.70)	NQ
Etiocholanolone Glucuronide	10 (5.70-55.70)	15.19
Etiocholanolone Sulphate	31 (1.4-44.1)	16.31
Androstenedione	8 (0.33-2.67)	23.39
17α–Hydroxyprogesterone	90 (0.07-6.37)	31.09

5. Conclusion and future perspectives

The current thesis covers certain analytical challenges that faces the analysis of drugs used in sports as performance enhancing agents. The use of HPLC and UHPLC with mass spectrometric detection facilitated the detection and quantification of drugs in biological fluids with high selectivity and sensitivity.

Chiral analysis of β-receptor blockers, such as propranolol and metoprolol, is a challenging task, especially that they are only available as racemates in the market. The enantiomers of each drug exhibit different pharmacological action which highlights the importance of their stereoselective analysis in biological fluids.

The chiral separation using HPLC was established using a polysaccharide chiral stationary phase which favors normal phase conditions. The low polarity solvents used to perform the chiral separation counteract the ability of the mass spectrometer to ionize the target analyte at the electrospray interface. Two approaches were tested to enhance the ionization intensity of target analytes with the mass spectrometric detection including post column addition of polar makeup solvents (OPSAI) or a direct introduction of makeup solvent to the ion source (SAESI).

With several tested solvent mixtures, better performance was achieved with OPSAI over the SAESI approach; thus, the OPSAI procedure was applied to the LC-MS chiral analysis of propranolol in human plasma and metoprolol in human plasma and saliva. As a future perspective, the longitudinal monitoring of both drugs constitutes an interesting approach to detect intake both in plasma and saliva samples.

The study of the pharmacokinetics parameters in both fluids can lead to better understanding of the therapeutic effect of the active enantiomer. On the other hand, saliva as a fast, simple and non-invasive sample can increase the feasibility of sampling, detection and quantification of drugs for different purposes as therapeutic monitoring and doping control.

Steroids are very challenging analytes and they gain high usability among athletes both in and out of sports competitions.

The current work describes the development of a new analytical method that allows the detection and quantification of endogenous anabolic androgenic steroids and their conjugated metabolites simultaneously in serum for the first time.

The method is aimed to be applied in anti-doping laboratories for routine analysis of endogenous steroids. Moreover, the method is applicable to the investigation of the potential biomarker ratios of the steroidal module as well as the longitudinal monitoring of endogenous anabolic androgenic steroids. The method also aims to investigate the steroidal module after the administration of different testosterone preparations such as testosterone gels.

The most challenging analytical task regarding the simultaneous steroid analysis was the different physicochemical properties between the unconjugated steroids and their conjugated metabolites. This reduced the sensitivity of detection as both positive and negative ESI conditions were needed for proper mass spectrometric detection. On the other hand, the mobile phase additives required in each condition (acids or buffers) were not compatible as either the conjugated or the unconjugated group of analytes were detected. Hence, the use of a high pH mobile phase with UHPLC-HRMS showed promising performance for all analytes whether

in chromatographic separation or mass spectrometric detection. The extraction of analytes from serum was performed using an SPE method based on two successive elutions with different solvent mixtures that suited the retention properties of each group of analytes. The method was applied to serum samples from healthy volunteers as an example of individual baseline values determination.

The investigation of the steroidal profile in human serum was performed to provide a suggested biomarker for GH doping. The UHPLC-HRMS method was applied to serum samples collected from subjects who has been administered recGH for 2 weeks. A negative correlation was found between IGF-1 and DHTS. Moreover, DHEA-S, DHT-S and T-S showed the lowest inter-subject baseline variations which suggests them as promising tools for GH doping detection. Further investigation will follow by the application of the method to a larger group with even consideration of male and females number. Moreover, confirmatory methods of steroids in correlations with GH intake are highly encouraged to improve the sensitivity and selectivity of the assay.

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