UNIVERSITEIT GENT

FACULTEIT GENEESKUNDE EN GEZONDHEIDSWETENSCHAPPEN

Improvements in anti-doping methods: a focus on cannabinoids

Nik De Brabanter

Thesis submitted in fulfillment of the requirements for the degree of
Doctor in Medical Sciences

2013

Promotor
Prof. Dr. ir. P. Van Eenoo
Co-promotor
Dr. K. Deventer

Promotor: Prof. Dr. ir. P. Van Eenoo
Ghent University, Belgium

Co-promotor: Dr. K. Deventer
Ghent University, Belgium

Members of the jury: Prof. Dr. J. Van de Voorde, Chairman
Ghent University, Belgium

Prof. Dr. A. Verstraete
Ghent University, Belgium

Prof. Dr. F. T. Delbeke
Ghent University, Belgium

Prof. Dr. C. Stove
Ghent University, Belgium

Prof. Dr. L. Vanhaecke
Ghent University, Belgium

Dr. T. Sobolevsky
Moscow Antidoping Centre, Russia

Dr. P. Van Renterghem
Ghent University, Belgium

Vooraf

Voor de allerlaatste versie van dit werk eindelijk naar de drukker kan,

een welgemeende dankjewel aan …

… Prof. Dr. ir. Peter Van Eenoo mijn promotor, Dr. Koen Deventer mijn copromotor en Prof. Dr. Frans Delbeke voor de vele kansen die ik in DoCoLab gekregen heb. Voor de begeleiding en het (bij)sturen van mijn onderzoek, voor het nalezen van al het schrijfwerk, van de eerste dag in de zomer van 2009 tot dit boekje vier zomers later.

… de DoCoLab collega's voor alle hulp, plezier en vertier in en rond het labo: van de uitkomst van experimenten ben je nooit zeker, van het niveau van de lokale humor daarentegen…

... de ganse familie en alle vrienden voor de steun en aanmoedigingen tijdens de voorbije 4 jaren.
En in het bijzonder aan Jolien en … voor zoveel, maar zeker voor de hopen geduld vooral tijdens de laatste maanden.

Nik

Table of contents

List of abbreviations

AAF	Adverse analytical finding
AAS	Anabolic-androgenic steroid
ABP	Athlete Biological Passport
ACN	Acetonitrile
BEWSD	Belgium Early Warning System for Drugs
BMCDDA	Belgian Monitoring Centre for Drugs and Drug addiction
CB	Cannabinoid receptor
CE	Collision energy
CEVAC	Center for Vaccinology
CID	Collision Induced Dissociation
Da	Dalton
DEA	American Drug Enforcement Agency
DL	Decision limit
DMSO	Dimethyl sulfoxide
DoCoLab	Doping Control Laboratory - Ghent University
EAAS	Endogenous anabolic androgenic steroids
EDND	European Database on New Drugs
EI	Electron impact
ELISA	Enzyme linked immunosorbent assays
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
EPO	Erythropoietin
EQAS	External quality assessment scheme
ESI	Electron spray ionisation
eV	Electron volt
EWS	European Early Warning System
GC	Gas chromatography
HCD	High-collision energy dissociation (trademark Thermo Fisher Scientific Inc.)
HLB	Hydrophilic-lipophilic balance
HLM	Human liver microsomes
HRMS	High resolution mass spectrometry
IAAF	International Association of Athletics Federations
IEC	International Electrotechnical Commission

IF	International Federation
IOC	International Olympic Committee
IRMS	Isotope ratio mass spectrometry
ISL	International Standard for Laboratories
ISO	Organisation for Standardisation
ISTD	Internal standard
IUPAC	International Union of Pure and Applied Chemistry
k	Coverage factor
KB	Koninklijk Besluit
LC	Liquid chromatography
LLE	Liquid liquid extraction
LOD	Limit of detection
LOI	Limit of identification
LOQ	Limit of quantification
m/z	Mass to charge ratio
MAD	Microwave Assisted Derivatisation
MeOH	Methanol
MRPL	Minimum required performance limit
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MSTFA	N-methyl-N-trimethylsilyltrifluoroacetamide
MU	Measurement uncertainty
MW	Molecular weight
NADO	National Anti-Doping Organisation
NADP	Nicotinamide adenine dinucleotide phosphate
NMR	Nuclear Magnetic Resonance
OFN	Oxygen free nitrogen
PBS	Phosphate Buffered Saline
PTV	Programmed Temperature Vaporization
QAS	Quality Assurance Steroid parameter
QqQ	Triple quadrupole
R2	Correlation coefficient
RA	Relative abundance

RI	Relative ion intensity
RSD	Relative standard deviation
RT	Retention time
S/N	Signal to noise ratio
SCID	Severe combined immuno deficient
SPE	Solid-phase extraction
SRM	Selected reaction monitoring
T/E	Testosterone to Epitestosterone ratio
TD	Technical document
THC	Δ9-tetrahydrocannabinol
THCA	11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid
TLC	Thin layer chromatography
TMS	Trimethylsilyl
$u_{c\ Max}$	maximum combined standard uncertainty
UCI	International Cycling Union
UNODC	United Nations Office on Drugs and Crime
uPA	Urokinase plasminogen activator
W	Watt
WADA	World Anti-Doping Agency

Part I

General introduction

Chapter 1

Introduction

1 Doping

The desire to win and the search to improve performance is inherent to sport. This endeavor has tempted the athlete for centuries to explore different ways to take a lead on his or her opponents. Nowadays, sports, whether professional or not, are often practiced using a scientific approach. Indeed, research conducted in sport related areas has led to more efficient training methods, targeted to the needs of the athlete. In addition to customized training schedules, attention is also paid to optimal diet and – if applicable – the best equipment and technical facilities.

However, there is a thin line between scientific optimization of sports and the misuse of scientific knowledge. Starting from the 20th century, rapid advances in the medical and pharmaceutical industry have led to (incorrect) use of drugs in sports. The doping story started with stimulating agents as amphetamine and strychnine to suppress pain and fatigue, mainly in sport disciplines where endurance is of great importance [1]. These products had been used for years without the public (authorities) paying attention to the impact on the health of the athlete. However, in the 1960s, the general public and the authorities became aware of the seriousness of the situation due to two fatalities in cycling. Although not officially confirmed [2], both the death of Knud Enemark Jensen (Olympic Games, 1960) and Tom Simpson (Tour de France, 1967) were linked with rumored stimulant abuse [3], although no hard evidence of the direct link can be provided, even today, as other factors were also involved. Nevertheless, triggered by these incidents the International Olympic Committee (IOC) formed a Medical Commission, in charge of testing and monitoring doping in sports and a (short) list of prohibited substances was drafted. As a result, tests for stimulants were developed based upon gas chromatographic separation [4] and used for the first doping controls during the Olympics in Mexico City in 1968 [1]. Later, other federations like the International Association of Athletics Federations (IAAF) and the International Cycling Union (UCI) introduced their own anti-doping rules and further tests were developed for anti-doping purposes [3].

In the following years, the scope of sports affected by doping and classes of doping agents grew and continuous efforts were made to tackle the doping problem. Similarly, anti-doping methods needed to be developed (Fig. 1.1). In the 1950s and 1960s anabolic steroids were introduced in sports mainly to improve the recovery after great efforts [5]. Over the following years the use of (synthetic) derivatives of testosterone increased in sports all over the world for their effect on the athlete's muscle mass and strength. Together with the development of detection strategies for these compounds, the IOC prohibited the use of anabolic steroids in competition in 1974. This measure resulted in a shift in use of steroids towards the training period to avoid detection in competition. It became clear after several high profile doping scandals, including the anabolic steroid program of the German Democratic Republic [6] and the famous stanozolol positive test of sprinter Ben Johnson at the Olympic Games of Seoul in 1988 [5], that the use of these products was well organized. To counter this evolution, the IAAF was the first organization to set up out of competition testing in 1991. However, then again, users adapted quickly to the new situation by changing to steroid precursors or specially synthesized designer steroids [7]. Effective tests for these substances were only gradually developed and only in the late 1980s, beginning of the 1990s an effective testing program could be implemented.

In addition to anabolic agents, which have been the major group of adverse analytical findings for years, other drugs found their way into sports as well. In disciplines where not the physical effort but the control of movement and calmness is required, beta-blockers were introduced. These products were inserted in the IOC list in 1985 together with diuretics, which are taken to effect a rapid reduction in weight and in that way reach weight class limits or to increase the excretion rate of urine and reduce urinary concentrations of prohibited drugs [8,9]. Also in the 1980s, recombinant human erythropoietin was introduced on the market for clinical purposes. Concerned with the potential performance enhancing capabilities and the severe side-effects of growth hormone and erythropoietin (EPO), the IOC added the class of peptide hormones to the prohibited list in 1989, however no direct test was available at that time for these substances. Only for human chorionic gonadotrophin (hCG) a test was available. Indeed, until the development of a screening test for EPO in 2000 [10], indirect tests based upon maximal haematocrit levels were the only indirect indicative tests which could be applied [11].

Next to the use of EPO, intravenous administration of red blood cells is another way to increase the blood oxygen carrying capacity. While homologous blood transfusion can be detected via flow cytometry based upon differences in minor blood group antigens [12], an autologous administration is harder to trace. Indirect indications can be given by the detection of metabolites of blood bags plasticizers in urine [13], but the only accepted indirect method is the hematological module of the Athlete Biological Passport (ABP, explanation below).

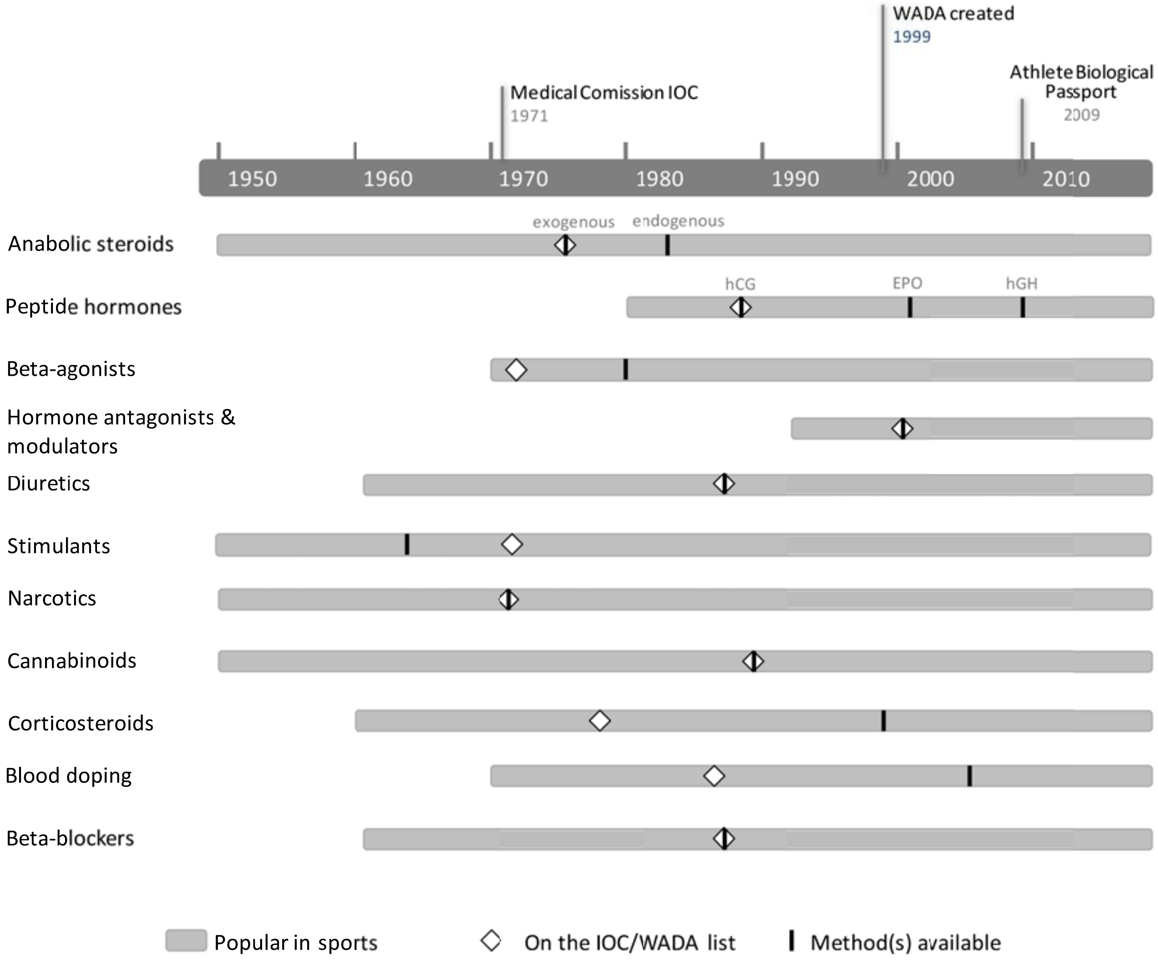


Fig. 1.1 Historical overview of the (estimated) use of prohibited substances, their prohibition by IOC/WADA and routine application of detection methods [4,5,8,11,14–19].

2 World Anti-Doping Agency

Gradually more partnerships arose to counter drugs in sports at different levels. In the early years there was, however, little international consistency, since most countries still worked independently on their own territory and every international federation applied its own rules. It seemed as if yet another incident was needed to find sufficient international support for the next necessary step. The public attention and appall for the doping scandal during the 1998 Tour de France, created a momentum that was used to gather all the partners in the fight against doping: the IOC, governments, (inter)national organizations and other public and private institutions [3]. The result of the World Conference on Doping in 1999 was the foundation of the independent World Anti-Doping Agency (WADA) [11]. The main aim was to harmonize the anti-doping rules in between international federations and national anti-doping organizations. Today, WADA has grown to the global leading organization, which develops protocols and guidelines, promotes research and education and coordinates the World Anti-Doping Program [20]. The best known achievement has been the implementation of the World Anti-Doping Code. This Code aims:

- *To protect the Athletes' fundamental right to participate in doping-free sport and thus promote health, fairness and equality for Athletes worldwide, and*
- *To ensure harmonized, coordinated and effective anti-doping programs at the international and national level with regard to detection, deterrence and prevention of doping* [21].

Further WADA annually publishes the Prohibited List, which contains all prohibited substances and prohibited methods divided into *In-Competition* and *Out-of-competition* (Table 1.1). A compound can be included in the list if it fulfills at least two out of the three following criteria: it has potential to enhance sport performance, it constitutes a health risk to the athlete or it violates the spirit of sport described in the Code [21].

Table 1.1: Categories of the 2013 Prohibited List

Prohibited at all times	
S0	Non-approved substances
S1	Anabolic agents
S2	Peptide hormones, growth factors and related substances
S3	Beta-2 agonists
S4	Hormone and metabolic modulators
S5	Diuretics and other masking agents
M1	Enhancement of oxygen transfer
M2	Chemical and physical manipulation
M3	Gene doping
Prohibited in competition	
S6	Stimulants
S7	Narcotics
S8	Cannabinoids
S9	Glucocorticosteroids
Prohibited in particular sports	
P1	Alcohol
P2	Beta-blockers

3 WADA-accredited laboratories

An essential part in setting up a proficient anti-doping system is the use of well-performing laboratories, responsible for the analyses of the doping control samples. Additionally, these labs are often also the key players in the research around doping and related fields.

3.1 Accreditation and quality control

The WADA Code stipulates that anti-doping tests can only be performed in WADA accredited laboratories. To obtain such accreditation, a laboratory needs the support of a National Anti-Doping Organisation (NADO), must hold 17025 accreditation of the International Organization for Standardization/International Electrotechnical Commission (ISO/IEC) and participate successfully in the external quality assessment scheme (EQAS) of WADA [22].

ISO17025 accreditation requires the implementation of a quality management system, including different aspects of the laboratory organization. Particular attention is paid to the documentation, on the one hand concerning the analytical procedures, on the other hand to enable traceability of all handlings. This ranges from installation of equipment, to its calibration and the appropriate training of staff members. The methods used should fit for the purpose and should be properly validated. According to the ISO definition this validation procedure is performed to “confirm by examination and provision of objective evidence that the particular requirements for a specified intended use are fulfilled” [23]. On a regular basis, the competence of the laboratory is tested by means of both internal and external audits and the participation in proficiency tests.

3.2 The instrumentation

In the beginning of sport drug testing in the 1970s, thin layer chromatography (TLC) and gas chromatography (GC) were the workhorses of the first anti-doping labs [24]. Over the years, the list of available techniques has grown at high speed. Although in the 1970s the first methods for anabolic steroids were based upon immunoassay technology [11], the switch was made to chromatographic methods only a few years later. Both gas chromatography and liquid chromatography (LC) were coupled to mass spectrometry (MS), whereupon this mass spectrometric detection was brought to higher levels with the implementation of tandem mass spectrometry (MS/MS), high resolution mass spectrometry (HRMS) and isotope ratio mass spectrometry (IRMS) [5]. The continuous implementation of these new techniques allows to detect (new) doping substances better and faster. And this is an absolute necessity, given the continuously increasing complexity and more stern demands imposed to doping control laboratories. Indeed, over the years the number of samples analyzed increased (Fig. 1.2) together with a growing list of substances to be screened for. Regarding these compounds, it should be noted that WADA has defined minimum required performance levels (MRPL) [25] to be met by every accredited lab. These MRPLs are neither thresholds nor reporting limits, but define the levels at or below which substances should be detected routinely. Also, the number of metabolites implemented in methods to detect the abuse of a certain compound increased as well. The latter is mainly the result of the research conducted in the anti-doping field. The fact that WADA laboratories play a key role in this is reflected in their scientific output describing the improved methodologies, new metabolism studies, etc. [26].

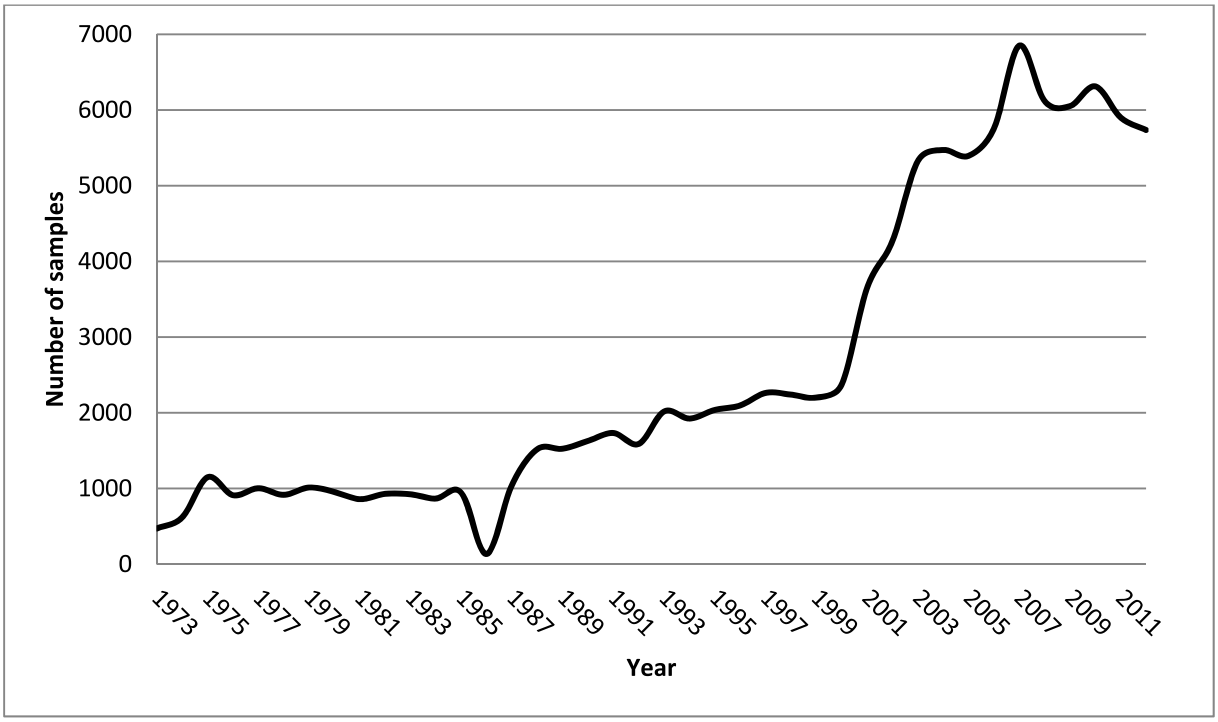


Fig. 1.2 Evolution in the number of samples analyzed at DoCoLab up to 2012.

Together with the analytical challenges, also economic concerns should be taken into account. During the period 2008 to 2011, the global number of analyzed doping control samples has stagnated. Much has to do with the economical situation lately, since doping control programs and corresponding research projects usually rely on government resources. To cope with this situation, laboratories aim towards increased efficiency to reduce costs: this goes from reducing the number of analytical methods over increasing the sample turn-around and throughput to automation of sample preparation procedures. It should be mentioned that - compared to 2011 – a 12% increase in analyzed doping control samples was observed in 2012. If this is related to an increase in testing for the 2012 London Olympic Games or really constitutes a turning-point, currently remains an open question.

3.3 Improved detection strategies in DoCoLab

In terms of analytical equipment, over the last years DoCoLab has invested with two objectives in view. Next to the above-mentioned challenges in terms of detection limits, the course was set towards the introduction of an open screening approach and an improved steroid profiling strategy.

In the LC-department, the open screening strategy has been made possible by the installation of a high resolution mass spectrometer based upon Orbitrap™ technology. The method developed on this Exactive mass spectrometer is capable of detecting over 200 compounds from the classes S1, S3, S4 (e.g. tamoxifen), S5, S6, S7, S8 (e.g. JWH-compounds), S9, P1 (e.g. ethylglucuronide) and P2 (Table 1.1) by acquiring high resolution accurate mass data in full scan mode [27]. This makes the traditional development of MS methods – containing specific ions or transitions for every compound – redundant. Indeed, the previously used tandem MS technology only acquired data for the predefined components, while the HRMS detector is capable of measuring full scan data after which the compounds of interest are extracted in the data processing part, based upon retention time and the accurate mass of the given compound. This enables the retrospective data analysis of all samples analyzed with the method in the case of a new detected doping agent, a new long-term metabolite, etc. Under the current regulations [22] these full scan data are stored for eight years, while the storage of the corresponding urine samples is between three months and eight years. Since this long time storage of urine samples in a secure location under a continuous chain of custody leads to logistical problems, this is currently only done for big events as the Olympic Games or on special request.

Detection of the abuse of endogenous steroids requires a different approach. It is known that administration of testosterone or one of its precursors alters the concentrations of the endogenous steroids excreted in urine. To detect this, methods were developed capable of quantifying testosterone (T), epitestosterone (E), androsterone (A), etiocholanolone (Etio), 5α-Androstane-3α,17β-diol (5ααβ-Adiol) and 5β-Androstane-3α,17β-diol (5βαβ-Adiol), together the so-called steroid profile [28]. Detecting an altered steroid profile however, is not that straightforward. To diagnose endogenous steroid abuse, the distinction between normal and elevated levels is based upon thresholds for every steroid, derived from population statistics [29].

Over the last years, it was possible to improve the existing steroid profiling methods in DoCoLab thanks to the installation of a new GC-system equipped with a triple quadruple mass spectrometer. The improved selectivity and specificity of this GC-MS/MS system together with the use of deuterated internal standards, the addition of markers to control the complete sample preparation procedure for every step and the additional quantification of minor metabolites [30,31], allows for a more accurate quantification of the - extended - steroid profile [32,33].

3.4 The athlete biological passport

Nevertheless, the disadvantages of using population based statistics to establish thresholds remain. It is known that urinary excretion of the above-mentioned endogenous steroids is subject to both intra-, inter-individual and genetic variations [29]. Since laboratories don’t have access to personal details of the athlete and his or her steroid profile, the current general threshold may allow steroid takers to slip through the net.

To avoid this, individual reference ranges should be established for every single athlete. This was already done in the 2000s, as follow-up studies conducted when a suspicious result was found [31], but can now be made possible in the framework of the ABP, in use from 2009 [34]. At the moment, this contains only blood passport data to detect the use of EPO or blood transfusion. Therefore, selected blood parameters obtained from hematological tests over time are registered in the athlete’s personal database. This enables the calculation of reference ranges for each parameter, specifically for every individual athlete in a way that abnormal values can be detected, which can result in intelligent and targeted testing.

The future steroid module in the athlete passport necessitates an accurate determination of endogenous steroid concentrations. To support this, more research projects are in the DoCoLab pipeline, both towards analytical methodologies and factors influencing this steroid profile [35].

4 Cannabis and doping

Classified under category S8 of the 2013 Prohibited List [36], the status of cannaboids in competition is described as:

"Natural (e.g. cannabis, hashish, marijuana) or synthetic delta 9-tetrahydrocannabinol (THC) and cannabimimetics (e.g. "Spice", JWH-018, JWH-073, HU-210) are prohibited."

Since the Medical Commission of the IOC placed marijuana on the list of doping substances in 1989 till this last update on the List of 2013, this decision has given rise to controversy. The inclusion of cannabis as a doping agent has been heavily debated. The supporters of the prohibition point out that cannabis can have an impact on the performance in particular sports and/or take the view that athletes serve as role models, while the opponents refute the argument of performance enhancement and state that the use of cannabinoids is only a private social issue [37].

The influence of cannabis on the athletes' *sport performance* has been the subject of research for many years and conflicting results have been reported. In most studies negative effects of cannabis use on the sporting achievements were found, ranging from increased heart rate and reduced peak performance to reduction of reaction times and psychomotor skills [37–40]. When the level of concentration is important, cannabis use can influence this in a positive way [37]. On the other hand, the sedative effects can be used to reduce the stress and anxiety of the athlete before and after competition [37,38]. Also the sleeping time and quality could be improved by cannabis use [39], making it a matter of indirect performance enhancing.

Moreover, these sedative properties have an impact on the estimation of risks, which results in a *health risk* for both the user and his or her fellow athletes. Indeed, especially for team sports, motorized disciplines or equipment handling, an athlete under the influence of cannabis may suffer reduced coordination, slowed reaction times and can misjudge situations, potentially leading to afflicting injuries upon the athlete itself, fellow competitors or even spectators. When using cannabis chronically different studies diagnose withdrawal symptoms and other serious health effects [37,41].

When considering the *spirit of sport*, the classification of cannabis in most countries is an important argument. Cannabis is still illegal in most countries and even in countries where its use has been legalized; it is - similar to alcohol use or smoking - not encouraged. Hence, the position of the athlete as a role model does not fit with the (illegal) status of cannabis.

4.1 A threshold substance

To date, these arguments are strong enough to maintain the status of cannabis on the Prohibited List. This means that WADA accredited laboratories have the task to report a concentration of 11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid (THCA), the major metabolite of cannabis in urine, higher than the decision limit as an Adverse Analytical Finding (AAF). The threshold of 15 ng/mL was installed to avoid positive testing after passive inhalation of cannabis smoke [39]. For this purpose, in our lab, confirmation procedures were developed and improved over time [42–44].

On May 11 2013, WADA published an update on the Technical Document [45] containing a tenfold increase of the threshold concentration for THCA in urine to 150 ng/mL. It was clear that there was a big discrepancy between the duration of the effect – which has an impact in competition – and the time period that out-of-competition use can be detected. Raising the threshold to 150 ng/mL (decision limit = 175 ng/ml) aims at reducing the chance for a positive test after (allowed) out-of-competition use and will probably reduce the high number of AAFs for cannabis. Indeed, in 2011 THCA was responsible for 7.9% of all positive findings reported by WADA accredited laboratories.

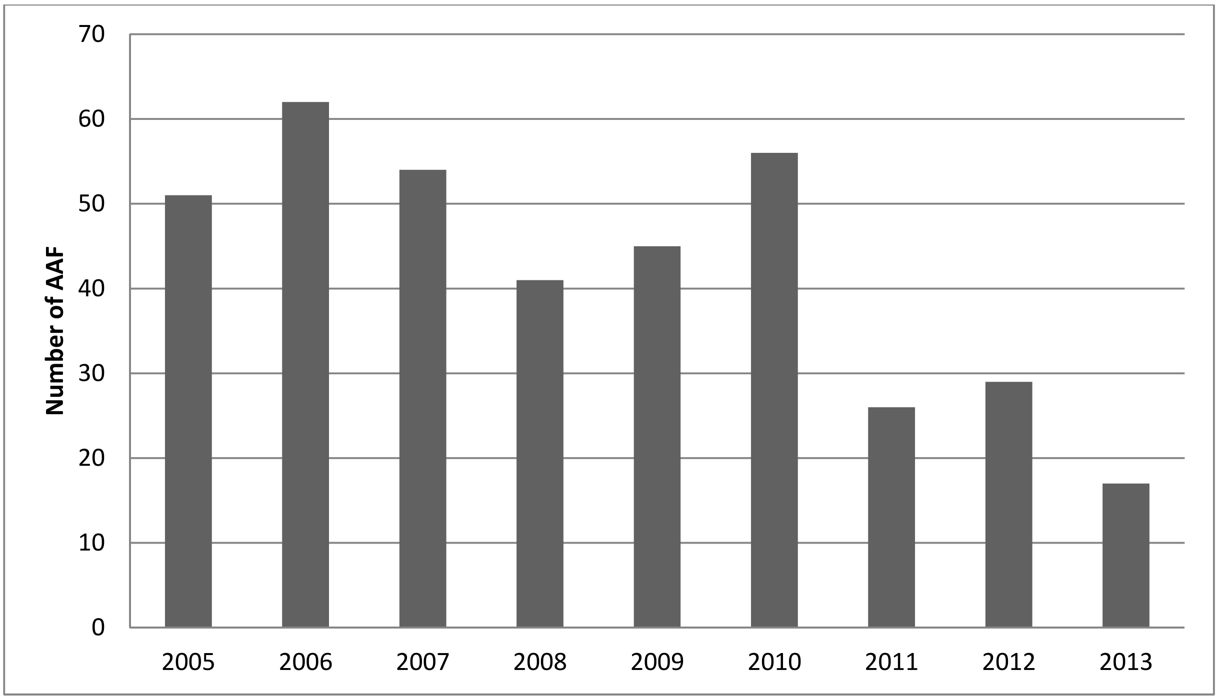


Fig. 1.3 Number of positive reported samples for cannabis in DoCoLab (threshold 15 ng/mL) until May 1, 2013.

From 2005 to mid-2013, 381 samples were reported positive for cannabis in DoCoLab (Fig. 1.3). When it comes to sport disciplines, DoCoLab statistics on the last eight years show that the prevalence is higher in team sports like soccer, baseball and basketball. Besides these, also in the category of martial arts and strength sports cannabis turns out to be more popular (Fig. 1.4).

When the new threshold of 150 ng/mL, with corresponding decision limit of 175 ng/mL, is applied for this time period, only 41.2% of this number would still be positive. However, changing the threshold entails more. Indeed, although the method in force can be maintained for the greater part (sample preparation, chromatographic and mass spectrometric conditions), a complete validation procedure should be performed in order to use the method for the newly installed threshold. The changes imply the adjustment of the range of calibration curves and a new estimation of the measurement uncertainty (MU), lower than the maximum combined standard uncertainty ($u_{c\ Max}$) imposed by WADA [45]. Given the short time period before the date of commencement (11 May 2013), validation and implementation of the updated methodology should be completed as fast as possible.

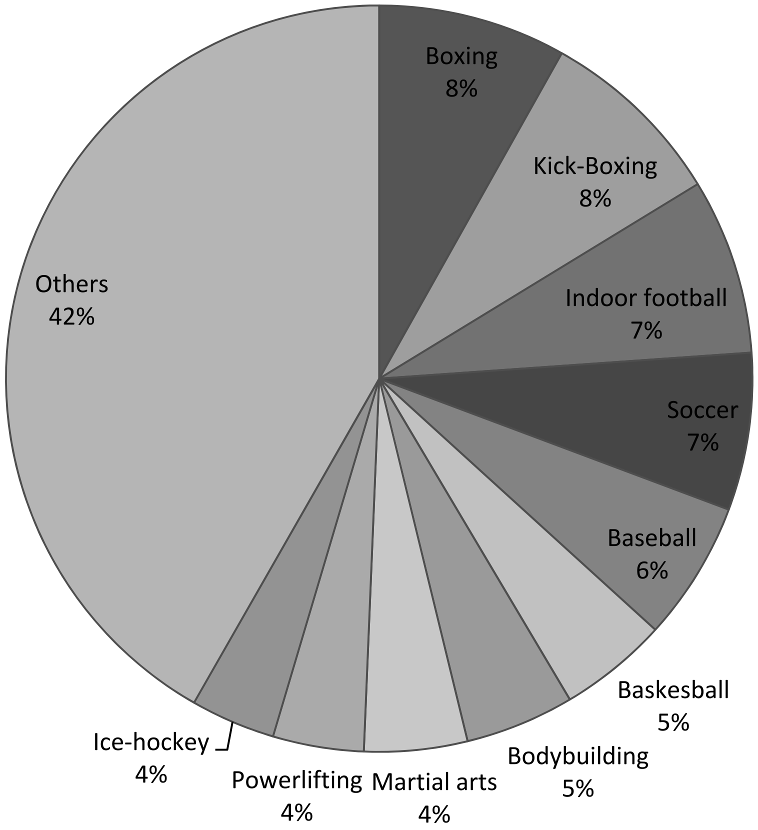


Fig. 1.4 Distribution of disciplines for positive cannabis samples in DoCoLab between 2005 and mid-2013.

4.2 The emergence of synthetic cannabinoids

The first synthetic cannabinoid - HU-210 - was mentioned on the Prohibited List of 2010 [46] and on the List of 2011 [47] the definition was extended to the above-mentioned, covering the broad range of cannabimimetics currently on the market. The arguments behind this run parallel to these of classical cannabis, while for the cannabimimetics even more importance can be attached to the health risk. Indeed, little or no information is available on the (side-)effects of these products on the human body (cfr. Chapter 6). Due to their higher potency compared to classical cannabis of *cannabis sativa*, the impact of these synthetic analogues is hard to predict and even life-threatening intoxications cannot be ruled out [48–52]. To this day the occurrence of synthetic cannabinoids in sports is rather small compared to traditional THC. Indeed, compared to the 398 adverse analytical findings for the latter in 2012, only 6 were found positive for JWH-018 and 2 for JWH-073 [53].

References

[1] L.D. Bowers. Anti-dope testing in sport: the history and the science. *FASEB journal: official publication of the Federation of American Societies for Experimental Biology*. 2012, *26*, 3933–6.

[2] A. Ljungqvist. Half a century of challenges. *Bioanalysis*. 2012, *4*, 1531–3.

[3] M. Kamber, P.-E. Mullis. The worldwide fight against doping: from the beginning to the World Anti-Doping Agency. *Endocrinology and metabolism clinics of North America*. 2010, *39*, 1–9, vii.

[4] P. Hemmersbach. History of mass spectrometry at the Olympic Games. *Journal of mass spectrometry□: JMS*. 2008, *43*, 839–53.

[5] R.K. Müller. History of doping and doping control. *Handbook of experimental pharmacology*. 2010, 1–23.

[6] W.W. Franke, B. Berendonk. Hormonal doping and androgenization of athletes: a secret program of the German Democratic Republic government. *Clinical chemistry*. 1997, *43*, 1262–79.

[7] R. Kazlauskas. Designer steroids. *Handbook of experimental pharmacology*. 2010, 155–85.

[8] M. Verroken. Drug use and abuse in sport. *Best Practice & Research Clinical Endocrinology & Metabolism*. 2000, *14*, 1–23.

[9] K. Deventer, P. Van Eenoo, F.T. Delbeke. Simultaneous determination of beta-blocking agents and diuretics in doping analysis by liquid chromatography/mass spectrometry with scan-to-scan polarity switching. *Rapid communications in mass spectrometry□: RCM*. 2005, *19*, 90–98.

[10] F. Lasne, J. de Ceaurriz. Recombinant erythropoietin in urine. *Nature*. 2000, *405*, 635.

[11] D.H. Catlin, K.D. Fitch, A. Ljungqvist. Medicine and science in the fight against doping in sport. *Journal of internal medicine*. 2008, *264*, 99–114.

[12] M. Nelson, H. Popp, K. Sharpe, M. Ashenden. Proof of homologous blood transfusion through quantification of blood group antigens. *Haematologica*. 2003, *88*, 1284–95.

[13] N. Monfort, R. Ventura, P. Platen, T. Hinrichs, K. Brixius, W. Schänzer, M. Thevis, H. Geyer, J. Segura. Plasticizers excreted in urine: indication of autologous blood transfusion in sports. *Transfusion*. 2012, *52*, 647–57.

[14] K.D. Fitch. Beta2-Agonists at the Olympic Games. *Clinical reviews in allergy & immunology*. *31*, 259–68.

[15] W.D. Knopp, T.W. Wang, B.R. Bach. Ergogenic drugs in sports. *Clinics in sports medicine*. 1997, *16*, 375–92.

[16] F.T. Delbeke. From amanita muscaria to somatotropine: The doping story. *Biology of Sport*. 2000, *17*, 81–86.

[17] M. Verroken. Ethical aspects and the prevalence of hormone abuse in sport. *The Journal of endocrinology*. 2001, *170*, 49–54.

[18] R.I.G. Holt, I. Erotokritou-Mulligan, P.H. Sönksen. The history of doping and growth hormone abuse in sport. *Growth hormone & IGF research : official journal of the Growth Hormone Research Society and the International IGF Research Society*. 2009, *19*, 320–26.

[19] E.H. De Rose. Doping in athletes--an update. *Clinics in sports medicine*. 2008, *27*, 107–30, viii–ix.

[20] WADA. World Anti-Doping Agency Website. http://www.wada-ama.org/en/

[21] WADA. World Anti-Doping Code. 2009, http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-The-Code/WADA_Anti-Doping_CODE_2009_EN.pdf

[22] WADA. International Standard for Laboratories, version 7.0. 2012, http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-IS-Laboratories/ISL/WADA_Int_Standard_Laboratories_2012_EN.pdf

[23] Eurachem. *The Fitness for Purpose of Analytical Methods A Laboratory Guide to Method Validation and Related Topics*. 1998,

[24] M. Donike. The detection of doping by means of chromatographic methods. 1966. *Drug testing and analysis*. 2011, *3*, 15–7.

[25] WADA. WADA Technical Document – TD2013MRPL. 2013, http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-IS-Laboratories/Technical_Documents/WADA-TD2013MRPL-Minimum-Required-Performance-Levels-v1-2012-EN.pdf

[26] M. Thevis, T. Kuuranne, H. Geyer, W. Schanzer. Annual banned-substance review: analytical approaches in human sports drug testing. *Drug testing and analysis*. 2010, *2*, 149–61.

[27] A.J. Girón, K. Deventer, K. Roels, P. Van Eenoo. Development and validation of an open screening method for diuretics, stimulants and selected compounds in human urine by UHPLC-HRMS for doping control. *Analytica chimica acta*. 2012, *721*, 137–46.

[28] P. Van Renterghem, P. Van Eenoo, W. Van Thuyne, H. Geyer, W. Schanzer, F.T. Delbeke. Validation of an extended method for the detection of the misuse of endogenous steroids in sports, including new hydroxylated metabolites. *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences*. 2008, *876*, 225–35.

[29] P. Van Renterghem, P. Van Eenoo, H. Geyer, W. Schanzer, F.T. Delbeke. Reference ranges for urinary concentrations and ratios of endogenous steroids, which can be used as markers for steroid misuse, in a Caucasian population of athletes. *Steroids*. 2010, *75*, 154–63.

[30] P. Van Renterghem, P. Van Eenoo, P.-E. Sottas, M. Saugy, F. Delbeke. A pilot study on subject-based comprehensive steroid profiling: novel biomarkers to detect testosterone misuse in sports. *Clinical endocrinology*. 2011, 134–40.

[31] P. Van Renterghem, P. Van Eenoo, P.-E. Sottas, M. Saugy, F. Delbeke. Subject-based steroid profiling and the determination of novel biomarkers for DHT and DHEA misuse in sports. *Drug testing and analysis*. 2011, *2*, 582–88.

[32] P. Van Eenoo, W. Van Gansbeke, N. De Brabanter, K. Deventer, F.T. Delbeke. A fast, comprehensive screening method for doping agents in urine by gas chromatography-triple quadrupole mass spectrometry. *Journal of chromatography. A*. 2011, *1218*, 3306–16.

[33] N. De Brabanter, W. Van Gansbeke, L. Geldof, P. Van Eenoo. An improved gas chromatography screening method for doping substances using triple quadrupole mass spectrometry, with an emphasis on quality assurance. *Biomedical chromatography*. 2012, *26*, 1416–35.

[34] WADA. Athlete biological passport - Operating guidelines. 2012, http://www.wada-ama.org/Documents/Science_Medicine/Athlete_Biological_Passport/WADA_ABP_OperatingGuidelines_version_3.0.pdf

[35] U. Mareck, H. Geyer, G. Opfermann, M. Thevis, W. Schanzer. Factors influencing the steroid profile in doping control analysis. *Journal of mass spectrometry□: JMS*. 2008, *43*, 877–91.

[36] WADA. The 2013 Prohibited List, International Standard. 2013, http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-Prohibited-list/2013/WADA-Prohibited-List-2013-EN.pdf

[37] M.A. Huestis, I. Mazzoni, O. Rabin. Cannabis in sport: anti-doping perspective. *Sports Medicine*. 2011, *41*, 949–66.

[38] M. Yonamine, D.R. Campos, R.L.D. Moreau. Marijuana as doping in sports. *Sports Medicine*. 2003, *33*, 395–99.

[39] M. Saugy, L. Avois, C. Saudan, N. Robinson, C. Giroud, P. Mangin, J. Dvorak. Cannabis and sport. *British Journal of Sports Medicine*. 2006, *40*, 13–15.

[40] A. Pokrywka, Z. Obmiński, D. Kwiatkowska, R. Grucza. Cannabinoids cases in polish athletes. 2009, http://core.kmi.open.ac.uk/display/5587219

[41] F. Grotenhermen. Pharmacokinetics and pharmacodynamics of cannabinoids. *Clin Pharmacokinet*. 2003, *42*, 327–60.

[42] K.J.S. De Cock, F.T. Delbeke, D. De Boer, P. Van Eenoo, K. Roels. Quantitation of 11 -nor-delta9-tetrahydrocannabinol-9-carboxylic acid with GC-MS in urine collected for doping analysis. *Journal of Analytical Toxicology*. 2003, *27*, 106–09.

[43] C. Chebbah, O.J. Pozo, K. Deventer, P. Van Eenoo, F.T. Delbeke. Direct quantification of 11-nor-Delta(9)-tetrahydrocannabinol-9-carboxylic acid in urine by liquid chromatography/tandem mass spectrometry in relation to doping control analysis. *Rapid Commun Mass Spectrom*. 2010, *24*, 1133–41.

[44] N. De Brabanter, W. Van Gansbeke, F. Hooghe, P. Van Eenoo. Fast quantification of 11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid (THCA) using microwave-accelerated derivatisation and gas chromatography–triple quadrupole mass spectrometry. *Forensic Science International*. 2012, 90–95.

[45] WADA. Decision limits for the confirmatory quantification of threshold substances. 2013, http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-IS-Laboratories/Technical_Documents/WADA-TD2013DL-Decision-Limits-for-the-Confirmatory-Quantification-Threshold-Substances-2.0-EN.pdf

[46] WADA. The 2010 Prohibited List, International Standard. 2010, http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-Prohibited-list/WADA_Prohibited_List_2010_EN.pdf

[47] WADA. The 2011 Prohibited List, International Standard. 2011, http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-Prohibited-list/To_be_effective/WADA_Prohibited_List_2011_EN.pdf

[48] U.S. Zimmermann, P.R. Winkelmann, M. Pilhatsch, J. a Nees, R. Spanagel, K. Schulz. Withdrawal phenomena and dependence syndrome after the consumption of “spice gold”. *Deutsches Ärzteblatt international*. 2009, *106*, 464–67.

[49] D. Hurst, G. Loeffler, R. McLay. Psychosis associated with synthetic cannabinoid agonists: a case series. *The American journal of psychiatry*. 2011, *168*, 1119.

[50] Belgian monitoring centre for drugs and drug addiction. Nieuwe psychoactieve substanties in België (2005-2011). 2012, 59. http://ewsd.wiv-isp.be/Shared documents/Reports/Nieuwe psychoactieve substanties in Belgi? 2005-2011.pdf

[51] EMCDDA. Understanding the “Spice” phenomenon. 2009, 1–34. http://www.emcdda.europa.eu/attachements.cfm/att_80086_EN_Spice Thematic paper□? final version.pdf

[52] UNODC. Synthetic cannabinoids in herbal products. http://www.unodc.org/documents/scientific/Synthetic_Cannabinoids.pdf

[53] WADA. 2012 Anti-Doping Testing Figures. 2012, http://www.wada-ama.org/Documents/Resources/Testing-Figures/WADA-2012-Anti-Doping-Testing-Figures-Report-EN.pdf

Chapter 2

Aim of the study

The research described in this work is carried out in DoCoLab, the only laboratory with WADA accreditation in the Benelux. It is built up around two main objectives and copes with the growing challenges in sport drug testing.

1 Routine screening

The first part focuses on the improvements made in the routine analysis of urine samples. According to WADA's International Standard for Laboratories [1], for the analysis of urine samples a two-step approach is used. The Initial Testing Procedure (also 'screening') should yield the information on the potential presence of substances mentioned on the Prohibited List [2], possibly via its metabolites or markers. Any so-called presumptive analytical finding is subsequently referred to a Confirmation Procedure, in which a new aliquot is analyzed to obtain more information in support of an Adverse Analytical Finding (AAF).

When a method for screening is developed in DoCoLab, the aim is always to cover as many prohibited substances as possible. This has several reasons. It is obvious that in terms of efficiency and the analysis costs per sample, reducing the number of analytical procedures is the way to go. Moreover, this is also a good evolution in terms of sample consumption since the amount of urine available to perform all necessary screening (and confirmation) procedures is limited (60 mL in the A-sample).

This thesis starts with the introduction of the new GC-MS/MS system in the gas chromatography department of the lab. This system was chosen as a successor of the older GC-MS systems, based upon its (claimed) good characteristics in terms of scan time, selectivity and sensitivity. These properties – which should be evaluated - allow for the development of a new GC-screening method incorporating the compounds previously screened by GC-MS.

The new screening method should be able to detect the increasing number of compounds and/or corresponding metabolites at decreasing MRPL's. Additionally, the correct quantification of endogenous steroids will become even more important in the future, when the steroid module in the Athlete Biological Passport is implemented. Therefore, the use and advantages of more and "better" internal standards for the construction of calibration curves and monitoring of other quality control aspects should be explored. Concerning the latter, it should be possible to follow-up every step of the sample preparation protocol and monitor the factors which could potentially influence the measured concentrations of the endogenous steroids.

In the first part of this thesis, the aim is to evaluate if the GC-MS/MS system is able to tackle the abovementioned challenges. Furthermore, attention should be paid to the efficiency of the methodology: the new screening method should cover all compounds previously screened for by two different GC-MS methods using the shortest possible sample turnaround time.

Similarly, the new GC-MS/MS instrument should be capable of improving existing confirmation procedures as well. Therefore research will aim at optimizing both the sample preparation part and the instrumental part of the procedure for the confirmation of THCA, the main metabolite of cannabis in urine.

2 Synthetic cannabinoids

Over the last years several new illicit drugs appeared on the market, complementing cannabis in the prohibited group S8. These so-called synthetic cannabinoids are a class of substances that is expanding rapidly. Around 2008, these products were detected for the first time as additives in smoking mixtures known as ‘Spice’. The packages, promised only to containing a few grams of herbal material, pretended to be the legal alternatives for the classical cannabis from *cannabis sativa*. This immediately explains their popularity all over the world: producing similar effects, these products do not trigger the well-known tests for cannabis abuse in urine [3–5], plasma [6] or oral fluid [7] and were for a long time not prohibited. Due to the increased abuse of these products, several national and international organizations started banning the first known compounds [8,9]. However, from the moment the first compounds were added to any list of forbidden drugs, the next herbal mixtures turned out to contain new cannabimimetics [10]. This steady increase in the number of synthetic cannabinoids - often only slightly chemically modified compared to first discovered - makes it hard for controlling instances to follow-up this new phenomenon. Furthermore, it is known that these compounds are excessively metabolized in the human body [11,12]. Since no parent compound is excreted in urine, knowledge of the metabolism of these cannabimimetics is necessary to design screening methods using this matrix.

Excretion studies are then used to reveal the metabolic pathways of the human body. Unfortunately, since for these compounds no pharmacological data are available, administering them to humans is ethically questionable. Therefore models are developed which allow circumventing this problem and can elucidate the metabolism of the compound. In DoCoLab, two different models are developed which could be used to tackle this problem. Since metabolism of xenobiotic compounds mainly occurs in the liver [13], both models mimic the properties of the human liver in terms of metabolomics.

The *in vitro* approach uses human liver microsomes (HLM). These sub-cellular fractions, isolated from human liver via differential centrifugation, contain the enzymes (cytochrome P450) involved in metabolism [13]. This model has the advantage that it is fast and easily applicable in analytical laboratories using the available equipment. On the other hand, it also contains some weaknesses. Since only cell fractions are used, factors like absorption, distribution and excretion are not taken into account [14]. Therefore *in vivo* models are still used to provide additional information. To reduce the inter-species differences between the metabolism of test animals and that of the human body and better mimic the human metabolism, a chimeric mice model was developed in cooperation with the Center for Vaccinology (CEVAC). This model was initially developed to study the infection and treatment of hepatitis virus [15] and is based on the transplantation of human hepatocytes to uPA-SCID mice. Therefore mice were used in which overexpression of the urokinase plasminogen activator (uPA) caused a chronic liver disease. This functional liver deficit creates a growth advantage for liver regeneration by transplanted hepatocytes. However, to avoid graft rejection when transplanting human hepatocytes, these mice were crossed with “severe immune deficient’’ mice. In that way a uPA-SCID mouse is obtained in which successful transplantation of human hepatocytes is possible. Previous research on steroid metabolism showed that these mice with humanized liver can be a good model for metabolic studies [16].

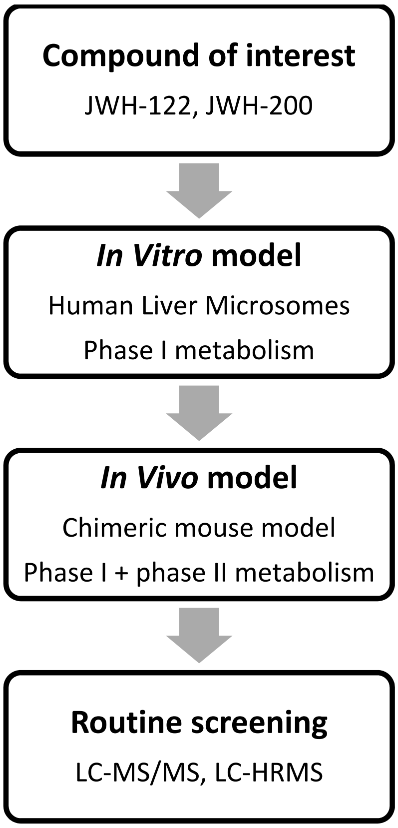


Fig. 2.1 The DoCoLab strategy: from metabolism investigation towards routine implementation.

Both models will be used to investigate the metabolism of some new synthetic cannabinoids, i.e. JWH-122 and JWH-200. Starting from the low-complexity model (*in vitro*), it will be tried to identify metabolites for both compounds, which can subsequently be confirmed *in vivo*. Using the *in vivo* model should also allow obtaining information on the phase II metabolism of the given compounds.

The final aim will be the selection of the most optimal metabolite to implement in the existing routine screening methods and in that way make detection of both JWH-122 and JWH-200 abuse in urine possible (Fig. 2.1).

References

[1] WADA. International Standard for Laboratories, version 7.0. 2012, http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-IS-Laboratories/ISL/WADA_Int_Standard_Laboratories_2012_EN.pdf

[2] WADA. The 2013 Prohibited List, International Standard. 2013, http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-Prohibited-list/2013/WADA-Prohibited-List-2013-EN.pdf

[3] C. Chebbah, O.J. Pozo, K. Deventer, P. Van Eenoo, F.T. Delbeke. Direct quantification of 11-nor-Delta(9)-tetrahydrocannabinol-9-carboxylic acid in urine by liquid chromatography/tandem mass spectrometry in relation to doping control analysis. *Rapid Commun Mass Spectrom*. 2010, *24*, 1133–41.

[4] N. De Brabanter, W. Van Gansbeke, F. Hooghe, P. Van Eenoo. Fast quantification of 11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid (THCA) using microwave-accelerated derivatisation and gas chromatography–triple quadrupole mass spectrometry. *Forensic Science International*. 2012, 90–95.

[5] S. Strano-Rossi, F. Molaioni, F. Rossi, F. Botre. Rapid screening of drugs of abuse and their metabolites by gas chromatography/mass spectrometry: application to urinalysis. *Rapid Communications in Mass Spectrometry*. 2005, *19*, 1529–35.

[6] C. Abbara, R. Galy, A. Benyamina, M. Reynaud, L. Bonhomme-Faivre. Development and validation of a method for the quantitation of Delta9 tetrahydrocannabinol in human plasma by high performance liquid chromatography after solid-phase extraction. *Journal of pharmaceutical and biomedical analysis*. 2006, *41*, 1011–16.

[7] A. Molnar, J. Lewis, P. Doble, G. Hansen, T. Prolov, S. Fu. A rapid and sensitive method for the identification of delta-9-tetrahydrocannabinol in oral fluid by liquid chromatography-tandem mass spectrometry. *Forensic science international*. 2012, *215*, 92–96.

[8] UNODC. Synthetic cannabinoids in herbal products. http://www.unodc.org/documents/scientific/Synthetic_Cannabinoids.pdf

[9] EMCDDA. Understanding the “Spice” phenomenon. 2009, 1–34. http://www.emcdda.europa.eu/attachements.cfm/att_80086_EN_Spice Thematic paper□? final version.pdf

[10] R. Lindigkeit, A. Boehme, I. Eiserloh, M. Luebbecke, M. Wiggermann, L. Ernst, T. Beuerle. Spice: a never ending story? *Forensic science international*. 2009, *191*, 58–63.

[11] M. Hutter, S. Broecker, S. Kneisel, V. Auwärter. Identification of the major urinary metabolites in man of seven synthetic cannabinoids of the aminoalkylindole type present as adulterants in “herbal mixtures” using LC-MS/MS techniques. *Journal of mass spectrometry□: JMS*. 2012, *47*, 54–65.

[12] T. Sobolevsky, I. Prasolov, G. Rodchenkov. Detection of JWH-018 metabolites in smoking mixture post-administration urine. *Forensic science international*. 2010, *200*, 141–47.

[13] S.C. Strom, J. Davila, M. Grompe. Chimeric mice with humanized liver: tools for the study of drug metabolism, excretion, and toxicity. *Methods Mol Biol*. 2010, *640*, 491–509.

[14] M.D. Tingle, N.A. Helsby. Can in vitro drug metabolism studies with human tissue replace in vivo animal studies? *Environmental Toxicology and Pharmacology*. 2006, *21*, 184–90.

[15] P. Meuleman, L. Libbrecht, R. De Vos, B. de Hemptinne, K. Gevaert, J. Vandekerckhove, T. Roskams, G. Leroux-Roels. Morphological and biochemical characterization of a human liver in a uPA-SCID mouse chimera. *Hepatology*. 2005, *41*, 847–56.

[16] L. Lootens, P. Meuleman, O.J. Pozo, P. Van Eenoo, G. Leroux-Roels, F.T. Delbeke. uPA(+/+)-SCID Mouse with Humanized Liver as a Model for In Vivo Metabolism of Exogenous Steroids: Methandienone as a Case Study. *Clinical Chemistry*. 2009, *55*, 1783–93.

PART II:

Implementation of GC-QqQ-MS in doping control

Chapter 3:

A fast, comprehensive screening method for doping agents in urine by gas chromatography - triple quadrupole mass spectrometry

Adapted from:

P. Van Eenoo, W. Van Gansbeke, N. De Brabanter, K. Deventer, F.T. Delbeke.

A fast, comprehensive screening method for doping agents in urine by gas chromatography-triple quadrupole mass spectrometry.

J Chromatogr A. 2011, 1218, 3306–16.

Abstract

The use of performance enhancing drugs in sports is prohibited. For the detection of misuse of such substances gas chromatography or liquid chromatography coupled to mass spectrometry are the most frequently used detection techniques. In this work the development and validation of a fast gas chromatography tandem mass spectrometric method for the detection of a wide range of doping agents is described.

The method can determine 13 endogenous steroids (the steroid profile), 19-norandrosterone, salbutamol and 11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid in the applicable ranges and is able to detect qualitatively over 140 substances in 1 mL of urine in accordance with the minimum required performance levels of the World Anti-Doping Agency. The classes of substances included in the method are anabolic steroids, beta-2-agonists, stimulants, narcotics, hormone antagonists and modulators and beta-blockers. Moreover, by using a short capillary column and hydrogen as a carrier gas the run time of the method is less than 8 min.

1 Introduction

The use of performance enhancing drugs in sports is prohibited by the World Anti-Doping Agency and a wide range of pharmacological classes of drugs can be found on the Prohibited List [1]. In the framework of the fight against doping, mostly urine samples are collected and analysed for the presence of prohibited drugs or metabolites. In general, except for peptide hormones, screening for the misuse of doping substances is performed using chromatography. Moreover, to combine the necessary selectivity with the required sensitivity to detect the different classes of prohibited substances at or below the minimum required performance limit (MRPL), hyphenated chromatographic mass spectrometric methods are preferred [2].

Within the group of low molecular weight doping agents (MW < 1000 Da), the anabolic steroids are probably the most challenging and important class. They are the most misused substances in doping control [3], they are intensively metabolized in humans and have the lowest MRPL [2]. Adequate screening for misuse of these substances therefore relies on the detection of metabolites in urine samples collected from athletes [4].

Most of the studies investigating the metabolism of pharmaceutically available steroids were performed in the 1980's and early 1990's [5,6]. This research resulted in the selection of appropriate metabolites for the detection of steroid misuse. Over the years the selection of metabolites was further elaborated to include several metabolites that can result in prolonged detection times [7]. Gas chromatography-mass spectrometry (GC-MS) was essential as a technique for these findings and was therefore also employed to screen doping control samples routinely for the misuse of anabolic steroids. GC-MS remained the technology of choice for the detection of anabolic steroids until the appearance of tetrahydrogestrinone (THG) as a designer steroid on the underground market, which was almost undetectable by the GC-MS screening methods used at that time. THG lead to the introduction of liquid chromatography tandem mass spectrometry (LC-MS^n) as a screening technology in doping control laboratories. As a result GC-MS and LC-MS are now used as complementary techniques in doping control [8–10].

Besides anabolic steroids, GC-MS is also routinely applied for many other groups of doping agents. Particularly for stimulants and narcotics, GC-MS is still the most employed technology [11,12].

For routine purposes quadrupole GC-MS methods are normally used in the selected ion monitoring mode (SIM) for anabolic steroids and in full scan or a combination of SIM/scan for narcotics and stimulants. SIM-methods monitor a limited number of m/z-values (typical for a substance) rather than a range of m/z-values (full scan mode). Although less structural information is obtained in the SIM mode, in this way higher sensitivity is achieved, which is typically required for anabolic agents. Most screening methods for the detection of anabolic steroids have a run time of 20-35 minutes [13,14]. In the past several fast tandem mass spectrometric methods, using ion trap technology, have been published. However, these methods normally lacked the combination of a quantitative determination of the steroid profile (a range of endogenous steroids monitored to detect use of natural steroids) and a qualitative analysis of a wide range of exogenous steroids and other doping agents [15,16].

In this paper the use of a gas chromatograph coupled to a triple quadrupole mass spectrometer for the detection of a wide range of exogenous anabolic steroids and other doping agents as well as the determination of a full steroid profile within a single run of less than 10 minutes was investigated.

2 Material and methods

2.1 Reagents

N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) was purchased from Chem. Fabrik Karl Bucher (Waldstedt, Germany) and the enzyme preparation β-glucuronidase from E. coli K12 was obtained from Roche Diagnostics GmbH (Mannheim, Germany). Diethyl ether was purchased from Acros (Geel, Belgium), methanol and sodium hydrogen carbonate ($NaHCO_3$) from Fisher Scientific (Loughborough, UK), potassium carbonate (K_2CO_3), disodium hydrogen phosphate (Na_2HPO_4), sodium dihydrogen phosphate (NaH_2PO_4), and sodium sulphate (Na_2SO_4) were all from Merck (Darmstadt, Germany).

The phosphate buffer (pH 7) was prepared by dissolving 7.1 g $Na_2HPO_4.2H_2O$ and 1.4 g $NaH_2PO_4.H_2O$ in 100 mL water. The carbonate buffer (pH=9.5) was prepared by dissolving 135 g K_2CO_3 and 111 g $NaHCO_3$ in 900 aqua bidest.

2.2 Reference standards

2.2.1 Internal standards

D3-testosterone glucuronide (d3-T-gluc), d3-epitestosterone glucuronide (d3-E-gluc), d4-androsterone glucuronide (d4-A-gluc), d5-etiocholanolone (d5-Etio), d3-dihydrotestosterone glucuronide (d3-DHT-gluc), d3-5α-androstane-3α,17β-diol (d3-ααβ-Adiol), d5-5β-androstane-3α,17β-diol (d5-βαβ-Adiol) and d3-salbutamol (d3-Sal) were from NMI (Pymble, Australia). 17α-methyltestosterone (MT) was a kind gift from Organon (Oss, The Netherlands). A mixture was made in methanol containing 2 µg/mL d4-A-gluc, d5-Etio and d3-Sal, 1 µg/mL d3-ααβ-Adiol, d5-βαβ-Adiol and MT, 1.2 µg/mL d3-T-gluc, 0.3 µg/mL d3-E-gluc and 0.4 µg/mL d3-DHT-gluc.

2.2.2 Natural steroids

Testosterone, epitestosterone, androsterone, etiocholanolone, 11β-hydroxyandrosterone (11β-OH-androsterone), 11β-hydroxyetiocholanolone (11β-OH-etiocholanolone), 5α-androstane-3α,17β-diol, 5α-androstane-3β,17β-diol, 5β-androstane-3α,17β-diol, 4-androstene-3,17-dione, 5β-androstane-3,17-dione and 5α-androstane-3,17-dione were obtained from Sigma (Bornem, Belgium), 19-norandrosterone from NMI (Pymble, Australia), dehydroepiandrosterone (DHEA) from Serva (Heidelberg, Germany) and dihydrotestosterone (DHT) from Piette International Laboratories (Drogenbos, Belgium).

2.2.3 Exogenous steroids

3α-Hydroxytibolone (tibolone metabolite) was a kind gift from Akzo Nobel (Oss, The Netherlands), fluoxymesterone was obtained from Ciba-Geigy, boldenone and oxymesterone were purchased from the Institut für Biochemie of the Deutsche

Sporthochschule (Cologne, Germany). 3α,5α-tetrahydronorethisterone, was a kind gift from the Institute of Organic Chemistry and Biochemistry (Academy of Sciences of the Czech Republic, Prague, Czech Republic) and metenolone from the Drug Control Centre of King's College (London, UK).

16α-OH-furazabol, 16α-OH-stanozolol, 17α-ethyl-5α-estrane-3α,17β-diol, 17α-ethyl-5β-estrane-3α,17β-diol, 17α-methyl-5α-androstane-3α,17β-diol, 17α-methyl-5β-androstane-3α,17β-diol, 17α-trenbolone, 17β-hydroxy-17α-methyl-5α-androst-1-ene-3-one, 1α-methyl-5α-androstan-3α-ol-17-one, 1-methylene-5α-androstan-3α-ol-17-one, 1-testosterone, 2α-hydroxymethyl-17α-methyl-1,4-androstadiene-11α,17β-diol-3-one, 2α-hydroxymethylethisterone, 2α-methyl-5α-androstan-3α-ol-17-one, 4-chloro-4-androsten-3α-ol-17-one, 4-OH-testosterone, 5β-androst-1-en-17β-ol-3-one, 5α-Androst-1-ene-3β,17β-diol, 6β-hydroxy-dehydrochloromethyltestosterone, 6β-hydroxyfluoxymesterone, 6β-hydroxymethandienone, epimetendiol, 7β,17α-dimethyl-5β-androstane-3α,17β-diol, 7β,17α-dimethyl-5β-androstane-3α,17β-diol, 9α-fluoro-17,17-dimethyl-18-nor-androstan-4,13-diene-11β-ol-3-one, 9α-fluoro-17α-methyl-4-androsten-3α,6β,11β,17β-tetra-ol, dehydrochloromethyltestosterone, epioxandrolone, methyldienolone, 13β,17α-diethyl-5β-gonane-3α,17β-diol, 13β,17α-diethyl-5α-gonane-3α,17β-diol, oxabolone were purchased from NMI (Pymble, Australia). Oxandrolone was a gift from Searle & Co (Chicago, Ill, USA), 1-androstene-3,17-dione, 4-androstene-17α-methyl-11α,17β-diol-3-one, 7β-OH-DHEA, calusterone and mibolerone were from Steraloids (Newport, RI, USA). Madol and 2α,17α-dimethyl-17β-hydroxy-5α-androstan-3-one were bought from TRC (Toronto, Canada). Bolasterone was a gift from Upjohn and danazol from Withrop.

2.2.4 Narcotics

11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid, normethadone, ((±)-2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolinium perchlorate (EDDP), fentanyl, norfentanyl, fenbutrazate, buprenorphine, morphine, codeine, ethylmorphine, 6-monoacetylmorphine (6-MAM), oxymorphone were purchased from Cerilliant. Pethidine, hydromorphone, oxycodone, heroin, dextromoramide and methadone were bought from Sigma (Bornem, Belgium). Pentazocine was a gift from Whintrop Laboratories (Newcastle, United Kingdom).

2.2.5 Stimulants

Pipradrol and pemoline were gifts from Merrell-Dow (Cincinnati, OH, USA) and Boehringer-Ingelheim (Brussels, Belgium), respectively. Cocaine and benzoylecgonine were purchased from Cerilliant. Fenethylline was a gift from Chemiwerk Hamburg (Germany) and methylphenidate from Ciba-Geigy (Groot-Bijgaarden, Belgium). Fencamine was obtained from Laboratoires Miquel S.A. (Barcelona, Spain), fenspiride from Laboratoires Servier (Orléans, France) and amineptine and amineptine C5 metabolite from Laboratoires Servier (Orleans, France). Strychnine and fencamfamine were donated by Merck. Carphedon, 6-OH-bromantan, crotethamide, cropropamide, cyclazodone and famprofazone were bought from NMI. Dimefline was from Recordate Industria Chemica & Farmaceutica (Milan, Italy) and furfenorex and clobenzorex from Roussel Uclaf (Romainville, France). Amiphenazole and octopamine were purchased from Sigma (Bornem, Belgium), while ethamivan, chloorphentermine and benzphetamine were gifts from Sinclair Pharmaceuticals (Godalmings, UK), Tropon Werke (Cologne) and Upjohn (Kalamazoo, USA), respectively 3,3-diphenylamine and prenylamine were gifts from the World Association of Anti-Doping Scientists (WAADS).

2.2.6 Beta-blockers

Beta-blockers obtained as reference substances were: acebutolol from Rhone-Poulenc (Brussels, Belgium), alprenolol from Astra Chemicals (Holstein, Germany), propranolol from ICI (Kortenberg, Belgium), betaxolol from Synthelabo (Brussels, Belgium), labetolol from Glaxo (Brussels, Belgium), metoprolol from Ciba-Geigy, nadolol from Squibb (Braine l'Alleud, Belgium), oxprenolol from CIBA (Dilbeek, Belgium), pindolol from Sandoz (Vilvoorde, Belgium), sotalol from Pfizer, timolol from MSD (Brussels, Belgium), bisoprolol from Merck (Overijse, Belgium), carvedilol from Roche (Mannheim, Germany). Levobunolol (l-bunolol), esmolol were a kind gift from the South African Doping Control Laboratory. Carteolol was a gift from the Portuguese Doping Control Laboratory. The following products were extracted from therapeutical preparations: celiprolol (Selectol, Pharmacia, Brussels, Belgium) and metipranolol (Beta-Ophtiole, Tramedic, Sint-Niklaas, Belgium).

2.2.7 Beta-2-agonists

Salbutamol, terbutaline and clenbuterol were purchased from RIVM. Salmeterol xinafoate was a gift from GlaxoSmithKline (Philadelphia, PA, USA). Fenoterol was a gift from Boehringer & Sohn (Ingelheim am Rhein and formoterol from Novartis (Arnhem, The Netherlands). Bambuterol was donated by the Instituto Nacional do Desporto (Lisbon, Portugal).

2.2.8 Hormone antagonists and modulators

Anastrazole, toremifene, exemestane, 17β-hydroxy-6-methylene-androsta-1,4-diene-3-one (exemestane metabolite), were generous gifts from Astra Zeneca (Macclesfield, UK), WAADS, Pfizer (Groton, UK) and the Faculty of Pharmacy of the Helsinki University (Helsinki, Finland), respectively. 6α-OH-androstenedione, 4-OH-androstenedione (formestane), 4-hydroxycyclofenil, 3-hydroxy-4-methoxytamoxifen and bis-(4-cyanophenyl)methanol (Letrozole metabolite) were purchased from NMI. 4-OH-tamoxifen was bought from Sigma-Aldrich.

2.2.9 Other substances

Zilpaterol and zeranol were purchased from NMI, probenecid from Federa (Brussels, Belgium) and 5-hydroxypentoxyfylline (5-OH-pentoxyfylline) from Hoechst (Frankfurt, Germany)

2.2.10 Excretion urines

Excretion urines from the stimulants prolintane (Catovit®) and sibutramine (Reductil®) were obtained after the controlled administration of an oral therapeutic dose (10 mg each) to healthy volunteers, which had given written consent, and were provided by other doping control laboratories. The samples were stored at -20°C awaiting analysis.

Steroid stripped urine was prepared by bringing urine from children over an XAD-2 (Serva, Heidelberg, Germany) column which was conditioned with methanol and water. The obtained steroid stripped urine was subsequently tested for the presence/absence of endogenous steroids.

2.3 Sample preparation

To 1 mL of urine, 50 µl of the internal standard mixture, 1 mL phosphate buffer (pH=7.0) and 50 µl of β-glucuronidase from *E. coli* were added. The samples were incubated for 1.5h at 56°C. 1 mL of a liquid mixture $NaHCO_3/K_2CO_3$ (pH=9.5) and 5 mL of diethyl ether were added to hydrolyzed urine samples and extracted for 20 minutes by rolling. The organic layer was evaporated to dryness under oxygen free nitrogen (OFN) at room temperature.

To the dried residues, 20 µl of acetonitrile was added, followed by derivatisation for 1 h at 80°C with 100 µl MSTFA/ethanethiol/NH_4I (500:4:2).

2.4 Instrumentation

An Agilent (Agilent Technologies, Palo Alto, USA) model GC 7890 gas chromatograph coupled with an Agilent 7000A triple quadrupole mass spectrometer (Agilent Technologies) and a MPS2 autosampler and PTV-injector from Gerstel (Mülheim an der Ruhr , Germany) were used.

The GC was equipped with a 12.5 m capillary column (HP-Ultra 1, column length 12.5m×0.2mm with a 0.11µm film thickness) from J&W Scientific (Agilent Technologies, USA).

The column temperature was programmed as follows: the initial temperature was 100°C (0.2 min), 90°C/min→185°C, 9°C/min→230°C, 90°C/min→310°C (0.95 min). The transfer line was maintained at 310°C. Hydrogen (α-gas1, Airliquide, Desteldonk, Belgium) was used as a carrier gas at a flow rate of 1 mL/min.

Five µl was injected and the PTV-injector settings were 100°C (0.15min), 12°C/s→280°C.

Helium was used as a quench gas at a flow of 2.25 mL/min and nitrogen as a collision gas at a flow of 1.5 mL/min.

2.5 Method validation

2.5.1 Quantitative

Six point calibration curves were made by spiking steroid stripped urine samples (3 replicates per concentration). Unweighted least squares regression was used to construct the calibration curves.

Accuracy and precision (repeatability) of the method were subsequently tested at every level (n=6). Acceptable tolerances (%) for precision were calculated from $2/3RSD_{max} = 2^{(1-0.5\log C)}$ [17]. Tolerances for the accuracy -expressed as bias- were set at a maximum 15% [18].

Additionally, 50 urine samples were analyzed using the traditional method on a single quadrupole instrument and on the triple quadrupole instrument.

2.5.2 Qualitative analysis

The method validation was performed according the Eurachem guidelines [19] on 10 different, randomly chosen urine samples.

To determine the limits of detection (LOD), 10 different urine samples were spiked with reference mixtures at different levels in the concentration range of 0.05, 0.1, 0.2, 0.5, 1 and 2 times the MRPL level. The LOD was defined as the lowest concentration at which a substance can be detected in all samples analysed ($n = 10$). Repeatability was assessed through the analysis of multiple samples spiked at different levels during the determination of the LOD. Selectivity and specificity were tested by the analysis of blank urine samples.

3 Results and discussion

3.1 Sample preparation

One of the main difficulties for doping control laboratories is that the methods used need to be able to detect very low levels of a wide variety of prohibited substances in a small volume of a dirty matrix (predominantly urine). Indeed, the volume of urine delivered to laboratories is currently 60 mL [20]. This amount needs to be sufficient for laboratories to screen for and eventually confirm (using a totally independent analysis) the presence of any prohibited substance. Therefore methods used in doping control laboratories need to be able to detect a wide variety of substances in as little urine as possible. The method in this work only uses 1 mL of urine for the screening of a wide range of doping agents. In general the volume is 2-5 times lower than the volume normally used for screening methods for anabolic steroids [13–16] and therefore constitutes a drastic reduction thanks to the PTV-injection. This is useful since in doping control a limited amount of urine is available for screening and confirmation of a wide range of substances.

The developed method is very comprehensive. Only a few anabolic agents for which GC-MS is not particularly suitable (e.g. tetrahydrogestrinone, methyltrienolone, stanozolol) and for which LC-MS offers a valuable alternative [21] are not included in the current method. The method also includes one or more metabolites of all prohibited narcotics, the most frequently used beta-2-agonists and hormone antagonists and modulators and beta-blockers. Additionally, the method contains a high number of stimulants and several substances from all other groups of prohibited substances (except peptide hormones and glucocorticosteroids).

The method encorporates a high number of quality assurance measures which cover the three basic steps in sample preparation: hydrolysis, extraction and derivatisation.

The use of a high amount of β-glucuronidase allows for an efficient hydrolysis after 1.5h at 56°C. Moreover, the use of both glucuronidated and free steroids with similar structure (d4-A-gluc, d5-Etio) allows for an adequate evaluation of hydrolysis efficiency.

By using a diverse mixture of internal standards, the differences in physicochemical properties, possibly leading to changes in extraction efficiency of the broad spectrum of target compounds in the current method is covered. This has several important benefits, besides the obvious advantages for the quantitation of the non-deuterated structural analogues.

The inclusion of transitions for mono-TMS derivatised androsterone and etiocholanolone in the method additionally allows for the evaluation of the derivatisation efficiency.

Hence, this integrated approach allows for a comprehensive evaluation of the sample preparation efficiency per sample (rather than per batch or solely at the time of validation) since all major sample preparation steps are monitored.

To evaluate possible microbiological sample degradation, 5α-androstane-3,17-dione and 5β-androstane-3,17-dione are monitored. This is important since microbiological degradation can affect the steroid profile (the combination of endogenous steroids quantified to detect misuse with natural steroids). In cases where elevated concentrations of 5α-androstane-3,17-dione or 5β-androstane-3,17-dione are detected, particular care must be taken in the evaluation of the steroid profile.

3.2 Gas chromatography

The aim of this study was to develop a fast GC-MS/MS method, capable of quantifying the endogenous steroids given in Table 3.1 and at the same time detecting a wide range of other substances qualitatively. Sufficient resolution between compounds is a prerequisite for adequate quantitation. In this method, the separation of the isomers androsterone and etiocholanolone, present at relatively large concentrations (Table 3.1), and to a minor extent the other isomers (11β-OH-A and 11β-OH-Etio and 5ααβ-Adiol and 5βαβ-Adiol) put restrictions on chromatographic speed and injected volumes. Nevertheless, in this method 5 µl of sample could be injected using a PTV-injector. This is substantially higher than in previous methods using split/splitless injection [22].

Table 3.1. Target substances for a quantitative analysis

Substance	Internal standard	calibrators (ng/mL)	Correlation coefficient (r^2)
testosterone	d3-T-gluc	2-5-20-50-100-200	0.9918
epitestosterone	d3-E-gluc	2-5-20-50-100-200	0.9933
androsterone	d4-A-gluc	48-120-600-1200-2400-4800	0.9903
etiocholanolone	d5-E	48-120-600-1200-2400-4800	0.9716
11β-OH-androsterone	d4-A-gluc	40-100-500-1000-2000-4000	0.9769
11β-OH-etiocholanolone	d5-E	40-100-500-1000-2000-4000	0.9877
dihydrotestosterone	d3-DHT-gluc	4-10-40-100-200-400	0.9755
dehydroepiandrosterone	d3-DHT-gluc	4-10-40-100-200-400	0.9927
4-androstene-3,17-dione	d3-DHT-gluc	4-10-40-100-200-400	0.9908
5α-androstane-3α,17β-diol	d3-ααβ-Adiol	4-10-40-100-200-400	0.9841
5β-androstane-3α,17β-diol	d5-βαβ-Adiol	4-10-40-100-200-400	0.9603
5α-androstane-3β,17β-diol	d3-ααβ-Adiol	4-10-40-100-200-400	0.9933
5α-androstane-3,17-dione	MT	4-10-40-100-200-400	0.9975
5β-androstane-3,17-dione	MT	4-10-40-100-200-400	0.9853
19-norandrosterone	MT	1-3-5-10-15-20	0.9902
salbutamol	d3-Sal	100-300-500-1000-1500-2000	0.9807
THCA	MT	5-15-25-50-75-100	0.9862

The use of a short capillary column compared to those used in previous methods [13–16,23], in combination with a high flow rate of hydrogen (instead of helium which is used traditionally as a carrier gas) resulted in a substantial reduction of the GC run time. Indeed, the current method has a run time of 7.98 min. However, even at high concentrations of 4.8 µg/mL, androsterone and etiocholanolone are sufficiently separated to allow for an adequate quantification (Fig. 3.1). This significant reduction in GC run time greatly improves sample turn around, which can be essential in those cases (e.g. Olympics) where stringent demands are put on sample reporting times (24-48 h).

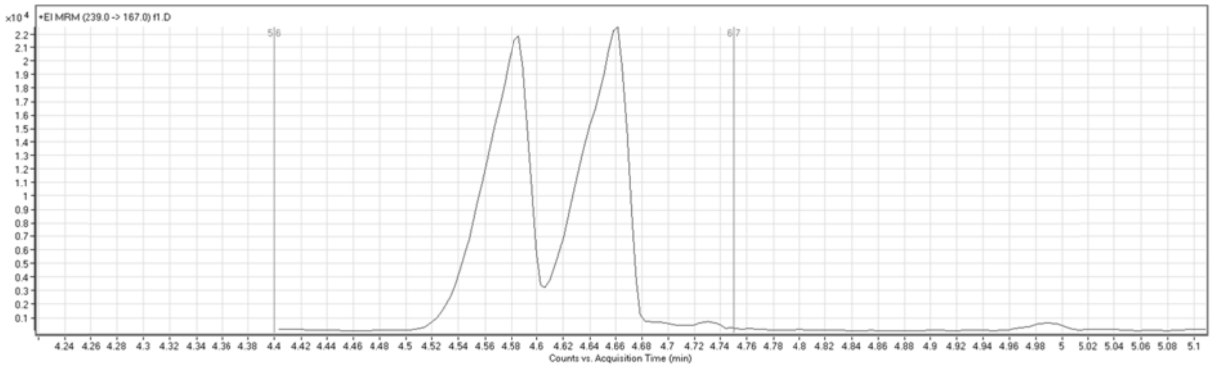


Fig. 3.1. Extracted ion chromatogram (m/z 239→167) for androsterone-bis-TMS and etiocholanolone-bis-TMS at the highest calibrator concentration (4.8 µg/mL).

3.3 Mass spectrometry

Determination and optimization of the mass spectrometric conditions were done in a multistep process. In the first step, full scan spectra were obtained for every (derivatised) compound. After selection of a suitable precursor ion, full product scan mass spectra were acquired at different collision energies (10 and 25 eV). Suitable product ions were selected and SRM transitions were set up. Selection of the final product ions (at least two transitions per substance) and optimization of the collision energy (5, 10, 15, 20, 30, 35 eV) was then performed both on reference standards and extracts from spiked urine samples. Per substance, the best S/N ratio was used for the final selection of the most appropriate transitions and collision energies.

Table 3.2 shows the final instrumental settings of the mass spectrometer for the investigated target analytes.

3.4 Method validation

3.4.1 Quantitative

The substances included in the quantitative part of the method include those steroids traditionally used in doping control to establish the use of a prohibited substance (T, E, A, Etio, DHT, DHEA, Adion, 5ααβ-Adiol, 5βαβ-Adiol). Additionally the method monitors other endogenous steroids which are not affected by the intake of natural anabolics (11bOH-A and 11b-OH-Etio) as well as markers of microbiological degradation (5α-androstanedione and 5β-androstanedione). The inclusion of these parameters can greatly assist in the evaluation process of atypical steroid profiles due to increased production of endogenous steroids or alteration by microbiological degradation. Besides these steroids the method also quantifies salbutamol, the most widely used beta-2-agonist, norandrosterone and the major metabolite of cannabis (11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid, THCA).

Although large differences in calibration ranges exist between the monitored compounds, correlation coefficients of 6-point calibration curves (3 replicates per calibrator) made in steroid stripped urine were acceptable. Additional analysis revealed that the residual standard deviations at every point of the calibration curves were lower than 2/3 of the maximum residual standard deviation as calculated by Horwitz. Moreover, the bias at each of these points was below 15%, showing acceptable accuracy as well. Therefore, in agreement with Eurachem guidelines, the method can be regarded as validated for quantitative purposes.

A comparison between the traditionally used GC-MS method in selected ion monitoring and the new methodology using selected reaction monitoring (SRM) for the testosterone to epitestosterone ratio is shown in Fig. 3.2. These results indicate an excellent correlation (r^2=0.95) and comparability (slope is almost equal to 1) between both methodologies.

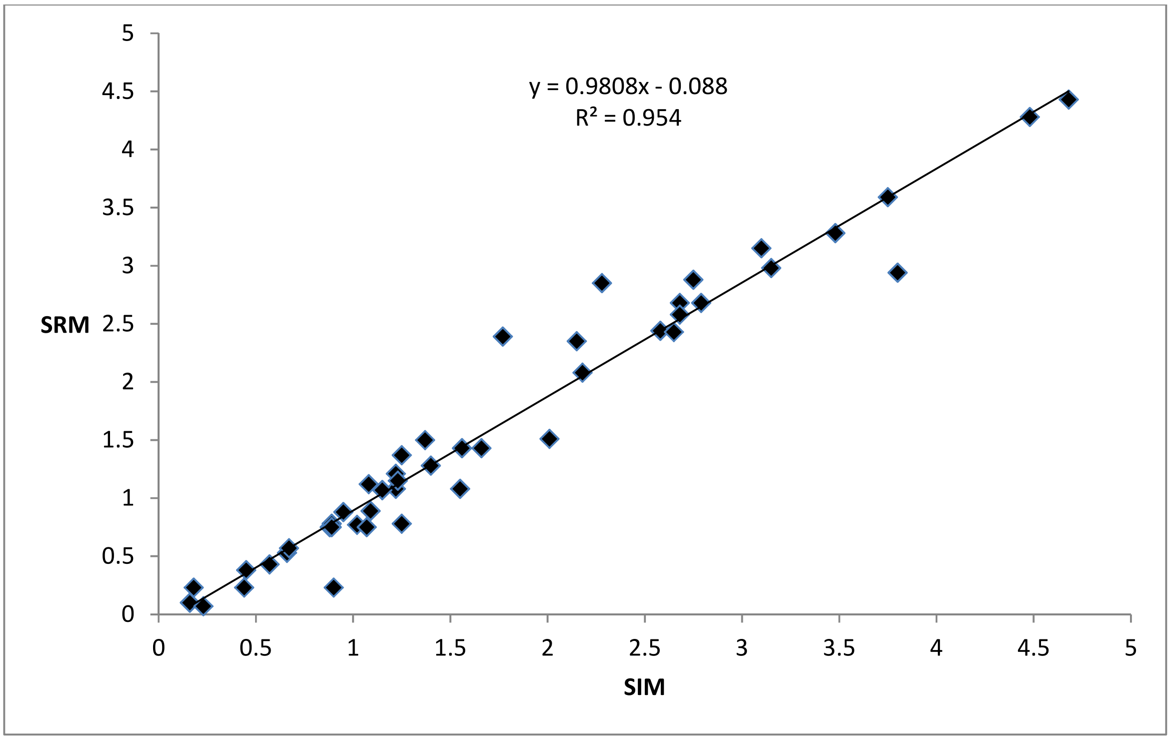


Fig. 3.2. Testosterone to epitestosterone ratio (T/E) in 50 urine samples using a single quadrupole mass spectrometer in Selected Ion Monitoring (SIM) and using a triple quadrupole mass spectrometer in selected reaction monitoring (SRM).

3.5 Qualitative analysis

The method validation for the non-threshold substances was also performed in accordance with Eurachem guidelines. Selectivity was tested by analyzing ten blank urine samples and verifying that there were no matrix interferences. Additionally these samples were spiked at different concentration levels. The lowest concentration at which concurrent signals (S/N>3) for each monitored transition were obtained at the expected retention time (± 1%) in all samples was assigned as the lower limit of detection (LOD). These LODs for the exogenous substances are given in Table 3.2. The method comprises 41(metabolites) of anabolic steroids, 4 other anabolic agents, 6 beta-2-agonists, 11 hormone antagonists and modulators, 19 narcotics, 16 stimulants and 15 beta-blockers.

It should be noted that in some cases, the observed LOD for a metabolite exceeds WADA's MRPL. For these substances, the method was regarded as non-validated, although they remained part of the method. For all such cases, the method includes another metabolite (of the same parent drug) that has an LOD at or below the MRPL. This is the case for e.g. fluoxymesterone: the LOD for 6β-hydroxyfluoxymesterone (Table 3.2) is 20 ng/mL, while WADA's MRPL is set at 10 ng/mL. However, the LOD of 9α-fluoro-17,17-dimethyl-18-nor-androstan-4,13-diene-11β-ol-3-one, another fluoxymesterone metabolite, is compliant with the MRPL. Because - except for a few substances - WADA's technical document does not specify which metabolites need to be monitored, the method can therefore still be considered as WADA-compliant for the detection of fluoxymesterone. Moreover the detection of multiple metabolites instead of a single metabolite to determine misuse of a doping agent has multiple advantages. Firstly, it can provide additional supporting evidence for misuse since in most cases the concentration in a "positive" sample will be clearly above the MRPL. Additionally, the inclusion of multiple metabolites can assist in the detection of a prohibited substance at different time points after use. Indeed, it is widely known that the excretion profile of metabolites is time and inter-individual dependent. Therefore, a metabolite which is the major metabolite in one individual after a definite post-administration time, might only be a minor metabolite in another individual which took the drug at another point in time. The current method is also capable of detecting all compounds from the class of "other anabolic agents", except the group of selected androgen receptor modulators for which it was not tested, as these compounds are still in clinical phase trials.

Table 3.2. Substances (as TMS-enol-TMS ether derivatives) included in the method according to WADA prohibited class, instrument settings, limit of detection and applicable minimum required performance limit (MRPL).

class	RT (min)	substance	transitions	collision energy (eV)	LOD (ng/mL)	MRPL (ng/mL)
S1a	4.14	5β-androst-1-en-17β-ol-3-one	432.0 -> 194.0	15	5	10
			432.0 -> 206.0	15		
	5.32	Boldenone	430.0 -> 206.0	10	10	10
			430.0 -> 191.0	30		

	5.09	1-Androstenediol	434.0 -> 195.0	20	5	10
			434.0 -> 127.0	20		
	5.05	1-testosterone	432.0 -> 194.0	5	10	10
			432.0 -> 206.0	10		
	5.09	17α-methyl-5α-androstane-3α,17β-diol	435.0 -> 255.0	20	2	2
			435.0 -> 213.0	20		
	5.12	17α-methyl-5β-androstane-3α,17β-diol	435.0 -> 255.0	20	5	2
			435.0 -> 213.0	20		
	6.7	oxymesterone	534.0 -> 389.0	20	10	10
			534.0 -> 444.0	20		
	4.15	epimetendiol	358.0 -> 301.0	15	2	2
			358.0 -> 196.0	5		
	6.57	6β-hydroxymethandienone	517.0 -> 229.0	20	5	10
			517.0 -> 337.0	15		
	5.63	Metenolone PC	446.0 -> 208.0	10	5	10
			446.0 -> 195.0	15		
	4.92	1-Methylene-5α-androstan-3α-ol-17-one (metenolone metab)	446.0 -> 341.0	15	20	10
			446.0 -> 195.0	5		
	5.64	17α-Ethyl-5β-estrane-3α,17β-diol (norethandrolone major metab)	421.0 -> 241.0	15	10	10
			421.0 -> 331.0	5		
	5.4	17α-Ethyl-5α-estrane-3α,17β-diol (norethandrolone minor metab)	421.0 -> 241.0	15	5	10
			421.0 -> 145.0	25		
	4.77	2α-methyl-5α-androstan-3α-ol-17-one (drostanolone metab)	448.0 -> 433.0	10	10	10
			448.0 -> 253.0	25		
	6.05	Bolasterone PC	460.0 -> 355.0	15	10	10
			460.0 -> 315.0	15		
	5.62	7α,17α-dimethyl-5β-androstane-3α,17β-diol (bolasterone metab)	284.0 -> 269.0	5	10	10
			284.0 -> 213.0	10		
	6.13	Calusterone PC	460.0 -> 355.0	15	10	10
			460.0 -> 315.0	15		
	5.45	7β,17α-dimethyl-5β-androstane-3α,17β-diol (calusterone metab)	229.0 -> 105.0	30	/	10
			269.0 -> 159.0	5		
	5.07	1α-Methyl-5α-androstan-3α-ol-17- one (mesterolone metab)	448.0 -> 433.0	10	5	10
			448.0 -> 253.0	20		
	5.63	4-Chloro-4-androsten-3α-ol-17-one (clostebol metab)	466.0 -> 181.0	20	10	10
			466.0 -> 431.0	15		
	6.47	norclostebol	452.0 -> 216.0	20	2	10
			452.0 -> 321.0	15		
	6.67	fluoxymesterone PC	552.0 -> 407.0	15	/	10
			552.0 -> 357.0	15		
			552.0 -> 319.0	15		
	6.93	6β-OH-fluoxymesterone	640.0 -> 640.0	10	20	10
			640.0 -> 143.0	25		
	5.04	9α-fluoro-17,17-dimethyl-18-nor-androstan-4,13-diene-11β-ol-3-one	462.0 -> 208.0	15	5	10
			462.0 -> 337.0	15		
	6.17	oxandrolone	363,0 -> 161,0	15	10	10
			308.0 -> 117.0	15		
	5.56	epioxandrolone	363,0 -> 161,0	15	20	10
			308.0 -> 117.0	15		

	6.68	dehydrochloromethyltestosterone PC	478.0 -> 285.0	20	10	10
			478.0 -> 353.0	5		
	6.82	6β-hydroxy-dehydrochloromethyltestosterone	315.0 -> 227.0	20	20	10
			315.0 -> 241.0	15		
	5.19	17α-trenbolone	307.0 -> 291.0	10	10	10
			307.0 -> 275.0	20		
	7.1	2-Hydroxymethyl-17α-methylandrostadiene-11α,17β-diol-3-one (formebolone metab)	444.0 -> 356.0	25	/	10
			367.0 -> 257.0	25		
	6.48	17α-methyl-4-androstene-11α,17β-diol-3-one (formebolone metab)	534.0 -> 389.0	15	10	10
			534.0 -> 339.0	25		
	5.85	mibolerone	446.0 -> 431.0	15	10	10
			446.0 -> 341.0	20		
	6.14	ethisterone	456.0 -> 316.0	15	1	10
			456.0 -> 301.0	15		
	4.76	3α,5α-tetrahydronorethisterone	431.0 -> 167.0	20	2	10
			431.0 -> 193.0	20		
	7.11	16-OH-furazabol	490.0 -> 231.0	15	10	10
			490.0 -> 143.0	35		
	5.94	methyldienolone	430.0 -> 285.0	10	10	10
			430.0 -> 325.0	10		
	5.97	13β,17α-diethyl-5α-gonane-3α, 17β-diol (norbolethone metab)	435.0 -> 255.0	10	20	10
			435.0 -> 159.0	15		
	6.14	13β,17α-diethyl-5β-gonane-3α, 17β-diol (norbolethone metab)	435.0 -> 255.0	20	5	10
			435.0 -> 345.0	5		
	3.68	madol	345.0 -> 255.0	15	10	10
			345.0 -> 201.0	15		
	6.11	2α,17α-dimethyl-17β-hydroxy-5α-androstane-3-one	462.0 -> 141.0	15	10	10
			462.0 -> 143.0	15		
	6.27	4-OH-nandrolone (oxabolone)	506.0 -> 147.0	20	2	10
			506.0 -> 93.0	25		
			506.0 -> 195.0	20		
	6.48	4-OH-testosteron	520.0 -> 225.0	15	2	10
			520.0 -> 431.0	15		
	6.33	6-OH-androstenedione	518.0 -> 319.0	15	1	10
			518.0 -> 413.0	15		
	5.19	7β-OH-DHEA	430.0 -> 325.0	10	20	10
			430.0 -> 220.0	10		
s1b	4.04	19-norandrosterone	405.0 -> 225.0	10	1	2
			405.0 -> 315.0	5		
	4.12	5β-Androstane-3,17-dione	290.0 -> 275.0	10	EAAS	/
			290.0 -> 185.0	10		
	4.64	5α-androstane-3α,17β-diol	256.0 -> 185.0	15		
			256.0 -> 157.0	15		
	4.71	5β-androstane-3α,17β-diol	256.0 -> 185.0	15		
			256.0 -> 157.0	15		
	4.58	androsterone	239.0 -> 167.0	35		
			239.0 -> 117.0	35		
	4.63	etiocholanolone	239.0 -> 167.0	35		
			239.0 -> 117.0	35		

	5.09	5α-Androstan-3,17-dione	290.0 -> 275.0	10		
			290.0 -> 185.0	10		
	4.98	DHEA	432.0 -> 327.0	10		
			432.0 -> 237.0	10		
	5.14	epitestosterone	432.0 -> 209.0	10		
			432.0 -> 327.0	10		
	5.13	5α-androstane-3β,17β-diol	421.0 -> 255.0	20		
			421.0 -> 213.0	20		
	5.29	4-androstenedione	430.0 -> 209.0	15		
			430.0 -> 234.0	15		
	5.24	DHT	434.0 -> 195.0	20		
			434.0 -> 182.0	20		
	5.41	testosteron	432.0 -> 209.0	10		
			432.0 -> 327.0	10		
	5.52	11β-OH-androsterone	522.0 -> 236.0	10		
			522.0 -> 324.0	10		
	5.6	11β-OH-etiocholanolone	522.0 -> 236.0	10		
			522.0 -> 324.0	10		
	4.13	Mono TMS Androsterone	347.0 -> 253.0	20	qas	/
S1c	3.37	zilpaterol	308.0 -> 218.0	10	5	10
			308.0 -> 203.0	15		
			291.0 -> 219.0	15		
	6.43	zeranol	433.0 -> 295.0	15	10	10
			433.0 -> 309.0	15		
	2.42	clenbuterol	335.0 -> 227.0	10	0,2	2
			335.0 -> 300.0	10		
	5.37	3α-hydroxytibolone	443.0 -> 193.0	35	5	10
			443.0 -> 167.0	30		
S3	2.17	salbutamol	369.0 -> 207.0	15	25	100
			369.0 -> 191.0	15		
	1.96	terbutaline	356.0 -> 267.0	25	50	100
			356.0 -> 355.0	25		
	6.07	fenoterol	322.0 -> 68.0	15	100	100
			322.0 -> 279.0	15		
	6.6	fenoterol C,N-methylene	308.0 -> 207.0	15	/	50
			308.0 -> 179.0	15		
	6.73	formoterol	178.0 -> 121.0	20	50	100
			178.0 -> 135.0	20		
	7.82	salmeterol	311.0 -> 149.0	15	100	100
			311.0 -> 121.0	25		
	5.02	bambuterol	354.0 -> 72.0	25	5	100
			354.0 -> 282.0	10		
S4	3.63	aminogluthetimide deriv.1	361.0 -> 206.0	30	5	50
			361.0 -> 221.0	10		
	5.26	aminogluthetimide deriv.2	580.0 -> 551.0	20	/	50
			580.0 -> 519.0	20		
	3.16	anastrazole	293.0 -> 70.0	10	50	50
			293.0 -> 209.0	15		
	3.17	letrozole metabolite	291.0 -> 160.0	15	2,5	50

			291.0 -> 217.0	20		
	6.94	exemestane PC	441.0 -> 307.0	20	/	50
			441.0 -> 193.0	20		
	6.94	17β-hydroxy-6-methylene-androsta-1,4-diene-3-one	443.0 -> 207.0	20	25	50
			443.0 -> 193.0	20		
	6.43	4-OH-androstene-3,17-dione (formestane)	518.0 -> 221.0	15	2	10
			518.0 -> 190.0	10		
	6.57	toremifene	405.0 -> 58.0	15	25	50
			405.0 -> 72.0	5		
	6.86	4-hydroxy-methoxytamoxifen 1	489.0 -> 72.0	5	25	50
			489.0 -> 58.0	15		
	7.02	4-hydroxy-methoxytamoxifen 2	489.0 -> 72.0	5	25	50
			489.0 -> 58.0	15		
	5.78	4-OH-tamoxifen	459.0 -> 72.0	5	2,5	50
			459.0 -> 58.0	15		
	7.74	raloxifene	578.0 -> 193.0	35	25	50
			578.0 -> 413.0	30		
	6.57	4-OH-cyclofenil	512.0 -> 422.0	10	2,5	50
			512.0 -> 343.0	5		
S5	3.13	probenecid	328.0 -> 103.0	25	12,5	250
			328.0 -> 193.0	15		
S6	2.16	carphedon	272.0 -> 104.0	25	50	500
			272.0 -> 229.0	15		
	4.98	6-OH-bromantan	395.0 -> 91.0	30	2,5	500
			393.0 -> 91.0	30		
	2.08	pemoline	178.0 -> 104.0	10	5	500
			392.0 -> 178.0	10		
	2.28	octopamine	174.0 -> 86.0	5	100	500
			426.0 -> 206.0	15		
			426.0 -> 179.0	15		
	7.14	strychnine	316.0 -> 144.0	15	100	200
			316.0 -> 220.0	10		
	1.37	crotethamide	154.0 -> 86.0	10	50	500
			154.0 -> 69.0	15		
	1.97	ethamivan	295.0 -> 223.0	25	50	500
			295.0 -> 265.0	20		
	1.36	fencamfamine	215.0 -> 186.0	5	50	500
			215.0 -> 98.0	15		
	4.24	fenspiride	241.0 -> 96.0	10	25	500
			241.0 -> 154.0	10		
	4.65	prenylamine	238.0 -> 58.0	20	50	500
			238.0 -> 91.0	20		
	1.94	clobenzorex	168.0 -> 125.0	20	100	500
			168.0 -> 89.0	35		
	2.51	cyclazodone	360.0 -> 178.0	15	10	500
			360.0 -> 247.0	15		
	6.57		286.0 -> 72.0	20		

		famprofazone	286.0 -> 214.0	15	50	500
	1.66	benzphetamine	148.0 -> 91.0	20	10	500
			148.0 -> 65.0	35		
	1.74	methylphenidate	156.0 -> 45.0	35	100	500
			156.0 -> 84.0	10		
	6.47	amineptine	193.0 -> 115.0	15	10	500
			193.0 -> 178.0	15		
	4.53	amineptine C5 metabolite	193.0 -> 115.0	15	50	500
			193.0 -> 178.0	15		
	2.7	cocaine	303.0 -> 82.0	15	50	500
			303.0 -> 198.0	5		
	3.07	benzoylecgonine	240.0 -> 82.0	20	100	500
			361.0 -> 82.0	20		
	3.56	prolintane metabolite14	322.0 -> 293.0	20	excr	500
			322.0 -> 205.0	20		
	2.28/2.34	prolintane metabolite 5a/b	304.0 -> 142.0	20	excr	500
			304.0 -> 75.0	20		
	2.67	prolintane metabolite 9	228.0 -> 158.0	20	excr	500
			228.0 -> 138.0	20		
	2.52	sibutramine metabolite 1	158.0 -> 116.0	10	excr	500
			158.0 -> 102.0	10		
	2.74/2.82	sibutramine metabolite 2/3	246.0 -> 156.0	20	excr	500
			246.0 -> 84.0	20		
S7	7.47	buprenorphine	554.0 -> 522.0	15	0,5	10
			554.0 -> 450.0	20		
	6.57	dextromoramide	265.0 -> 166.0	15	20	200
			265.0 -> 98.0	10		
	4.91	heroin	369.0 -> 327.0	10	2,5	200
			369.0 -> 268.0	25		
	4.66	MAM	399.0 -> 287.0	15	20	200
			399.0 -> 340.0	10		
	5.37	fentanyl	245.0 -> 189.0	10	/	10
			245.0 -> 146.0	15		
	2.19	norfentanyl	175.0 -> 120.0	5	/	10
			175.0 -> 56.0	15		
	4.32	hydromorphone	429.0 -> 234.0	15	100	200
			429.0 -> 357.0	25		
	2.73	methadone	296.0 -> 191.0	20	10	200
			296.0 -> 281.0	10		
	2.93	methadone 2	296.0 -> 191.0	20	40	200
			296.0 -> 281.0	10		
	2.37	normethadone 1	224.0 -> 103.0	35	100	200
			224.0 -> 191.0	35		
	2.73	normethadone 2	296.0 -> 191.0	20	10	200
			296.0 -> 252.0	20		
	2.14	EDDP	277.0 -> 105.0	25	40	200
			277.0 -> 220.0	20		
	4.42	morphine	429.0 -> 287.0	20	10	200
			429.0 -> 220.0	35		

	4.37	oxycodone	459.0 -> 368.0	15	200	200
			459.0 -> 312.0	15		
	4.76	oxymorphone	502.0 -> 70.0	30	40	200
			517.0 -> 355.0	15		
	3.12	pentazocine	357.0 -> 246.0	15	100	200
			357.0 -> 289.0	15		
	1.47	pethidine	247.0 -> 71.0	5	4	200
			247.0 -> 173.0	5		
	3.97	codeine	371.0 -> 229.0	5	10	200
			371.0 -> 234.0	5		
	4.21	ethylmorphine	385.0 -> 214.0	35	10	200
			385.0 -> 234.0	10		
	2.51	pipradrol	239.0 -> 161.0	20	5	200
			239.0 -> 221.0	20		
	5.25	fenbutrazate	261.0 -> 103.0	35	50	200
			261.0 -> 175.0	15		
S8	6.06	THCA	371.0 -> 289.0	15	<5	7,5
			371.0 -> 265.0	15		
P2	1.91	oxprenolol	150.0 -> 109.0	15	50	500
			221.0 -> 72.0	15		
	3.62	betaxolol	364.0 -> 209.0	10	100	500
			364.0 -> 172.0	10		
	2.94	bisoprolol	405.0 -> 56.0	25	100	500
			405.0 -> 172.0	15		
	3.07	pindolol 1	204.0 -> 133.0	15	500	500
			220.0 -> 75.0	15		
	3.65	pindolol 2	205.0 -> 130.0	15	50	500
			292.0 -> 218.0	15		
	3.05	esmolol	352.0 -> 193.0	5	100	500
			352.0 -> 56.0	15		
	3.02	metipranolol	366.0 -> 281.0	5	25	500
			366.0 -> 239.0	15		
	2.64	propanolol	316.0 -> 231.0	5	25	500
			316.0 -> 75.0	15		
	3.15	timolol	373.0 -> 186.0	15	50	500
			373.0 -> 70.0	35		
	4.12	carteolol	421.0 -> 186.0	15	50	500
			421.0 -> 365.0	5		
	4.12	levobunolol	234.0 -> 233.0	5	25	500
			234.0 -> 217.0	10		
	2	celiprolol 1	319.0 -> 129.0	15	/	500
			205.0 -> 89.0	15		
			205.0 -> 117.0	15		
	3.45	celiprolol 2	200.0 -> 128.0	15	500	500
			200.0 -> 144.0	15		
	4.53	nadolol	510.0 -> 70.0	35	250	500
			510.0 -> 186.0	20		

	6.2	acebutolol 1 + 2	278.0 -> 166.0	30	500	500
			278.0 -> 208.0	30		
	1.72	alprenolol	321.0 -> 72.0	15	250	500
			306.0 -> 203.0	15		
	6.67	labetolol	383.0 -> 265.0	15	100	500
			383.0 -> 251.0	15		
ISTD	4.66	5β-Androstane-3α,17β-diol-d5	246.0 -> 190.0	15	ISTD	/
			246.0 -> 164.0	15		
	4.62	5α-Androstane-3α,17β-diol-d3	244.0 -> 202.0	15		
			244.0 -> 188.0	15		
	4.51	androsterone-d4	423.0 -> 333.0	20		
			423.0 -> 243.0	20		
	4.56	etiocholanolone-d5	424.0 -> 334.0	20		
			424.0 -> 244.0	20		
	5.12	epitestosterone-d3	435.0 -> 330.0	5		
			435.0 -> 209.0	20		
	5.38	testosterone-d3	435.0 -> 330.0	20		
			435.0 -> 209.0	20		
	5.17	DHT-d3	437.0 -> 205.0	15		
			437.0-> 195.0	15		
	2.16	salbutamol-d3	372.0 -> 210.0	20		
			372.0-> 193.0	20		
	5.97	17α-methyltestosterone	446.0 -> 301.0	25		
			446.0-> 198.0	20		

Besides the anabolic agents, a wide variety of hormone antagonist and modulators can be detected at or below the MRPL. This list includes substances with a steroidal structure (formestane, 6α-OH-androstenedione and the metabolite of exemestane: 17β-hydroxy-6-methylene-androsta-1,4-diene-3-one) as well as non steroidal compounds (aminogluthetimide, anastrazole, letrozole metabolite, raloxifene, toremifene, 4-OH-cyclofenil, 4-OH-tamoxifen and the isomers of 4-OH-methoxytamoxifen). Moreover, as androsta-1,4,6-triene-3,17-dione also metabolizes to boldenone and its metabolites [24], the only substances from this class which are not included in the method are testolactone, clomiphene and fulvestrant, due to lack of reference standards for the metabolites of these substances.

Similar as for the previous groups, most prohibited narcotics undergo extensive Phase I and Phase II metabolism. Therefore all WADA prohibited narcotics and/or metabolites were included in the current method. Except for fentanyl, which shows superior detection by LC-MS, all LOD's were lower than WADA's MRPL and therefore the methodology is very well suited for monitoring the misuse of narcotics. In addition to the prohibited narcotics, the method also screens for codeine, because the use of codeine can result in the detection of morphine. In cases where the detection of morphine can be attributed to the use of codeine however, a laboratory should not report such cases [2]. Although in general, urine is not well suited to determine the post-administration time of sample collection, the current method offers some possibilities for several substances by monitoring multiple metabolites for which the excretion profile is time dependent. This is e.g. the case for heroin for which not only the parent substance but also morphine and 6-monoacetylmorphine (MAM) are monitored. Besides these substances, which are also important in forensic science, toxicology and laboratories working in the field of drugs of abuse and work place testing, the method is also capable of simultaneously quantifying 11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid (THCA), the major metabolite of cannabis and one of the most detected doping agents world-wide.

In contrast to the narcotics, most stimulants are not excreted as conjugates. Therefore, the inclusion of these substances was not the focus of this research. Nevertheless, a wide range of stimulants (or metabolites), including cocaine and its metabolite benzoylecgonine are included in the method.

The method covers the most frequently used β-agonists in sports. Moreover, in the case of fenoterol both the parent drug (O-TMS tetrakis derivatised) and a degradation product, C,N-methylene fenoterol-tetrakis-TMS derivatives were monitored [25]. Although the degradation product was not detected in the validation study, its inclusion in the method will increase the detection capability of the method for real samples substantially since fenoterol can be rapidly degraded.

Although beta-blockers are only prohibited in particular sports, many drugs belonging to this group of substances are excreted as glucuronide conjugates. Therefore 15 beta-blockers were included in the method. The inclusion of this group has the advantage, that in case their detection is requested, no additional analysis needs to be performed and hence this leads to an optimized laboratory efficiency.

Although the derivatisation procedure uses an optimized protocol [26] and the method monitors the effectiveness by the detection of mono-TMS derivatised androsterone and etiocholanolone, the formation of multiple derivatives of several compounds (e.g. celiprolol, pindolol) is still possible. Although in general one of the derivatives gives a better signal than the other, the inclusion of the second derivative can be regarded as a safety precaution. Taking into account the scan speed of the instrument (500 scans/s), this addition of transitions does not decrease the overall performance of the method.

4 Conclusion

A fast GC-MS/MS method for the quantitative determination of the steroid profile, salbutamol, THCA and norandrosterone as well as the qualitative detection of 142 doping agents (or their metabolites) was developed and validated. Using hydrogen as a carrier gas and a short (12.5 m) capillary column all doping agents could be detected within a single run of less than 8 minutes. The use of a wide range of internal standards allows for an evaluation of the sample preparation efficiency.

The current method shows that the combination of triple quadrupole technology and large volume injection can greatly improve the detection capabilities of target substances in complex matrices as biological fluids.

Acknowledgments

This research was performed with support of the World Anti-Doping Agency. The technical assistance of Dr.ir. B. Tienpont (Research Institute for chromatography, Kortrijk, Belgium) is gratefully acknowledged.

References

[1] WADA. The 2009 Prohibited List, International Standard. 2009, http://www.wada-ama.org/rtecontent/document/2009_List_En.pdf

[2] WADA. Technical Document: Minimum Required Performance Limits for Detection of Prohibited Substances: TD2010MRPL. 2010, http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-IS-Laboratories/WADA_TD2010MRPLv1.0_Minimum Required Performance Levels_Sept 01 2010_EN.pdf

[3] WADA. Laboratory statistics 2010. 2010, http://www.wada-ama.org/Documents/Resources/Testing-Figures/WADA_2010_Laboratory_Statistics_Report.pdf

[4] W. Schänzer, M. Donike. Metabolism of anabolic steroids in man: synthesis and use of reference substances for identification of anabolic steroid metabolites. *Analytica Chimica Acta*. 1993, *275*, 23–48.

[5] H. Bi, P. Du, R. Massé. Studies on anabolic steroids—8. GC/MS characterization of unusual seco acidic metabolites of oxymetholone in human urine. *The Journal of Steroid Biochemistry and Molecular Biology*. 1992, *42*, 229–42.

[6] W. Schänzer, H. Geyer, M. Donike. Metabolism of metandienone in man: Identification and synthesis of conjugated excreted urinary metabolites, determination of excretion rates and gas chromatographic-mass spectrometric identification of bis-hydroxylated metabolites. *The Journal of Steroid Biochemistry and Molecular Biology*. 1991, *38*, 441–64.

[7] P. Van Eenoo, F.T. Delbeke. Metabolism and excretion of anabolic steroids in doping control--new steroids and new insights. *The Journal of steroid biochemistry and molecular biology*. 2006, *101*, 161–78.

[8] D.H. Catlin, M.H. Sekera, B.D. Ahrens, B. Starcevic, Y.-C. Chang, C.K. Hatton. Tetrahydrogestrinone: discovery, synthesis, and detection in urine. *Rapid communications in mass spectrometry□: RCM*. 2004, *18*, 1245–49.

[9] K. Deventer, P. Van Eenoo, F.T. Delbeke. Screening for anabolic steroids in doping analysis by liquid chromatography/electrospray ion trap mass spectrometry. *Biomedical chromatography□: BMC*. 2006, *20*, 429–33.

[10] O.J. Pozo, P. Van Eenoo, K. Deventer, F.T. Delbeke. Ionization of anabolic steroids by adduct formation in liquid chromatography electrospray mass spectrometry. *Journal of Mass Spectrometry*. 2007, 497–516.

[11] W. Van Thuyne, P. Van Eenoo, F.T. Delbeke. Implementation of gas chromatography combined with simultaneously selected ion monitoring and full scan mass spectrometry in doping analysis. *Journal of chromatography. A*. 2008, *1210*, 193–202.

[12] W. Van Thuyne, P. Van Eenoo, F.T. Delbeke. Comprehensive screening method for the qualitative detection of narcotics and stimulants using single step derivatisation. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences*. 2007, *857*, 259–65.

[13] C. Ayotte, D. Goudreault, A. Charlebois. Testing for natural and synthetic anabolic agents in human urine. *Journal of Chromatography B: Biomedical Sciences and Applications*. 1996, *687*, 3–25.

[14] M. Saugy, C. Cardis, N. Robinson, C. Schweizer. Test methods: anabolics. *Bailliere's best practice & research. Clinical endocrinology & metabolism*. 2000, *14*, 111–33.

[15] J. Munoz-Guerra, D. Carreras, C. Soriano, C. Rodriguez, A.F. Rodriguez. Use of ion trap gas chromatography-tandem mass spectrometry for detection and confirmation of anabolic substances at trace levels in doping analysis. *Journal of chromatography. B, Biomedical sciences and applications*. 1997, *704*, 129–41.

[16] J. Marcos, J.A. Pascual, X. de la Torre, J. Segura. Fast screening of anabolic steroids and other banned doping substances in human urine by gas chromatography/tandem mass spectrometry. *Journal of mass spectrometry□: JMS*. 2002, *37*, 1059–73.

[17] A. van W. Verwaal, M. van Bavel, A. Boot, J. Bravenboer, F. de Goei, C. Maas, P. Der. *De Ware(n) Chemicus*. 1996, 106.

[18] F. Bressolle, M. BrometPetit, M. Audran. Validation of liquid chromatographic and gas chromatographic methods - Applications to pharmacokinetics. *Journal of Chromatography B-Biomedical Applications*. 1996, *686*, 3–10.

[19] Eurachem. *The Fitness for Purpose of Analytical Methods A Laboratory Guide to Method Validation and Related Topics*. 1998,

[20] WADA. International standard for laboratories, version 6.0. 2009, http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-IS-Laboratories/WADA_Int.Standard_Laboratories_2009_EN.pdf

[21] O.J. Pozo, P. Van Eenoo, K. Deventer, F.T. Delbeke. Development and validation of a qualitative screening method for the detection of exogenous anabolic steroids in urine by liquid chromatography-tandem mass spectrometry. *Analytical and bioanalytical chemistry*. 2007, *389*, 1209–24.

[22] P. Van Renterghem, P. Van Eenoo, W. Van Thuyne, H. Geyer, W. Schanzer, F.T. Delbeke. Validation of an extended method for the detection of the misuse of endogenous steroids in sports, including new hydroxylated metabolites. *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences*. 2008, *876*, 225–35.

[23] F. Botre, M. Mazzarino, M. Orengia. Application of fast gas chromatography/mass spectrometry for the rapid screening of synthetic anabolic steroids and other drugs in anti-doping analysis. *Rapid communications in mass spectrometry*. 2007, *21*, 4117–24.

[24] M.K. Parr, G. Fusshöller, N. Schlörer, G. Opfermann, T. Piper, G. Rodchenkov, W. Schänzer. Metabolism of androsta-1,4,6-triene-3,17-dione and detection by gas chromatography/mass spectrometry in doping control. *Rapid communications in mass spectrometry□: RCM*. 2009, *23*, 207–18.

[25] M.K. Henze, G. Opfermann, H. Spahn-Langguth, W. Schänzer. Screening of beta-2 agonists and confirmation of fenoterol, orciprenaline, reproterol and terbutaline with gas chromatography-mass spectrometry as tetrahydroisoquinoline derivatives. *Journal of chromatography. B, Biomedical sciences and applications*. 2001, *751*, 93–105.

[26] P. Kiousi, Y.S. Angelis, E. Lyris, M. Koupparis, A.C. Calokerinos, J. Atta-Politou, C.G. Georgakopoulos. Two-step silylation procedure for the unified analysis of 190 doping control substances in human urine samples by GC-MS. *Bioanalysis*. 2009, *1*, 1209–24.

Chapter 4:

Gas chromatography screening for doping substances using triple quadrupole mass spectrometry: an improved methodology.

Adapted from:

N. De Brabanter, W. Van Gansbeke, L. Geldof, P. Van Eenoo.

An improved gas chromatography screening method for doping substances using triple quadrupole mass spectrometry, with an emphasis on quality assurance.

Biomed Chromatogr. 2012, *26***,** 1416–35.

Abstract

In the fight against doping gas chromatography (GC) coupled to mass spectrometry (MS) is by far the most used technique. Recently a method using the combination of GC and triple quadrupole technology was developed for the detection of a wide range of prohibited substances, leading to improved selectivity and sensitivity. Here, this system was used to detect over 150 compounds from different classes (steroids, narcotics, stimulants, beta-blockers, beta-2-agonists, hormone antagonists) in a qualitative way.

In the quantitative part, the traditional steroid profile with most important endogenous steroids is expanded with 6 minor metabolites (4-hydroxy-androst-4-ene-3,17-dione, 6α-hydroxy-androst-4-ene-3,17-dione, 16α-hydroxy-androst-4-ene-3,17-dione, 3β,7β-dihydroxy-androst-5-en-17-one and 3β,16α-dihydroxy-androst-5-en-17-one) which further improves the detection and identification of endogenous steroid abuse. Besides these also norandrosterone, salbutamol and the major metabolite of cannabis are quantified.

Taking into account the tremendous implications an adverse analytical finding can have on the (professional) life of an athlete, methods developed for anti-doping purposes should be subjected to the highest level of quality. Here, the addition of a combination of (deuterated) internal standards allows for an accurate quality control of every single step of the whole methodology: hydrolysis efficiency, derivatisation efficiency and microbiological degradation are individually monitored in every single sample. Additionally, evaluation of the pre-analytical phase is important. Therefore, special attention is paid to the relation between parameters indicating degradation by micro-organisms and the reliability of the steroid profile. The impact of the degradation is therefore studied by evaluation of the quantities and percentages of 5α-androstane-3,17-dione and 5β-androstane-3,17-dione.

Additionally, the concept of measurement uncertainty was introduced for the evaluation of relative abundances of mass-to-charge ratios and the obtained ranges were compared with the World Anti-Doping Agency (WADA) regulations on tolerance windows for relative ion intensities. The results indicate that both approaches are very similar.

1 Introduction

Since 1999 the fight against doping in sports is regulated by the World Anti-Doping Agency (WADA), responsible for annual publication of the prohibited list [1,2]. This list contains hundreds of different specified compounds, each of them with other chemical properties, divided in different classes. The WADA accredited laboratories have the difficult task to screen for this wide range of compounds and report the data in a short period of time.

Gas chromatography coupled to mass-spectrometry (GC-MS) is still the most popular technique for screening and confirmation of these compounds [1,3–5]. Using selected ion monitoring (SIM) mode the required limits can be detected in a GC run of about 30 min [6]. Now, with the introduction of GC-MS triple quadrupole (QqQ) systems, these existing methods can be improved in terms of detection limits and run times.

Normal screening methods for anabolic steroids in doping control laboratories consume 2 to 5 mL of urine and sample preparation contains several steps: enzymatic hydrolysis, liquid-liquid extraction and evaporation of the organic solvent [1,7,8]. To improve the characteristics of the compounds in terms of chromatography, silylation reagents are used for derivatisation [9]. Since both alcohol and carbonyl functionalities need derivatisation, the reactive trimethyliodosilane is chosen. This is formed *in situ* when mixing of N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA), ammonium iodide and ethanethiol [10].

While in earlier described methods a single internal standard is used [1,6,11], multiple deuterated internal standards have been introduced to allow for an improved correction of losses during sample preparation steps. The combination of glucuronidated and free internal standards also allows for the determination of hydrolysis efficiency.

Besides the screening for exogenous drugs, also the abuse of endogenous compounds should be detected. For the determination of the abuse of testosterone and related compounds steroid profiling is necessary. Traditionally, the steroid profile contains the concentrations of the most important endogenous steroids such as testosterone (T), epitestosterone (E), androsterone (A), etiocholanolone (Etio), 5α-androstane-3α,17β-diol (5ααβ-Adiol), 5β-androstane-3α,17β-diol (5βαβ-Adiol), androstenedione (Adion), dehydroepiandrosterone (DHEA) and dihydrotestosterone

(DHT). Population-based reference ranges are set to distinguish natural steroid profiles from the ones influenced by steroid administration. According to the WADA technical document samples need to be submitted to isotope ratio mass spectrometry (IRMS) analysis if the T/E ratio is greater than 4, the concentration of T and E greater than 200 ng/mL and A greater than and 10 000 ng/mL respectively and the concentration of DHEA greater than 100 ng/mL [12]. The concentrations of these steroids can however show large interindividual variations, and these reference ranges, unfortunately, do not cover all natural variations. Additionally there are more steroids available today of which the intake does not always influence the traditional steroid profile to an important extent. This was already shown for oral administration of DHEA [13] and Adion [14]. Recent studies showed that the discriminating power of steroid profiling can be increased by adding minor steroid metabolites [15,16]. These are formed after administration of endogenous steroids, when the major metabolism pathways are saturated. Those minor metabolites are mostly hydroxylated and oxygenated at C4, C6, C7 and C16 positions, are more specific and can allow for a better determination of the abused steroid [8]. Hence, inclusion of some of these metabolites in a routine screening method should improve detection of misuse with natural steroids.

Since a lot of factors can cause variations in the endogenous concentrations, ratios of these steroidal hormones concentrations can be regarded as more stable alternatives. Ratios including T/E, A/Etio, A/T, 5ααβ-Adiol/5βαβ-Adiol showed to be less susceptible to variables like exercise, physical performance, volume and density of the urine, etc. [17]

In this paper a GC-QqQ-MS screening method is described for the determination of the traditional steroid profile together with the minor metabolites and corresponding concentration ratios. Further the method is able to detect over 150 components qualitatively, at concentration levels at or below WADA’s minimum required performance limit (MRPL) [18]. Next to this, an extensive set of quality control measures is implemented. In this way quality control can be done for every individual sample and per batch.

Under quality control, special attention is paid to microbiological degradation of urine samples. It is shown that micro-organisms can be present in urine and can cause alterations in the steroid profile [19]. Micro-organism activity can in a first step induce deconjugation of steroids and thereafter cause modifications in the steroidal structure by oxidoreductive reactions. The conversion of androsterone and etiocholanolone to 5α-androstane-3,17-dione (5α-dion) and 5β-androstane-3,17-dione (5β-dion) respectively by consecutively deconjugation and bacterial 3-hydroxysteroid dehydrogenase activity is well-known [17,20] (Fig. 4.1). Similarly, unconjugated testosterone concentration can increase by bacterial conversion of 5-androstene-3β,17β-diol, leading to an elevation of T/E ratios [17].

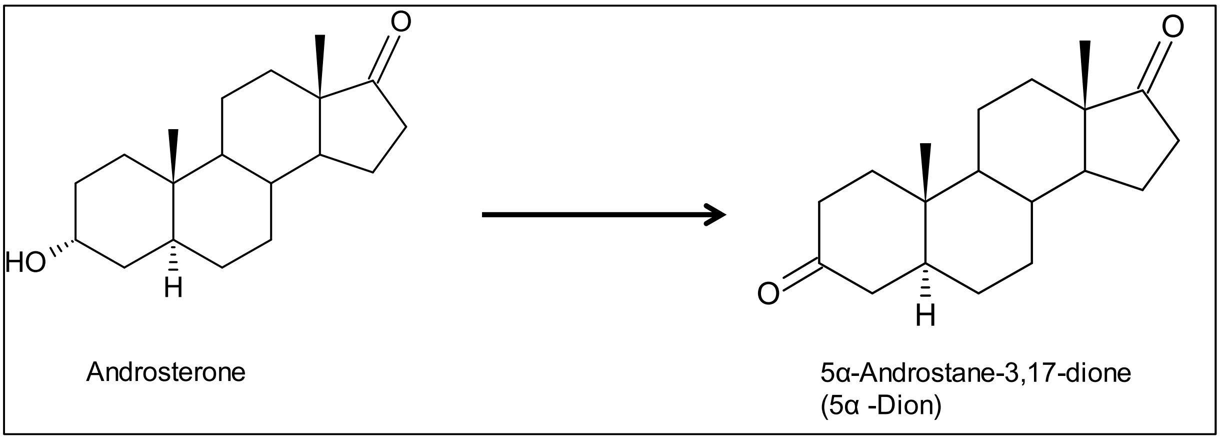


Fig. 4.1 Microbiological conversion of androsterone tot 5α-androstane-3,17-dione.

To avoid reporting atypical findings in steroid parameters caused by these effects, the WADA technical document TD2004EAAS [12] rejects results if the concentration of free testosterone and/or epitestosterone in the specimen exceeds 5% of the respective glucuroconjugates. Because screening for steroids is usually done in the total (free + glucuronidated) fraction, additional analyses are required if these concentrations needs to be determined. Elevated amounts of 5α-androstane-3,17-dione and 5β-androstane-3,17-dione also indicate microbiological degradation. Recently it was shown that the 5% rule for free testosterone correlates well with threshold values for 5α-dion and 5β-dion in order to control microbiological degradation in the screening methods [21]. This paper describes the effect of these parameters on the complete steroid profile.

2 Material and methods

2.1 Reagents

The enzyme β-glucuronidase from E. Coli, used for hydrolysis was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Methanol, sodium hydrogen carbonate ($NaHCO_3$) were obtained from Fisher Scientific (Loughborough, UK). Potassium carbonate (K_2CO_3), disodium hydrogen phosphate (Na_2HPO_4), sodium dihydrogen phosphate (NaH_2PO_4) and sodium sulphate (Na_2SO_4) were from Merck (Darmstadt, Germany)

The phosphate buffer solution was prepared by dissolving 7.1 g $Na_2HPO_4.2H_2O$ and 1.4 g $NaH_2PO_4.H_2O$ in 100 mL water. The carbonate buffer was prepared by dissolving 135g K_2CO_3 and 111g $NaHCO_3$ in 900 mL aqua bidest.

Diethylether en ethanethiol were purchased from Acros (Geel, Belgium). Acetonitrile was from Biosolve (The Netherlands) and ammoniumiodide (NH_4I) from Sigma Aldrich (Bornem, Belgium). MSTFA was obtained from Chem. Fabrik Karl Bucher (Waldfstedt, Germany) and sodiumsulphate from Merck (Darmstadt, Germany).

2.2 Internal standards

17α-Methyltestosterone (IS) was a kind gift from Organon (Oss, The Netherlands). Following deuterated internal standards were from NMI (Pymble, Australia): d3-testosterone glucuronide (d3-T-gluc), d3-epitestosterone glucuronide (d3-E-gluc), d4-androsterone glucuronide (d4-A-gluc), d5-etiocholanolone (d5-Etio), d3-dihydrotestosterone glucuronide (d3-DHT), d3-5α-androstane-3α,17β-diol (d3-ααβ-Adiol), d5-5β-androstane-3α,17β-diol (d5-βαβ-Adiol) and d3-salbutamol (d3-Sal). D6-dehydroepiandrosterone (d6-DHEA) was from Sigma-Aldrich (Bornem, Belgium)

The internal standard mixture was prepared in methanol, containing 91.5 µg/mL d3-ααβ-Adiol and d5-βαβ-Adiol, 88.9 µg/mL d4-A-gluc, 48.7 µg/mL d3-DHT, 83 µg/mL d3-E-gluc, 52.8 µg/mL d5-Etio, 91.8 µg/mL IS, 100 µg/mL d3-Sal, 101 µg/mL d3-T-gluc and 51.8 µg/mL d6-DHEA. This stock solution was diluted (4:1) prior to usage.

2.3 Endogenous steroids

Testosterone, epitestosterone, androsterone, etiocholanolone, 11β-hydroxyandrosterone, 11β-etiocholanolone, 5α-androstane-3α,17β-diol, 5β-androstane-3α,17β-diol, 4-androstene-3,17-dione, 5β-androstane-3,17-dione, 5α-androstane-3,17-dione and pregnanediol were purchased from Sigma (Bornem, Belgium), 19-norandrosterone, 4-hydroxy-androst-4-ene-3,17-dione (4OH-Adion) and 3β,7β-dihydroxy-androst-5-en-17-one (7βOH-DHEA) from NMI (Pymble, Australia), dehydroepiandrosterone (DHEA) from Serva (Heidelberg, Germany) and dihydrotestosterone (DHT) from Piette International Laboratories (Drogenbos, Belgium). 6α-Hydroxy-androst-4-ene-3,17-dione (6αOH-Adion), 16α-Hydroxy-androst-4-ene-3,17-dione (16αOH-Adion) and 3β,16α-dihydroxy-androst-5-en-17-one (16αOH-DHEA) were purchased from Steraloids (Newport, RI, USA).

2.4 Exogenous steroids

16α-OH-furazabol, 16α-OH-stanozolol,17α-ethyl-5α-estrane-3α,17β-diol (norethandrolone metab.), 17α-methyl-5α-androstane-3α,17β-diol (α-methyltestosterone metab.), 17α-methyl-5β-androstane-3α,17β-diol (β-methyltestosterone metab.), 17α-trenbolone, 17β-hydroxy-17α-methyl-5α-androst-1-ene-3-one (methyl-1-testosterone), 1α-methyl-5α-androstan-3α-ol-17-one (mesterolone metab.), 1-methylene-5α-androstan-3α-ol-17-one (methenolone metab.), 1-testosterone, 2α-methyl-5α-androstan-3α-ol-17-one (drostanolone metab.), 4-chloro-4-androsten-3α-ol-17-one (clostebol metab.), 4-OH-testosterone, 5β-androst-1-en-17b-ol-3-one (boldenone metab.), 5α-Androst-1-ene-3β,17β-diol (1-androstenediol), 6β-hydroxy-dehydrochloromethyltestosterone (oral turinabol metab.), 6β-hydroxymethandienone, epimetendiol, 7α,17α-dimethyl-5β-androstane-3α,17β-diol (bolasterone metab.), 7β,17α-dimethyl-5β-androstane-3α,17β-diol (calusterone metab.), 9α-fluoro-17,17-dimethyl-18-nor-androstan-4,13-diene-11β-ol-3-one (fluoxy-18-nor), dehydrochloromethyltestosterone (oral turinabol), epioxandrolone, methyldienolone, 13β,17α-diethyl-5β-gonane-3α,17β-diol (norbolethone metab 2), 13β,17α-diethyl-5α-gonane-3α,17β-diol (norbolethone metab 1), 3'-hydroxystanozolol, 4-OH-nandrolone, 2-hydroxymethyl-17α-methylandrosta-1,4-dione-11α,17β-diol-3-one (formebolone metab) and 17α-ethyl-5α-estrane-3α,17β-diol (norethandrolone minor metab.) were purchased from NMI (Pymble, Australia). Oxandrolone was a gift from Searle & Co (Chicago, IL, USA), 1-androstene-3,17-dione, calusterone, mibolerone and norclostebol were from Steraloids (Newport, RI, USA). Bolasterone was a gift from Upjohn, danazol from Whintrop, oxymetholone from Parke Davis and mesterolone from Shering. 3α-hydroxytibolone (tibolone metab.) was a kind gift from Akzo Nobel (Oss, The Netherlands), fluoxymesterone was obtained from Ciba-Geigy, boldenone and oxymesterone were purchased from the Institut für Biochemie of the Deutsche Sporthochschule (Cologne, Germany). 3α,5α-tetrahydronorethisterone (norethisteron metab.), was a kind gift from the Institute of Organic Chemistry and Biochemistry (Academy of Sciences of the Czech Republic, Prague, Czech Republic), metenolone from the Drug Control Centre of King's College (London, UK), mestanolone from Doping Control Laboratory Barcelona, ethisteron from the Doping Control Laboratory ADOP (Lisbon, Portugal), 17β-hydroxy-6-methyleneandrosta-1,4-dien-3-one (exemestane metab.) from Helsinki University, 6β-hydroxyfluoxymesterone from the Laboratory for Doping Control Cologne and desoxymethyltestosterone (madol) from the Doping Control Laboratory Montreal. 2α,17α-dimethyl-5α-androsta-17β-ol-3-one (superdrol) was from TRC (North York, Canada).

2.5 Narcotics

11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid (THCA), normethadone, (±)-2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolinium perchlorate (EDDP), fentanyl, fenbutrazate, buprenorphine, morphine, codeine, ethylmorphine, 6-monoacetylmorphine (MAM), oxymorphone were purchased from Cerilliant. Pethidine, hydromorphone, oxycodone, heroine, dextromoramide and methadone were bought from Sigma (Bornem, Belgium). Pentazocine was a gift from Whintrop Laboratories (Newcastle, United Kingdom).

2.6 Stimulants

Pipradrol and pemoline were gifts from Merrell-Dow (Cincinnati, OH, USA) and Boehringer-Ingelheim (Brussels, Belgium), respectively. Cocaine and benzoylecgonine were purchased from Cerilliant. Fenethylline was a gift from Chemiwerk Hamburg (Germany) and methylphenidate from Ciba-Geigy (Groot-Bijgaarden, Belgium). Fencamine was obtained from Laboratoires Miquel S.A. (Barcelona, Spain), fenspiride from Laboratoires Servier (Orléans, France) and amineptine and amineptine C5 metabolite from Laboratoires Servier (Orleans, France). Strychnine and fencamfamine were donated by Merck. Carphedon, 6-OH-bromantane, crotethamide, cropropamide, cyclazodone and famprofazone were bought from NMI (Australia). Dimefline was from Recordate Industria Chemica & Farmaceutica (Milan, Italy) and furfenorex and clobenzorex from Roussel Uclaf (Romainville, France). Amiphenazole, pholedrine, niketamide, 1-benzylpiperazine and octopamine were purchased from Sigma (Bornem, Belgium), while ethamivan and benzphetamine were gifts from Sinclair Pharmaceuticals (Godalmings, UK), Tropon Werke (Cologne) and Upjohn (Kalamazoo, USA), respectively. 3,3-diphenylpropylamine and prenylamine were gifts from the World Association of Anti-Doping Scientists (WAADS). 3,4-Methylenedioxy-N-ethylamphetamine (MDEA) was purchased from Cerilliant (Round Rock, Texas). Selegiline was from Orion Pharma (Diegem, Belgium) and mefenorex from Roche (Mannheim, Germany).

2.7 Beta-blockers

Beta-blockers obtained as reference substances were: acebutolol from Rhone-Poulenc (Brussels, Belgium), alprenolol from Astra Chemicals (Holstein, Germany), propranolol from ICI (Korten- berg, Belgium), betaxolol from Synthelabo (Brussels, Belgium), labetolol from Glaxo (Brussels, Belgium), nadolol from Squibb (Braine l’Alleud, Belgium), oxprenolol from CIBA (Dilbeek, Belgium), pindolol from Sandoz (Vilvoorde, Belgium), timolol from MSD(Brussels, Belgium), bisoprolol from Merck (Overijse, Belgium), carvedilol from Roche (Mannheim, Germany). Levobunolol (l-bunolol), esmolol were a kind gift from the South African Doping Control Laboratory. following products were extracted from therapeutical prepa- rations: celiprolol (Selectol, Pharmacia, Brussels, Belgium) and metipranolol (Beta-Ophtiole, Tramedic, Sint-Niklaas, Belgium).

2.8 Beta-2-agonists

Salbutamol, terbutaline and clenbuterol were purchased from RIVM. Salmeterol xinafoate was a gift from GlaxoSmithKline (Philadelphia, PA, USA). Fenoterol was a gift from Boehringer&Sohn (Ingelheim am Rhein) and formoterol from Novartis (Arnhem, The Netherlands). Bambuterol was donated by the Instituto Nacional do Desporto (Lisbon, Portugal).

2.9 Hormone antagonists and modulators

Anastrazole, toremifene, exemestane, 17β-hydroxy-6-methylene-androsta-1,4-diene-3-one (exemestane metab.), were generous gifts from Astra Zeneca (Macclesfield, UK), WAADS, Pfizer (Groton, UK) and the Faculty of Pharmacy of the Helsinki University (Helsinki, Finland), respectively. 6α-OH-androstenedione, 4-OH-androstenedione, 4-hydroxycyclofenil (cyclofenyl metab.), 3-hydroxy-4-methoxytamoxifen and bis-(4-cyanophenyl)methanol (Letrozole metabolite) were purchased from NMI. 4-OH-tamoxifen and raloxifene was bought from Sigma–Aldrich.

2.10 Other substances

Zilpaterol and zeranol were purchased from NMI, probenecid from Federa (Brussels, Belgium) and 5-hydroxypentoxyfylline (5-OH-pentoxyfylline) from Hoechst (Frankfurt, Germany).

2.11 Urine samples

For the detection of prolintane, sibutramine and metabolites excretion urines were used. These urines were collected from healthy people after administration of 10 mg oral dose, in accordance with the Helsinki declaration on ethical principles [22].

For the stability tests concerning the microbiological degradation, urine samples from 6 healthy volunteers were collected. Each sample was divided in 3 containers and each of these was stored under different conditions: refrigerator (2-8°C), room temperature (21-24°C) and an incubator at 37°C. Every day during a period of 14 days, pH and specific gravity was measured and 3 aliquots were collected out of every container and stored at -80°C awaiting analysis.

2.12 Sample preparation

1 mL of urine was spiked with 25 µL of the internal standard mixture. For hydrolysis samples were incubated in an oven at 56°C for 1.5h after adding 1 mL of 0.1M phosphate buffer solution (pH = 7) and 50 µL of β-glucuronidase. After adding 2 mL $NaHCO_3/K_2CO_3$ solution (pH = 9.5) the mixture was extracted for 20 min with and 5 mL diethylether by rolling. The organic phase was centrifuged for 5 min at approximately 650g, dried with Na_2SO_4 and evaporated under OFN at room temperature.

20 µL of acetonitrile and 50 µL MSTFA was added to the dried residues, followed by derivatisation for 30 min at 80°C. Afterwards 50 µL of MSTFA/ethanethiol/NH_4I (500:4:2) was added for derivatisation at 80°C during another 30 min [9]. 1.3 µL of this mixture was injected in the GC.

2.13 Instrumentation

An Agilent (Agilent Technologies, Palo Alto, USA) GC 7890 gas chromatograph coupled with an Agilent 7000B triple quadrupole mass spectrometer (Agilent Technologies) and a MPS2 autosampler and PTV-injector from Gerstel (Mülheim and der Ruhr, Germany) were used.

The GC column – 12.5 m x 200 µm and 0.11 µm film thickness – was a HP-Ultra 1 from J&W Scientific (Agilent Technologies, USA). The temperature programme was as follows: the initial temperature was 80°C (0.2min), increased at 70°C/min to 183°C, then at 5.1°C/min to 220°C and at 50°C/min to a final temperature of 310°C (held for 1.8min). The transfer line was set at 310°C. Helium (Air Liquide) was used as carrier gas at a flow rate of 0.8 mL/min.

In splitless mode 1.3 µL was injected using following PTV-temperature settings: 25°C (0.20 min), 12°C/min → 310°C.

In the QqQ collision cell He was used as a quench gas at 2.25 mL/min and N_2 as collision gas at 1.5 mL/min.

2.14 Method Validation

2.14.1 Quantitative

To construct calibration curves, steroid stripped urine samples were spiked at 6 concentration levels. The least squares method was used for fitting the calibration curves.

At every level accuracy and precision were tested over 3 replicates. The tolerances for precision were calculated following the Horwitz equation ($RSD_{max}= 2^{(1-0,5\log C)}$). The accuracy, expressed as bias, may not exceed 15%.

2.14.2 Qualitative

The limit of detection (LOD) was determined by analyzing 10 different, randomly chosen urine samples spiked with the analyte at a range of concentration levels. These levels were chosen according the WADA MRPL's: i.e. 0.05, 0.1, 0.2, 0.5, 1 and 2 times the MRPL level [18]. The LOD was set at the lowest level where the analyte could be detected and complied with WADA's criteria for identification [23] in all samples.

For every compound precursor ions were selected with mass resolution of the first quadrupole set at unity. The collision energy (CE) was optimized, generating characteristic product ions. Selectivity and specificity of these transitions were tested by the analysis of ten blank urine samples. At least two precursor-product ion transitions are monitored and the relative abundance of these transitions are determined from the peak area of the integrated selected reaction monitoring (SRM) chromatograms. To ensure correct identification, maximum tolerance windows for these relative ion intensities (RI) are set by WADA (Table 4.1).

Table 4.1: Maximum tolerance windows for relative ion intensities to ensure appropriate confidence in identification [23].

Relative abundance (% of base peak)	GC-MS/MS
>50%	10% (absolute)
25% to 50%	20% (relative)
5% to <25%	5% (absolute)
<5%	50% (relative)

3 Results and discussion

3.1 Method development

The B-version of the Agilent Triple Quadrupole GC-MS/MS used in this study is equipped with a new EI-source. This source has an additional extractor lens, which improves sensitivity across the entire mass range resulting in lower limits of detection in comparison to a previous study [24]. The results for several examples from different classes are shown in Table 4.2.

Table 4.2: LOD values of some components to illustrate the improving sensitivity when using the extractor lens (source B).

	Source A	Source B
Compound	LOD (ng/mL)	LOD (ng/mL)
Pethidine	4	2
Bolasterone	10	5
Zeranol	10	5
Fenoterol	100	50
Raloxifene	25	5
Oxycodone	200	50
Esmolol	100	25

In this method helium is used as a carrier gas. This increases the run time compared to hydrogen as a carrier gas [24], but has also some considerable advantages. The extended temperature program allows for separation of some more volatile compounds. In that way some stimulants could be added to the method. Further the system showed to be more robust with the He configuration compared to the one on H_2. Using the He configuration it is sufficient to clean the source every 1000 injections, while on the H_2 configuration this maintenance needed to be performed every 250 injections. So less maintenance was needed for He, resulting in less downtime.

When changing the carrier gas, transitions and corresponding collision energies of all compounds were re-evaluated. After performing a full scan analysis the best precursor ions - with highest masses and abundances - were selected for every component. Next a product ion scan was performed on the selected precursor ions to select the most abundant product ions. The resulting transitions were optimized using different collision energies (5, 10, 15, 20, 25, 30, 35). Signal to noise was used as a criterion for the selection. No differences in fragmentation were observed. However, small differences in relative abundances and signal intensities were noticed.

In some cases for this He method another transition was found to give highest response compared to the H_2 method, but in general the largest difference was found in collision energies (Fig. 4.2). The explanation of this can be found at the level of the collision cell. When using low energy collision (1 to 100 eV), the collision induced dissociation (CID) may occur through vibrational excitation by momentum transfer [25]. This momentum transfer is calculated from the momenta of both colliding particles, given by the product of the mass (m) and velocity (v). This makes the choice of the gasses important. Hence the difference in mass for He and H_2 could explain for the difference in CID efficiency for a given gas velocity.

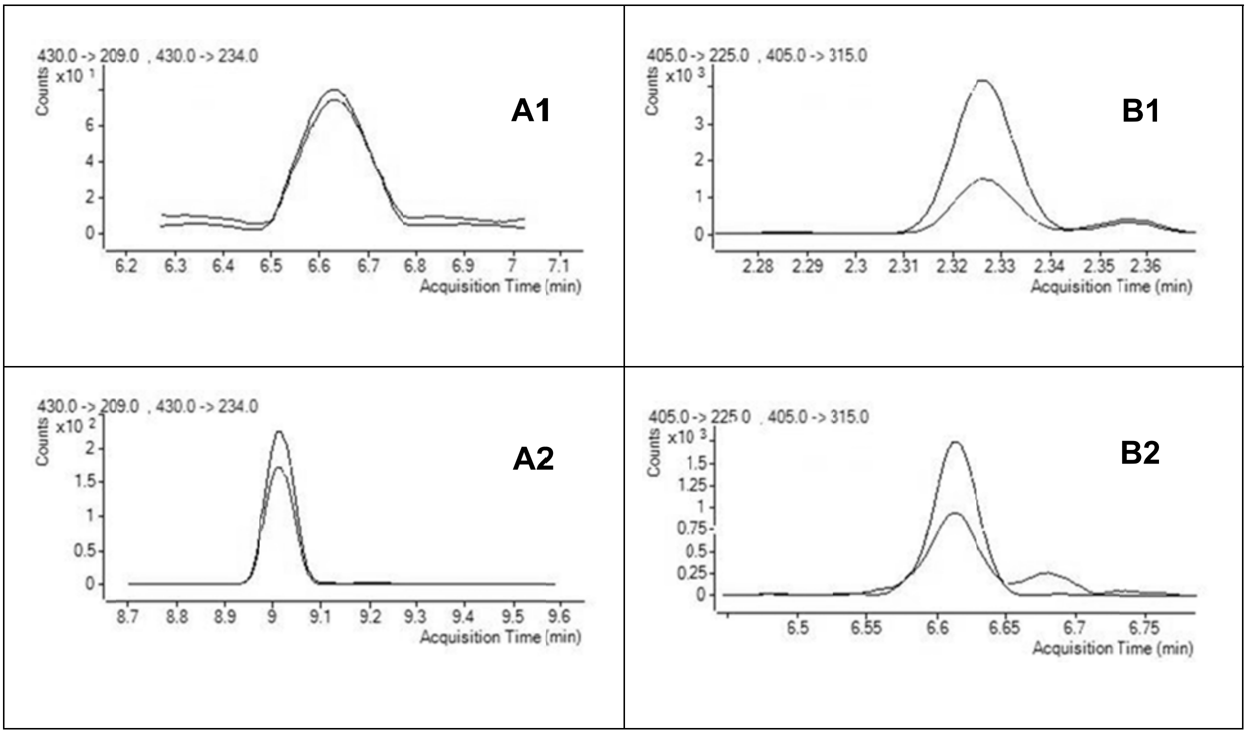


Fig. 4.2 Comparison between transitions for Adion (A1) and norandrosterone (B1) in the H_2-method and Adion (A2) and norandrosterone (B2) in the He-method.

3.2 Method Validation

3.2.1 Quality controls

In previous screening methods for doping substances [1,6,11], 17α-methyltestosteron was commonly used as internal standard. In this method additional deuterated internal standards to correct better for losses during sample preparation steps were added. Since, in general internal standards are dissolved in methanol and it is known that the activity of enzymes like β-glucuronidase is reduced in organic solvents [26], it is important to minimize the volume of solvent added. When using only 1 mL of urine, adding similar amounts of methanolic solutions as in previously described methods [6,24], leading to inhibition of hydrolysis could become problematic. Therefore, all internal standards solutions were prepared at relatively high concentrations, in order to limit the spiked volume to 25 µL.

The use of both free and glucuronidated steroids as internal standards makes evaluation of the hydrolysis efficiency possible. Androsterone-d4 is added in every sample as glucuronide conjugate at 486.87 ng in 1 mL of urine (concentration in androsteron-d4 equivalents). The degree of deconjugation after the 1.5h hydrolysis step with β-glucuronidase allows estimation of the efficiency for each sample individually. To correct for variations during sample preparation the area of transition 423→333 (CE 20) for free androsteron-d4 is divided by the area of transition 424→334 (CE 20) for etiocholanolone-d5. This compound is added free (non-conjugated) at 459 ng/mL. Experiments with both substances spiked as free standards in equivalent proportions have shown that the mean ratio of androsterone-d4 over etiocholanolone-d5 at a theoretical 100% hydrolysis efficiency is 0.85 under the given conditions. Analysis of a large number of samples (n > 200) showed that minor hydrolysis efficiency causes problems in quantification of compounds from an d4-A/d5-Etio ratio below 0.7, i.e. at 80% hydrolysis efficiency.

The addition of multiple deuterated internal standards (d3-E, d3-T, d5-5βαβ-Adiol, d3-5ααβ-Adiol, d3-DHT, d4-A, d5-Etio and d6-DHEA), more similar to the target compounds, improves correction for variations caused by sample preparation and extraction and also for variations in performance of the used analytical instruments. Although this phenomenon is known, most methods do not take this into account [8,27,28].

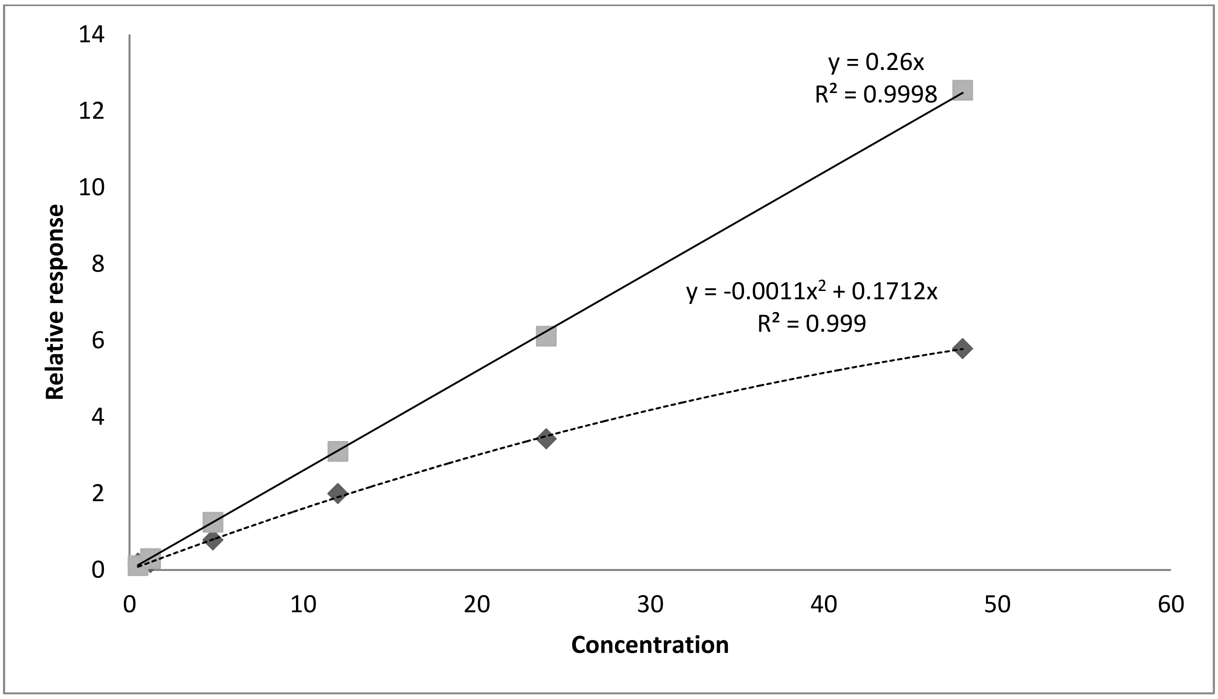


Fig. 4.3 Calibration curves for androsterone using as internal standard 100 ng/mL A-d4 (dotted line) and 500 ng/mL (solid line).

However, when using deuterated analogues, problems can arise from the natural abundances of the heavier isotopes in the target analyte. First of all the degree of isotopic substitution in the internal standard should be sufficiently large, making the probability of observing the same mass in the analyte as low as possible. Fulfilling this condition becomes hard for bigger molecules since for carbon atoms the relative abundances (RA) for ^{13}C are 1.11%. In this research all target molecules are silylated before analysis. Next to increasing the amount of carbon atoms, the TMS-groups introduce a Si atom with 3 different stable isotopes: ^{28}Si, ^{29}Si (RA 4.67%) and ^{30}Si (RA 3.10%). The carbon and silicon atoms together are responsible for the higher abundances of heavier isotopes. The higher the concentration of the silylated analyte, the bigger the contribution of the isotope with the same mass as the internal standard. This is the case for the isomers androsterone and etiocholanolone, which can be present in relatively large concentrations. For the highest calibration level of 4800 ng/mL the natural abundance of mass 438 in the bis silylated androsterone corresponds already to 12.36 ng/mL (Table 4.3). When spiking the internal standard A-d4 at 100 ng/mL this additional presence of mass 438 causes a 12% overestimation of the internal standard. This effect can be reduced by using a higher concentration for the deuterated analogue, spiking at 500 ng/mL lowers the error to 2.4%. Fig. 4.3 shows a deflection in the high concentration range of the calibration curve for androsterone when using internal standard A-d4 at 100 ng/mL (dotted line). When spiking 500 ng/mL A-d4 for the same concentrations of androsterone this deflection does not occur (solid line), additionally the slope of the curve increases, making the quantification more sensitive.

Table 4.3: Contribution of natural abundant stable isotopes of analytes

	Compound	Concentration (ng/mL)					
	Androsterone-bisTMS	48	120	600	1200	2400	4800
	absolute abundance mass 438	0.124	0.309	1.545	3.091	6.182	12.363
IS - Low conc	Androsterone-d4 -bisTMS	100	100	100	100	100	100
	Total abundance mass 438	100.124	100.309	101.545	103.091	106.182	112.363
	% contribution mass 438	0.123	0.308	1.522	2.998	5.822	11.003
IS - High conc	Androsterone-d4 -bisTMS	500	500	500	500	500	500
	Total abundance mass 438	500.124	500.309	501.545	503.091	506.182	512.363
	% contribution mass 438	0.025	0.062	0.308	0.614	1.221	2.413

Also transitions for mono-TMS derivatised androsterone and etiocholanolone were included to monitor derivatisation efficiency. Mono-TMS derivatised androsterone showed to be more sensitive than the etiocholanolone equivalent and is in that way a better parameter for derivatisation efficiency. The cut-off percentage for efficient derivatisation was set at 10% mono-TMS.

3.2.2 Microbiological degradation

For the stability tests, pH and density were measured every day during 14 days. For the density there were no changes observed. There were also no changes in urinary pH measured for all samples stored in the refrigerator and at room temperature.

For samples, stored in the incubator at 37°C, the urinary pH didn't change for urine 1 and 2 over the 14 days period of time. However, for urine 3 to 6, pH values increased after 4 days of incubation and all reached a steady state at pH 8.9 and 9.2. (Fig. 4.4) after 3-6 days. These changes in urinary pH are linked to urease activity of different micro-organisms [19] and provided evidence that the growth of these organisms is affected by the urine composition.

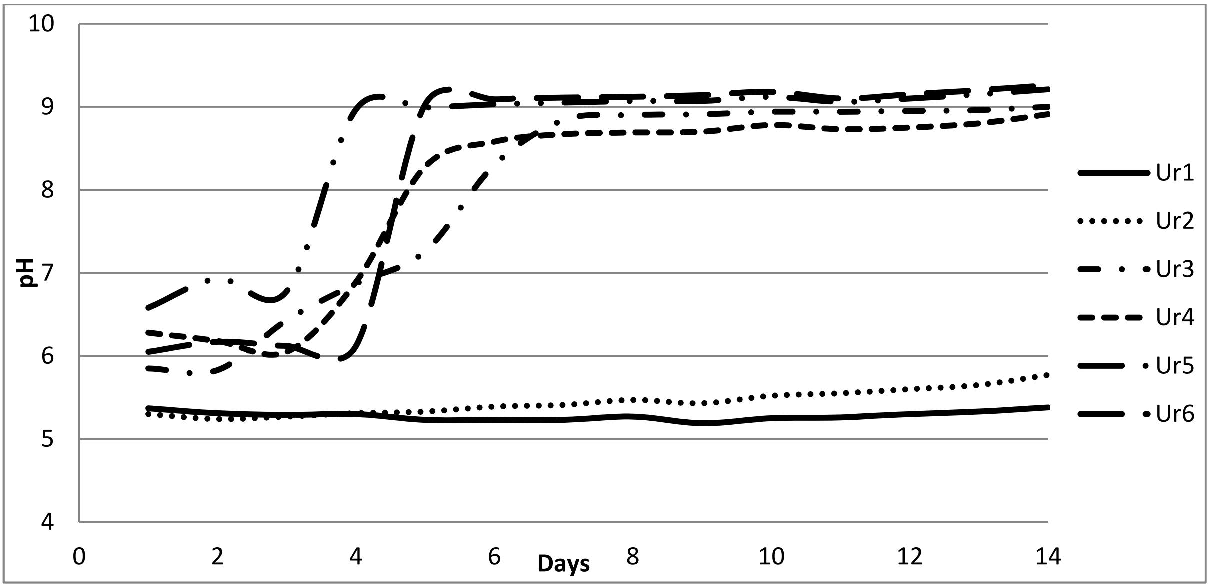


Fig. 4.4 Urinary pH changes of 6 different urines in function of the number of days incubated at 37°C.

Production of 5α-dion and 5β-dion out of androsterone and etiocholanolone, respectively, has also been identified as a marker of microbiological degradation. Therefore, these steroids were also quantified.

For aliquots stored in the refrigerator no observations indicating degradation were detected, except for urine 4. In this sample, concentrations of 5α-dion and 5β-dion were around 10 ng/mL at the beginning of the experiment. After 11 days these concentrations had increased to 25 ng/mL. However, no significant changes in the steroid profile (T, E, A, Etio) were found. Since absolute concentrations of steroids are affected by urinary flow, differences in production, etc, the percentage of 5α-dion to androsterone (%5α) and the percentage of 5β-dion to etiocholanolone (%5β) were also calculated to evaluate the relative importance of the degradation. In urine 4, these percentages were very small, i.e. 0.5 %5α and 2.2 %5β. This illustrates that the use of absolute concentrations of these steroids might not be the best indicator to monitor alterations of the steroid profile due to microbiological degradation. Hence, it can be concluded that although increases in 5α-dion and 5β-dion, indicating microbiological activity, can be noticed, samples can be stored in the refrigerator for 2 weeks without a significant impact on the steroid profile. pH values for all urines were constant during the duration of the experiment. These results also further supports previous research showing that storage in fridges as required by WADA's ISL [29] is a sufficient measure to adequately prevent degradation.

Analysis of urine 1, stored at room temperature, showed no formation of 5α/5β-dion and did not result in changes in the steroid profile. In urine 2, no changes neither in steroid profile nor in 5α/β-dion concentrations were noticed in the first week . However, after ten days significant increases in 5α-dion and 5β-dion (up to 85 ng/mL and 630 ng/mL or 2% and 15% of androsterone and etiocholanolone concentrations, respectively) were noticed. Concurrently, the T/E ratio increased from 0.05 to 0.1. For urine 3, changes in 5α-dion and 5β-dion concentrations were noticed from day 2 onwards (starting at 30 ng/mL and 20 ng/mL for 5α-dion and 5β-dion, respectively), but only after 14 days a change in steroid profile was noticed (T/E ratio decreases from 0.8 to 0.45). The %5α and %5β had increased by then to 5 and 15% respectively. Storage of urine 5 at room temperature resulted in significant formation of 5α-dion and 5β-dion. Indeed, already at day 4 these concentrations were 65 ng/mL and 500 ng/mL for 5α-dion and 5β-dion respectively. These values represent 1.5% and 15% of androsterone and etiocholanolone. Calculated percentages increase on day 7 where 14 %5α and 20 %5β were found. Very similar results were obtained for urine 4 and 6.

In general, when storing samples at room temperature, microbial degradation was observed and could be detected by monitoring 5α-dion and 5β-dion concentrations. pH values again were constant in function of time, making this an unreliable parameter for microbiological degradation. Since endogenous production and urinary flow can result in significant differences in basal endogenous steroid concentrations, the use of absolute threshold concentrations of 5α-dion and 5β-dion to establish significance of the microbial degradation on the steroid profile does not seem to be a good parameter. Instead, the use of a % of 5α-dion to androsterone and 5β-dion to etiocholanolone seems to be a better indicator. Based upon these preliminary experiments a threshold of 1% for 5α-dion and 5% of 5β-dion was established.

Storing samples in the incubator at 37°C accelerated these degradation processes. Concentrations around 30 ng/mL of 5α-dion and 200 ng/mL of 5β-dion were already measured after a few days: from 1 day for urine 5, 2 days for urine 2, 4 and 6 to 4 days for urine 3. Only urine 1 remained largely unaffected from microbiological degradation and only showed increasing 5α/5β-dion concentrations after 12 days. In contrast to the pH, which was constant, 5α-dion and 5β-dion were formed in urine 1 and 2. This indicates again that there is not always a correlation between pH elevation and formation of degradation products. Large changes in the concentrations of endogenous steroids were observed during the test period. In urine 4, concentrations of androsterone decreased from 2100 ng/mL at the beginning to 10 ng/mL after 6 days. For etiocholanolone, the same trend was found: from 1000 ng/mL at day 1 to 15 ng/mL after 7 days. In urine 6, the impact of degradation was best reflected in the testosterone concentration which decreased from 55 ng/mL at the start of the experiment, over 20 ng/mL after 3 days to 5 ng/mL after 10 days. Because epitestosterone concentrations remained constant, the T/E ratio decreased from 2.3 to 0.6 after 3 days. Evaluation of all samples stored at 37°C also confirmed the proposed threshold values of 1% for 5α-dion and 5% of 5β-dion.

In all samples it was found that Adion concentrations followed the same trend as 5α/5β-dion concentrations. From constant levels for samples stored in the refrigerator to a tenfold increase in concentrations for samples stored at room temperature and at 37°C. However, Adion concentrations cannot be used as a parameter for microbiological degradation because of natural fluctuations.

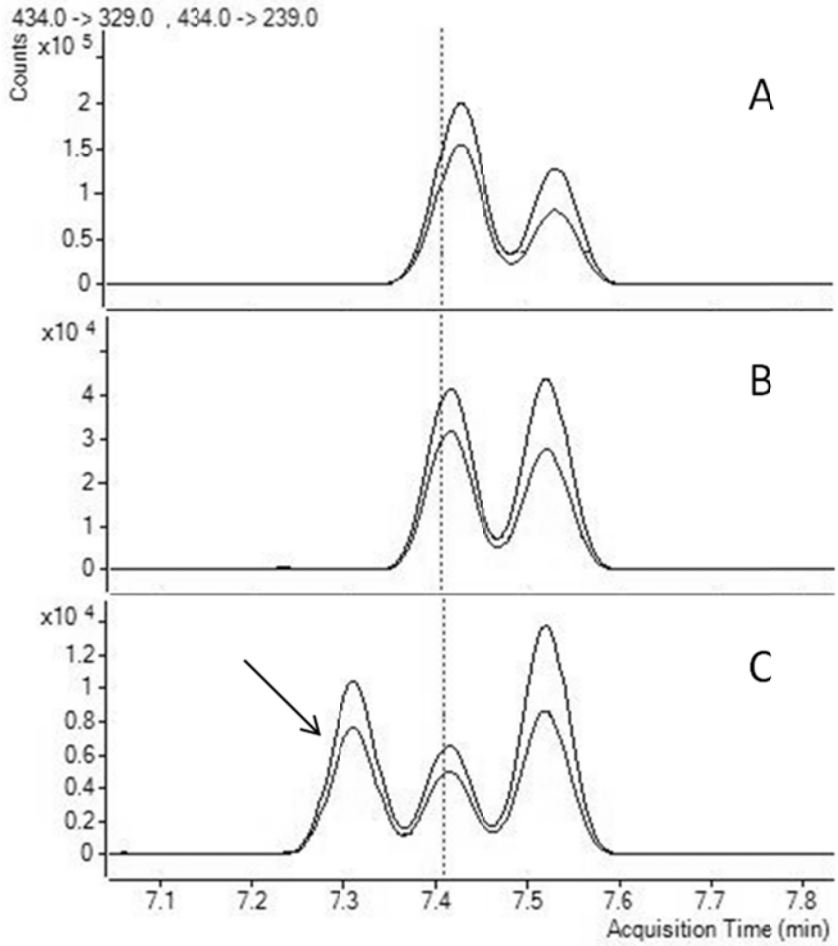


Fig. 4.5 EIC of androsterone (7.4min) and etiocholanolone (7.5min) of urine 5, stored for 5 days in the refrigerator (A), at roomtemperature (B) and in the incubator at 37°C (C).

It should also be noted that - next to the degradation of the endogenous steroids - measuring the steroid profile can be hindered due to interferences with degradation products. An example for androsterone is given in Fig. 4.5. When comparing storage of the same urine for 5 days in the refrigerator (5A) and at room temperature (5B) only a small difference in steroid concentrations can be found. However, when stored at 37°C, an interfering compound 0.1min before androsterone hinders proper integration. Investigation of the mass spectrum (Fig. 4.6) suggests that the unidentified substance is an isomer of androsterone, but this substance is neither etiocholanolone nor epiandrosterone. Further research is required to identify this substance.

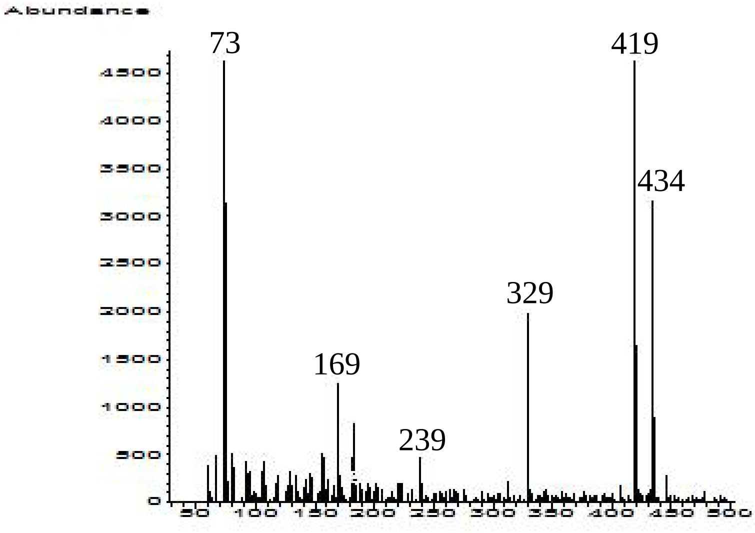


Fig. 4.6 Full scan mass spectrum of the interfering isomer of androsterone.

3.2.3 Quantitative

All the endogenous steroids traditionally included in the steroid profile used for doping control purposes (T, E, A, Etio, DHT, DHEA, Adion, 5ααβ-Adiol and 5βαβ-Adiol) are included in the quantitative part of the method. However, to improve the detection of administration of endogenous steroids, several minor metabolites were added to the method; namely 4OH-Adion, 6αOH-Adion, 16αOH-Adion, 7βOH-DHEA and 16αOH-DHEA. The endogenous concentrations of these metabolites are lower than those of the steroids monitored in the traditional steroid profile. Hence, a small increase in concentration after administration of a natural steroid will be more easily detected compared to elevations in the concentration of the traditionally monitored steroids which can occur in a wide concentration range. In previous research, upper reference limits based on population statistics were set for each of these minor and major metabolites [30].

Additionally, several other steroids are also quantified to obtain more information. These include 11β-OH-androsteron and 11β-OH-etiocholanolone which are not affected by intake of anabolic agents and act as indicators of elevated endogenous steroid production and/or urinary flow. 5α-androstande-3,17-dione and 5β-androstande-3,17-dione were added to monitor microbiological activity in urine during the pre-analytical phase. Finally salbutamol, an important beta-2-adrenergic agonist, the major metabolite of cannabis (11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid) and norandrosterone were also included.

The method of least squares has been used to construct the calibration curves over 6 levels for every quantitative component. All correlation coefficients (R^2) were above 98%. The precision was calculated and evaluated using the RSDmax values obtained via the Horwitz equation. All obtained relative standard deviations (RSD) were lower than the calculated RSD_{max}. The accuracy, expressed as bias, was below 15% for every calibration level.

3.2.4 Qualitative

Selectivity of the method was tested by analyzing a blank aliquot of the different urines, in order to check for possible naturally occurring interfering substances. These results showed that there are no interferences of the urinary matrix with the target compounds.

For every compound screened qualitatively limits of detection (LOD) were traditionally determined as the lowest level at which the compound could be detected concerning the right retention times and signal to noise ratio (S/N > 3). When using tandem mass spectrometry (MS^n), other criteria for detection and identification of the analyte are specified. This is done by determination of maximum tolerance windows for the relative ion intensities. The most abundant transition is taken as reference on which the second (and sometimes third) transition is scaled. Traditionally, the allowed tolerance windows for the relative abundances are stipulated in technical documents by governing bodies in the specified field. In this case the tolerance levels were calculated according to the requirements set by WADA in the applicable technical document (Table 4.1).

Although ILAC-G17-2002 specifies that for the time being the application of the concept of measurement uncertainty is not a requirement [31], recently criticism has been uttered which targeted the use of tolerance windows for relative abundance of the mass to charge ratios (*m/z*) which were not based upon measurement uncertainty [32]. Indeed, according to the criticism the regulations set by many fields, but in particular the sports drug testing authorities, were ad-hoc. Therefore, in this paper a second set of the tolerance levels was calculated using the general principles of measurement uncertainty (MU). In general random measurement errors cause statistical variations on the outcome of analytical measurements. The possible deviation between the measured value and the true value is expressed in measurement uncertainty (MU). This can be expressed as standard deviation or as an interval containing the true value with a given certainty. The range of these confidence intervals are calculated using a coverage factor (k). The value of this factor depends on the desired confidence level: k = 2 for 95% confidence level and k = 3 for a 99.7% confidence level. After the calculation of the standard deviation, this value will be multiplied by the coverage factor to determine the margins of the confidence levels. Both ways of calculating are used to ensure correct identification and determine the LOD of all qualitative compounds in this method.

In general MU is composed of a bias factor and a precision factor. In this study, only the influence of precision on the uncertainty of the intensities of mass spectrometric signals was evaluated as a first approach to measurement uncertainty in mass spectrometry. Hence, when all uncertainty factors would be taken into account, it can be expected that the tolerance windows would become larger. In this case the intervals are only set by the standard deviation of the relative ion intensities over the ten urines multiplied by a coverage factor of 3 (99.7% confidence level is chosen). So the maximum tolerance windows for the statistical point of view are given by [x-3sd, x+3sd], where x is the mean value and sd the standard deviation.

In this research both perspectives, explained above, are evaluated as criteria for measurement uncertainty in mass spectrometry.

When following the WADA criteria there are 4 categories in relative ion intensities (Table 4.1). In the lowest category, relative intensities (RI) do not exceed 5%. In this study, several transitions fall into this category, e.g. for oxprenolol, where the ratio between the transitions 221→72 (CE 20) and 221→56 (CE 10) is 4% (Table 4.4). It could be observed that for most of these cases the tolerance levels are slightly tighter when calculated with the principles of MU i.e. in the case of oxprenolol [3.11, 4.89], compared to the [2, 6] for the WADA criteria. However it is clear that these differences are only minor. A similar conclusion can be made for the second category (5% < RI < 25%), e.g. benzphetamine (RI 20%) or ethamivan (RI 8%) for given transitions and collision energies. In the third category (25% < RI < 50%) e.g. EDDP (RI 46.4%) and mesterolone metabolite (RI 30.5%), in most cases the tolerance levels when using the WADA criteria are more strict, but the differences are almost in general neglectable, e.g. bisoprolol. Compounds like amiloride (RI 89%) and cyclazodone (RI 92.5%) are situated in the last category (RI > 50%) and show that for one compound the WADA rules are slightly stricter while for another the MU tolerance levels are smaller, again no big differences between both were found.

In the past some critics concerning these tolerance windows was published, to point out that MU should be used instead of the levels fixed by WADA [32]. This research demonstrates that both perspectives are very similar and that practice shows that the tolerance levels imposed by WADA are therefore not arbitrary.

4 Conclusions

An improved screening method for detecting over 150 compounds from different classes in a qualitative way and the complete steroid profile expanded with minor metabolites in a quantitative way was developed. During validation, both WADA criteria and general principles of measurement uncertainty were used for correct identification of compounds in tandem mass spectrometry. It was found that both approaches are very similar.

The addition of both free and glucuronidated (deuterated) internal standards allows for quality control in every individual sample i.e. hydrolysis efficiency and derivatisation efficiency. Special attention was paid to microbiological degradation and the impact on the steroid profile. It was found that samples can be stored in the refrigerator for 2 weeks without impact on the steroid profile. Instead of 5α/5β-dion concentrations as absolute threshold values, relative threshold values of 1 %5α and 5 %5β were proposed, better reflecting the real impact on the concentrations of endogenous steroids in the steroid profile.

Acknowledgments

This project was carried out with the financial support of the World Anti-Doping Agency (WADA).

Table 4.4: Substance ordered by increasing retention times, all relative to 17α-Methyltestosterone (ISTD, RT=9.59min).

Compound	RRT (min)	Quantifier	Collision energy (eV)	LOD (ng/mL)	MRPL (ng/mL)	Relative Abundance (%)	Stdev	MU Un_L	MU UP_L	Wada Criterium	WADA UN_L	WADA UP_L
Methylamphetamine	0.163	206 -> 59	15	25	50							
		206 -> 88	15			3.8	1.41	-0.43	8.03	1.9	1.9	5.7
Selegeline	0.168	96 -> 56	15	25	50							
		96 -> 70	15			17.5	1.00	14.51	20.49	5	12.5	22.5
		96 -> 80	15			20.9	6.69	0.84	40.96	5	15.9	25.9
Phendimetrazine	0.174	85 -> 42	15	10	50							
		85 -> 70	15			23.7	1.61	18.87	28.53	5	18.7	28.7
		85 -> 56	15			20.6	4.92	5.83	35.37	5	15.6	25.6
Mefenorex	0.196	120 -> 44	15	25	50							
		120 -> 41	15			18.8	2.90	10.11	27.49	5	13.8	23.8
Prolintane	0.208	126 -> 97	15	5								
		126 -> 84	15			77	7.47	54.59	99.41	10	67	87
		126 -> 42	15			47.2	5.62	30.35	64.05	9.44	37.76	56.64
Furfenorex	0.216	138 -> 81	5	10	50							
		138 -> 53	30			36.6	3.11	27.27	45.93	7.32	29.28	43.92
Fencamfamine	0.217	215 -> 186	10	25	50							
		215 -> 198	20			22	1.06	18.83	25.17	5	17	27
Crothetamide	0.219	154 -> 86	10	25	50							
		154 -> 69	20			34.1	1.50	29.60	38.60	6.82	27.28	40.92

Compound	RRT (min)	Quantifier	Collision energy (eV)	LOD (ng/mL)	MRPL (ng/mL)	Relative Abundance (%)	Stdev	MU Un_L	MU UP_L	Wada Criterium	WADA UN_L	WADA UP_L
1-Benzylpiperazine	0.219	248 -> 102	15	10	50							
		248 -> 116	10			24.3	1.51	19.78	28.82	5	19.3	29.3
		248 -> 130	10			24.7	3.14	15.29	34.11	5	19.7	29.7
Cropropamide	0.225	168 -> 100	10	2.5	50							
		168 -> 69	15			24	0.19	23.42	24.58	5	19	29
Pholedrine	0.229	294 -> 263	15	25	50							
		294 -> 189	15			42.9	6.65	22.95	62.85	8.58	34.32	51.48
Pethidine	0.234	247 -> 71	10	20	200							
		247 -> 173	10			11.4	0.73	9.21	13.59	5	6.4	16.4
Lidocaine	0.242	220 -> 204	15	40	200							
		220 -> 245	15			44.1	2.43	36.82	51.38	8.82	35.28	52.92
MDEA	0.249	264 -> 204	15	25	50							
		264 -> 102	15			41.4	4.39	28.23	54.57	8.28	33.12	49.68
		264 -> 219	15			55.1	7.11	33.78	76.42	10	45.1	65.1
Benzphetamine	0.26	148 -> 91	20	2.5	50							
		148 -> 65	35			20.1	0.68	18.05	22.15	5	15.1	25.1
Alprenolol	0.277	321 -> 72	15	50	500							
		306 -> 203	15			68	3.81	56.58	79.42	10	58	78
Methylphenidate	0.279	156 -> 45	10	25	50							
		156 -> 84	10			72.3	5.93	54.51	90.09	10	62.3	82.3
oxprenolol	0.306	221 -> 72	20	50	500							
		221 -> 56	10			4	0.30	3.11	4.89	2	2	6

Compound	**RRT (min)**	**Quantifier**	**Collision energy (eV)**	**LOD (ng/mL)**	**MRPL (ng/mL)**	**Relative Abundance (%)**	**Stdev**	**MU Un_L**	**MU UP_L**	**Wada Criterium**	**WADA UN_L**	**WADA UP_L**
Clobenzorex	0.306	168 -> 125	15	5	50							
		168 -> 89	35			15.5	2.57	7.78	23.22	5	10.5	20.5
Ethamivan	0.315	295 -> 223	20	2.5	50							
		295 -> 265	20			8	0.33	7.02	8.98	5	3	13
Terbutaline	0.318	356 -> 267	25	10	100							
		356 -> 355	25			70.3	3.37	60.18	80.42	10	60.3	80.3
Pemoline	0.331	392 -> 178	15	2.5	50							
		392 -> 163	15			25.1	2.33	18.10	32.10	5.02	20.08	30.12
		392 -> 104	15			15.4	1.63	10.50	20.30	5	10.4	20.4
EDDP	0.344	277 -> 105	30	100	200							
		277 -> 220	25			46.4	4.50	32.90	59.90	9.28	37.12	55.68
Carphedon	0.351	272 -> 104	15	2.5	50							
		272 -> 198	15			25.7	0.69	23.62	27.78	5.14	20.56	30.84
Octopamine	0.353	174 -> 86	15	2.5	50							
		174 -> 100	10			55.4	6.09	37.12	73.68	10	45.4	65.4
Metroprolol	0.373	324 -> 207	15	250	500							
		324 -> 221	15			21.5	3.05	12.34	30.66	5	16.5	26.5
Normethadon deriv. 1	0.38	224 -> 103	35	40	200							
		224 -> 191	35			44.5	5.16	29.01	59.99	8.9	35.6	53.4

Compound	**RRT (min)**	**Quantifier**	**Collision energy (eV)**	**LOD (ng/mL)**	**MRPL (ng/mL)**	**Relative Abundance (%)**	**Stdev**	**MU Un_L**	**MU UP_L**	**Wada Criterium**	**WADA UN_L**	**WADA UP_L**
Clenbuterol	0.394	335 -> 227	10	0.2	2							
		337 -> 229	15			68.7	3.78	57.36	80.04	10	58.7	78.7
		335 -> 300	10			35.6	1.92	29.83	41.37	7.12	28.48	42.72
		335 -> 262	15			46.4	4.50	32.90	59.90	9.28	37.12	55.68
Pipradrol	0.407	239 -> 161	15	25	50							
		239 -> 221	15			52.1	6.62	32.25	71.95	10	42.1	62.1
Cyclazodone	0.411	360 -> 178	15	10	50							
		360 -> 247	15			92.5	1.30	88.62	96.39	10	82.5	102.5
Bupivacaine	0.412	140 -> 84	15	10	50							
		140 -> 98	15			49.3	3.30	39.39	59.21	9.86	39.44	59.16
Penbutolol	0.421	348 -> 70	15	10	200							
		348 -> 201	15			57.3	1.03	54.21	60.39	10	47.3	67.3
		348 -> 186	15			29	1.13	25.61	32.39	5.8	23.2	34.8
3,3-diphenylpropylamine	0.423	174 -> 86	10	10	50							
		174 -> 100	10			40.1	0.88	37.45	42.75	8.02	32.08	48.12
Amiphenazole	0.426	407 -> 187	10	25	50							
		407 -> 221	15			21.7	2.86	13.11	30.29	5	16.7	26.7
		407 -> 187	10			75.7	6.43	56.40	95.00	10	65.7	85.7
Methadone	0.428	296 -> 191	10	40	200							
		296 -> 281	10			69.4	2.43	62.11	76.69	10	59.4	79.4
Propanolol	0.432	316 -> 231	10	50	500							
		316 -> 208	15			30.5	1.86	24.92	36.08	6.1	24.4	36.6

Compound	RRT (min)	Quantifier	Collision energy (eV)	LOD (ng/mL)	MRPL (ng/mL)	Relative Abundance (%)	Stdev	MU Un_L	MU UP_L	Wada Criterium	WADA UN_L	WADA UP_L
cocaine	0.444	182 -> 82	10	5	50							
		182 -> 122	10			17.8	0.46	16.41	19.19	5	12.8	22.8
		182 -> 108	10			15.9	0.64	13.99	17.81	5	10.9	20.9
Normethadone deriv. 2	0.454	296 -> 191	20	10	200							
		296 -> 252	20			30.4	0.36	29.31	31.49	6.08	24.32	36.48
Bisoprolol	0.489	405 -> 56	25	100	500							
		405 -> 172	15			30.2	1.90	24.49	35.91	6.04	24.16	36.24
Amiloride	0.491	388 -> 147	10	125	250							
		388 -> 298	10			89	7.57	66.31	111.70	10	79	99
Metipranolol	0.502	366 -> 281	10	100	500							
		366 -> 239	15			74.1	2.22	67.43	80.77	10	64.1	84.1
Anastrazole	0.505	209 -> 141	30	10	50							
		209 -> 194	15			76.1	3.65	65.14	87.06	10	66.1	86.1
		209 -> 115	30			44	1.19	40.44	47.56	8.8	35.2	52.8
Benzoylecgonine	0.506	240 -> 82	20	25	50							
		361 -> 82	20			22.3	3.55	11.66	32.94	5	17.3	27.3
Bupivacaine deriv. 2	0.512	140 -> 84	15	10	50							
		140 -> 98	15			38.7	5.81	21.29	56.12	7.74	30.96	46.44
		140 -> 41	15			23.7	1.61	18.87	28.53	5	18.7	28.7
Pentazocine	0.515	289 -> 45	10	10	200							
		289 -> 110	15			22.4	0.22	21.73	23.07	5	17.4	27.4

Compound	RRT (min)	Quantifier	Collision energy (eV)	LOD (ng/mL)	MRPL (ng/mL)	Relative Abundance (%)	Stdev	MU Un_L	MU UP_L	Wada Criterium	WADA UN_L	WADA UP_L
Letrozole metab.	0.521	291 -> 160	20	2.5	50							
		291 -> 217	20			51.3	3.08	42.07	60.53	10	41.3	61.3
Timolol	0.525	373 -> 186	10	25	500							
		373 -> 70	35			65.6	1.97	59.70	71.50	10	55.6	75.6
Probenecid	0.527	328 -> 103	25	12.5	250							
		328 -> 193	15			47.3	1.80	41.91	52.69	9.46	37.84	56.76
Zilpaterol	0.567	308 -> 218	15	5	10							
		308 -> 235	15			24.6	2.44	17.29	31.91	5	19.6	29.6
		308 -> 203	25			4.5	1.17	1.00	8.00	2.25	2.25	6.75
Celiprolol	0.581	200 -> 128	15	100	500							
		200 -> 144	15			20.7	1.97	14.80	26.60	5	15.7	25.7
Aminogluthetimide deriv. 1	0.608	361 -> 206	30	5	50							
		361 -> 221	10			75	3.38	64.88	85.13	10	65	85
Betaxolol	0.611	364 -> 209	10	50	500							
		364 -> 172	10			42.2	3.21	32.58	51.82	8.44	33.76	50.64
Pindolol	0.619	205 -> 130	10	100	500							
		292 -> 218	15			48.5	3.93	36.71	60.29	9.7	38.8	58.2
Codeine	0.672	371 -> 229	15	10	200							
		371 -> 234	10			75.7	0.98	72.75	78.65	10	65.7	85.7
5-OH-pentoxyfylline	0.703	337 -> 237	15	5	50							
		337 -> 193	15			8.5	0.50	7.00	10.00	5	3.5	13.5

Compound	RRT (min)	Quantifier	Collision energy (eV)	LOD (ng/mL)	MRPL (ng/mL)	Relative Abundance (%)	Stdev	MU Un_L	MU UP_L	Wada Criterium	WADA UN_L	WADA UP_L
Levobunolol	0.703	234 -> 233	5	25	500							
		234 -> 217	10			101.6	0.20	100.99	102.21	10	91.6	111.6
Boldenone metab.	0.705	194 -> 179	10	0.5	10							
		194 -> 105	15			57.6	0.63	55.70	59.50	10	47.6	67.6
Epimethenediol	0.706	358 -> 301	15	1	2							
		358 -> 196	20			9.5	1.65	4.54	14.46	5	4.5	14.5
Ethylmorphine	0.713	385 -> 214	35	10	200							
		385 -> 234	10			60.6	1.21	56.96	64.24	10	50.6	70.6
Fenspiride	0.715	241 -> 96	20	2.5	50							
		241 -> 154	15			71.1	1.14	67.69	74.51	10	61.1	81.1
		241 -> 108	15			32.1	0.61	30.27	33.93	6.42	25.68	38.52
Hydromorphone	0.735	429 -> 234	25	20	200							
		429 -> 357	15			61.6	0.86	59.01	64.19	10	51.6	71.6
Oxycodone	0.745	459 -> 368	15	50	500							
		459 -> 312	15			88.2	0.53	86.61	89.79	10	78.2	98.2
Amineptine metab.	0.764	192 -> 191	20	5	50							
		192 -> 165	35			20.9	1.71	15.76	26.04	5	15.9	25.9
Nadolol	0.782	510 -> 70	35	25	500							
		510 -> 186	20			67.9	6.31	48.96	86.84	10	57.9	77.9
Prenylamine	0.792	238 -> 58	20	10	50							
		238 -> 167	10			12.6	2.36	5.53	19.67	5	7.6	17.6
		238 -> 91	20			1.1	0.35	0.04	2.16	0.55	0.55	1.65

Compound	**RRT (min)**	**Quantifier**	**Collision energy (eV)**	**LOD (ng/mL)**	**MRPL (ng/mL)**	**Relative Abundance (%)**	**Stdev**	**MU Un_L**	**MU UP_L**	**Wada Criterium**	**WADA UN_L**	**WADA UP_L**
MAM	0.797	399 -> 287	15	20	200							
		399 -> 340	10			77.1	1.46	72.71	81.49	10	67.1	87.1
Oxymorphone	0.816	502 -> 70	30	40	200							
		517 -> 355	15			97	4.95	82.16	111.84	10	87	107
Norethisteron metab.	0.82	431 -> 167	20	1	10							
		431 -> 193	20			68.4	1.98	62.45	74.35	10	58.4	78.4
Drostanolone metab.	0.822	448 -> 433	10	5	10							
		448 -> 343	25			39.4	2.05	33.25	45.55	7.88	31.52	47.28
		448 -> 253	25			23.9	3.35	13.86	33.94	5	18.9	28.9
Heroin	0.843	327 -> 215	15	2.5	50							
		327 -> 268	15			30.6	1.90	24.91	36.29	6.12	24.48	36.72
Metenolone metab.	0.849	446 -> 341	15	5	10							
		446 -> 195	20			31	3.35	20.96	41.04	6.2	24.8	37.2
1-Androstenedion	0.851	415 -> 221	20	5	10							
		415 -> 193	20			13.7	1.96	7.82	19.58	5	8.7	18.7
6-OH-Bromantane	0.861	395 -> 91	30	2.5	50							
		393 -> 91	30			96.9	0.97	93.99	99.81	10	86.9	106.9
Fluoxy-18-nor	0.869	462 -> 208	15	2	10							
		462 -> 337	15			25.9	1.40	21.70	30.10	5.18	20.72	31.08
Bambuterol	0.872	354 -> 72	25	5	100							
		354 -> 282	10			21.2	0.40	19.99	22.41	5	16.2	26.2

Compound	RRT (min)	Quantifier	Collision energy (eV)	LOD (ng/mL)	MRPL (ng/mL)	Relative Abundance (%)	Stdev	MU Un_L	MU UP_L	Wada Criterium	WADA UN_L	WADA UP_L
1-testosterone	0.873	194 -> 179	5	2	10							
		206 -> 191	10			28.7	2.96	19.83	37.57	5.74	22.96	34.44
		194 -> 105	10			63.7	3.89	52.04	75.36	10	53.7	73.7
Mesterolone metab.	0.874	448 -> 433	10	1	10							
		448 -> 253	20			30.5	2.41	23.27	37.73	6.1	24.4	36.6
1-Androstenediol	0.883	434 -> 195	10	2	10							
		434 -> 377	10			35.1	2.42	27.83	42.37	7.02	28.08	42.12
		434 -> 143	10			27.7	2.96	18.81	36.59	5.54	22.16	33.24
β-Methyltestosterone metab.	0.884	270 -> 213	15	1	2							
		255 -> 245	15			18.6	1.58	13.86	23.34	5	13.6	23.6
α-Methyltestosterone metab.	0.892	270 -> 199	20	1	2							
		255 -> 105	20			79.5	6.44	60.18	98.82	10	69.5	89.5
17α-Trenbolone	0.906	412 -> 322	15	5	10							
		412 -> 307	15			79.8	5.91	62.08	97.52	10	69.8	89.8
		412 -> 296	15			29.9	3.56	19.23	40.57	5.98	23.92	35.88
Dimefline	0.909	279 -> 163	15	25	50							
		279 -> 133	15			27	3.29	17.12	36.88	5.4	21.6	32.4
		279 -> 105	15			17.4	1.91	11.66	23.14	5	12.4	22.4
Fenbutrazate	0.912	261 -> 103	35	2.5	50							
		261 -> 175	15			53	3.18	43.46	62.54	10	43	63

Compound	RRT (min)	Quantifier	Collision energy (eV)	LOD (ng/mL)	MRPL (ng/mL)	Relative Abundance (%)	Stdev	MU Un_L	MU UP_L	Wada Criterium	WADA UN_L	WADA UP_L
Boldenone	0.926	206 -> 191	10	0.5	10							
		430 -> 206	15			10.2	1.14	6.77	13.63	5	5.2	15.2
		206 -> 165	15			23.6	0.87	20.98	26.22	5	18.6	28.6
Tibolone metab.	0.937	443 -> 193	35	2	10							
		443 -> 167	30			58.1	2.73	49.91	66.29	10	48.1	68.1
Norethandrolone metab.	0.938	421 -> 241	15	1	10							
		421 -> 145	25			77.8	7.08	56.56	99.04	10	67.8	87.8
Epioxandrolone	0.946	363 -> 161	20	5	10							
		363 -> 213	25			54.2	5.15	38.75	69.65	10	44.2	64.2
		363 -> 273	5			76.5	6.12	58.14	94.86	10	66.5	86.5
Calusterone metab.	0.949	374 -> 269	15	5	10							
		374 -> 229	15			67.6	4.60	53.81	81.39	10	57.6	77.6
Fentanyl	0.956	245 -> 146	20	0.5	10							
		245 -> 189	10			48.7	1.07	45.49	51.91	9.74	38.96	58.44
Me-1-Testosterone	0.967	356 -> 231	20	5	10							
		356 -> 206	5			77.4	5.96	59.52	95.28	10	67.4	87.4
		356 -> 192	5			54.3	4.67	40.29	68.31	10	44.3	64.3
Bolasterone metab.	0.969	269 -> 213	15	5	10							
		269 -> 173	15			45.1	5.46	28.73	61.47	9.02	36.08	54.12
Norethandrolone	0.969	421 -> 331	10	5	10							
		421 -> 241	15			77.8	7.08	56.56	99.04	10	67.8	87.8

Compound	**RRT (min)**	**Quantifier**	**Collision energy (eV)**	**LOD (ng/mL)**	**MRPL (ng/mL)**	**Relative Abundance (%)**	**Stdev**	**MU Un_L**	**MU UP_L**	**Wada Criterium**	**WADA UN_L**	**WADA UP_L**
Metenolone	0.971	446 -> 208	10	0.5	10							
		446 -> 195	25			69.2	3.60	58.40	80.00	10	59.2	79.2
Clostebol metab.	0.972	466 -> 431	15	5	10					0	0	0
		466 -> 181	25			66.2	4.37	53.09	79.31	10	56.2	76.2
7βOH-DHEA	0.984	430 -> 299	15	5	10							
		430 -> 325	15			76.1	3.42	65.83	86.37	10	66.1	86.1
Mibolerone	0.984	431 -> 341	15	1	10							
		431 -> 431	15			27	1.38	22.87	31.13	5.4	21.6	32.4
Butorphanol	0.997	416 -> 326	15	25	50							
		416 -> 103	15			69.4	5.48	52.95	85.85	10	59.4	79.4
Methyldienolone	0.997	430 -> 285	20	5	10							
		430 -> 340	15			38.7	3.99	26.74	50.66	7.74	30.96	46.44
Norbolethone metab.	1	435 -> 255	10	1	10							
		435 -> 345	5			36.1	3.61	25.27	46.93	7.22	28.88	43.32
Fenethylline deriv. 1	1.001	250 -> 207	15	25	50							
		250 -> 70	15			11.4	1.28	7.57	15.23	5	6.4	16.4
THCA	1.004	371 -> 289	20	7.5	15							
		371 -> 265	15			92.9	1.95	87.05	98.75	10	82.9	102.9
Bolasterone	1.007	460 -> 355	15	5	10							
		460 -> 315	20			60.3	4.22	47.64	72.96	10	50.3	70.3
Calusterone	1.007	460 -> 355	15	5	10							
		460 -> 315	20			65.8	4.34	52.77	78.83	10	55.8	75.8

Compound	**RRT (min)**	**Quantifier**	**Collision energy (eV)**	**LOD (ng/mL)**	**MRPL (ng/mL)**	**Relative Abundance (%)**	**Stdev**	**MU Un_L**	**MU UP_L**	**Wada Criterium**	**WADA UN_L**	**WADA UP_L**
Oxandrolone	1.01	363 -> 273	10	5	10							
		363 -> 213	15			18.1	2.70	10.01	26.19	5	13.1	23.1
		363 -> 161	20			35.1	3.47	24.68	45.52	7.02	28.08	42.12
Ethisteron	1.01	456 -> 316	15	0.5	10							
		456 -> 301	20			90.8	4.63	76.91	104.69	10	80.8	100.8
Acebutolol	1.015	278 -> 208	20	250	500							
		278 -> 236	15			69.7	6.27	50.88	88.52	10	59.7	79.7
		278 -> 166	20			14.1	1.93	8.30	19.90	5	9.1	19.1
4-OH-Nandrolone	1.022	506 -> 147	25	5	10							
		506 -> 195	20			20.4	1.67	15.38	25.42	5	15.4	25.4
		506 -> 93	20			17.1	2.50	9.61	24.59	5	12.1	22.1
Norbolethone metab. 1	1.022	435 -> 255	20	0.5	10							
		435 -> 345	5			36.1	3.61	25.27	46.93	7.22	28.88	43.32
6α-OH-Androstenedione	1.027	518 -> 319	25	5	10							
		518 -> 413	15			54.2	6.29	35.34	73.06	10	44.2	64.2
Triamterene	1.031	454 -> 340	15	125	250							
		454 -> 171	15			15.9	1.32	11.94	19.86	5	10.9	20.9
Zeranol	1.034	433 -> 295	20	5	10							
		433 -> 309	20			58.3	2.39	51.13	65.47	10	48.3	68.3
Formebolone metab. 2	1.036	534 -> 389	15	5	10							
		534 -> 339	25			28.2	2.57	20.50	35.90	5.64	22.56	33.84

Compound	RRT (min)	Quantifier	Collision energy (eV)	LOD (ng/mL)	MRPL (ng/mL)	Relative Abundance (%)	Stdev	MU Un_L	MU UP_L	Wada Criterium	WADA UN_L	WADA UP_L
4-OH-Androstenedione	1.038	518 -> 147	30	0.5	10							
		518 -> 169	30			23.9	1.48	19.45	28.35	5	18.9	28.9
		518 -> 107	30			57	4.39	43.83	70.17	10	47	67
Norclostebol	1.041	452 -> 216	15	5	10							
		452 -> 321	5			28.7	1.69	23.62	33.78	5.74	22.96	34.44
Amineptine	1.042	192 -> 191	15	10	50							
		192 -> 165	15			2.7	0.15	2.26	3.14	1.35	1.35	4.05
4-OH-Testosteron	1.043	520 -> 225	25	5	10							
		520 -> 431	15			65.3	7.51	42.77	87.83	10	55.3	75.3
Cyclofenil metab.	1.053	512 -> 422	15	2.5	50							
		512 -> 343	20			10.9	0.24	10.18	11.62	5	5.9	15.9
Toremifene	1.053	405 -> 58	15	25	50							
		405 -> 72	5			71.7	3.01	62.67	80.73	10	61.7	81.7
6β-OH-methandienone	1.055	517 -> 229	25	5	10							
		517 -> 337	15			8.9	0.80	6.50	11.30	5	3.9	13.9
Dextromoramide	1.055	265 -> 166	20	100	200							
		265 -> 98	20			78.4	1.88	72.76	84.04	10	68.4	88.4
Labetolol	1.064	383 -> 265	15	50	500							
		383 -> 251	15			52.5	6.67	32.50	72.50	10	42.5	62.5
		383 -> 223	15			28.7	3.10	19.40	38.00	5.74	22.96	34.44

Compound	RRT (min)	Quantifier	Collision energy (eV)	LOD (ng/mL)	MRPL (ng/mL)	Relative Abundance (%)	Stdev	MU Un_L	MU UP_L	Wada Criterium	WADA UN_L	WADA UP_L
Fluoxymesterone	1.065	552 -> 407	15	5	10							
		552 -> 357	20			36.9	4.72	22.73	51.07	7.38	29.52	44.28
		552 -> 319	25			40.9	4.34	27.89	53.91	8.18	32.72	49.08
Oral-Turinabol	1.065	240 -> 225	5	5	10							
		240 -> 189	20			22.7	1.93	16.91	28.49	5	17.7	27.7
		240 -> 93	15			29.2	1.28	25.35	33.05	5.84	23.36	35.04
Oxymesterone	1.069	534 -> 389	25	5	10							
		534 -> 444	20			13.8	2.79	5.44	22.16	5	8.8	18.8
Formoterol	1.07	277 -> 216	10	50	100							
		277 -> 233	10			49.3	6.80	28.89	69.71	9.86	39.44	59.16
Fenethylline deriv. 2	1.076	322 -> 207	15	25	50							
		322 -> 253	15			32.1	1.38	27.96	36.24	6.42	25.68	38.52
		322 -> 45	15			32.9	2.04	26.78	39.02	6.58	26.32	39.48
4-OH-Tamoxifen	1.077	459 -> 72	10	25	50							
		459 -> 58	20			40.1	0.88	37.45	42.75	8.02	32.08	48.12
Fenoterol	1.078	308 -> 207	25	50	100							
		308 -> 179	30			21.1	2.15	14.64	27.56	5	16.1	26.1
		308 -> 116	30			15.8	3.13	6.41	25.19	5	10.8	20.8
Famprofazone	1.082	286 -> 72	20	25	50							
		286 -> 214	15			18	2.45	10.66	25.34	5	13	23

Compound	RRT (min)	Quantifier	Collision energy (eV)	LOD (ng/mL)	MRPL (ng/mL)	Relative Abundance (%)	Stdev	MU Un_L	MU UP_L	Wada Criterium	WADA UN_L	WADA UP_L
Oral-Turinabol metab.	1.086	315 -> 227	15	5	10							
		317 -> 243	15			52.2	6.99	31.22	73.18	10	42.2	62.2
		315 -> 241	15			23	4.12	10.65	35.35	5	18	28
Exemestane metab.	1.098	443 -> 207	20	25	50							
		443 -> 353	10			33.8	3.45	23.46	44.14	6.76	27.04	40.56
Fencamine deriv. 1	1.098	365 -> 308	15	10	50							
		365 -> 251	15			12.4	1.98	6.45	18.35	5	7.4	17.4
		365 -> 84	15			3.6	0.74	1.39	5.81	1.8	1.8	5.4
Danazol	1.1	466 -> 167	20	5	10							
		466 -> 193	20			41.1	3.29	31.24	50.96	8.22	32.88	49.32
Canrenone	1.113	412 -> 397	15	25	250							
		412 -> 383	15			15.7	1.70	10.61	20.79	5	10.7	20.7
16-OH-furazabol	1.117	490 -> 231	15	1	10							
		490 -> 143	35			47.8	2.10	41.49	54.11	9.56	38.24	57.36
Strychnine	1.123	316 -> 220	15	10	50							
		316 -> 144	15			23.1	2.82	14.65	31.55	5	18.1	28.1
		316 -> 115	25			15.5	1.07	12.29	18.71	5	10.5	20.5
3’-OH-Stanozolol	1.128	545 -> 147	25	10	2							
		488 -> 182	15			33.2	3.35	23.14	43.26	6.64	26.56	39.84
16-OH-Stanozolol	1.128	560 -> 143	15	10	2							
		560 -> 231	15			10	2.86	1.42	18.58	5	5	15

Compound	**RRT (min)**	**Quantifier**	**Collision energy (eV)**	**LOD (ng/mL)**	**MRPL (ng/mL)**	**Relative Abundance (%)**	**Stdev**	**MU Un_L**	**MU UP_L**	**Wada Criterium**	**WADA UN_L**	**WADA UP_L**
Fencamine deriv. 2	1.146	293 -> 236	15	25	50							
		293 -> 84	15			2.7	0.83	0.21	5.19	1.35	1.35	4.05
		293 -> 58	15			23	2.02	16.93	29.07	5	18	28
Fluticasone	1.162	644 -> 224	15	15	30							
		644 -> 461	15			44.9	3.19	35.34	54.46	8.98	35.92	53.88
		644 -> 371	15			14.9	2.37	7.79	22.01	5	9.9	19.9
Buprenorfine	1.172	506 -> 84	25	0.5	10							
		506 -> 55	30			39.8	0.72	37.65	41.95	7.96	31.84	47.76
Raloxifene	1.216	578 -> 193	35	5	50							
		578 -> 413	30			30.9	2.69	22.84	38.96	6.18	24.72	37.08
Carvedilol	1.224	183 -> 154	15	250	500							
		183 -> 155	20			90.9	5.27	75.08	106.72	10	80.9	100.9
Salmeterol	1.227	311 -> 149	15	20	100							
		311 -> 121	25			22.6	0.95	19.75	25.45	5	17.6	27.6

Internal standard	RRT (min)	Quantifier	Collision energy (eV)	LOD (ng/mL)	MRPL (ng/mL)	Relative Abundance (%)	Stdev	MU Un_L	MU UP_L	Wada Criterium	WADA UN_L	WADA UP_L
Salbutamol-d3	0.3629	372 -> 210	20									
		372 -> 193	20			20	1.28	16.16	23.84	5	15	25
Androsterone-d4	0.7789	423 -> 333	20									
		423 -> 243	20			100	3.30	90.10	109.90	10	90	110
Etiocholanolone-d5	0.7894	424 -> 334	20									
		424 -> 244	20			65	1.82	59.54	70.46	10	55	75
5α-And-3α,17β-diol-d3	0.8029	244 -> 188	15									
		244 -> 202	15			96.8	0.00	96.80	96.80	10	86.8	106.8
		244 -> 145	15			55	0.00	55.00	55.00	10	45	65
5β-And-3α,17β-diol-d5	0.8081	246 -> 164	15									
		246 -> 190	15			62.1	0.62	60.24	63.96	10	52.1	72.1
		246 -> 176	15			47.3	1.37	43.18	51.42	9.46	37.84	56.76
DHEA-d6	0.8571	438 -> 333	20									
		438 -> 307	20			7.8	0.00	7.80	7.80	5	2.8	12.8
		438 -> 243	20			68.9	0.00	68.90	68.90	10	58.9	78.9
Epitestosterone-d3	0.8895	435 -> 209	15									
		435 -> 330	15			50	1.60	45.20	54.80	10	40	60
DHT-d3	0.902	437 -> 195	15									
		437 -> 143	15			73.9	5.03	58.82	88.98	10	63.9	83.9
		437 -> 205	15			30.5	1.95	24.64	36.36	6.1	24.4	36.6
Testosterone-d3	0.9397	435 -> 209	20									
		435 -> 330	20			35	0.74	32.80	37.21	7	28	42

Internal standard	RRT (min)	Quantifier	Collision energy (eV)	LOD (ng/mL)	MRPL (ng/mL)	Relative Abundance (%)	Stdev	MU Un_L	MU UP_L	Wada Criterium	WADA UN_L	WADA UP_L
17α-Methyltestosterone	1	446 -> 301	15									
		446 -> 198	20			7.9	0.70	5.81	9.99	5	2.9	12.9

References

[1] J. Marcos, J.A. Pascual, X. de la Torre, J. Segura. Fast screening of anabolic steroids and other banned doping substances in human urine by gas chromatography/tandem mass spectrometry. *Journal of mass spectrometry□: JMS*. 2002, *37*, 1059–73.

[2] WADA. The 2011 Prohibited List, International Standard. 2011, http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-Prohibited-list/To_be_effective/WADA_Prohibited_List_2011_EN.pdf

[3] C.G. Georgakopoulos, P. Kiousi, Y.S. Angelis, E. Lyris, M. Koupparis, A.C. Calokerinos, J. Atta-Politou. Two-step silylation procedure for the unified analysis of 190 doping control substances in human urine samples by GC-MS. *Bioanalysis*. 2009, *1*, 1209–24.

[4] S. Strano-Rossi, F. Molaioni, F. Rossi, F. Botre. Rapid screening of drugs of abuse and their metabolites by gas chromatography/mass spectrometry: application to urinalysis. *Rapid Communications in Mass Spectrometry*. 2005, *19*, 1529–35.

[5] W. Van Thuyne, P. Van Eenoo, F.T. Delbeke. Implementation of gas chromatography combined with simultaneously selected ion monitoring and full scan mass spectrometry in doping analysis. *Journal of chromatography. A*. 2008, *1210*, 193–202.

[6] F. Botre, M. Mazzarino, M. Orengia. Application of fast gas chromatography/mass spectrometry for the rapid screening of synthetic anabolic steroids and other drugs in anti-doping analysis. *Rapid communications in mass spectrometry*. 2007, *21*, 4117–24.

[7] L.D. Bowers. Analytical advances in detection of performance-enhancing compounds. *Clinical Chemistry*. 1997, *43*, 1299–304.

[8] P. Van Renterghem, P. Van Eenoo, W. Van Thuyne, H. Geyer, W. Schanzer, F.T. Delbeke. Validation of an extended method for the detection of the misuse of endogenous steroids in sports, including new hydroxylated metabolites. *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences*. 2008, *876*, 225–35.

[9] P. Kiousi, Y.S. Angelis, E. Lyris, M. Koupparis, A.C. Calokerinos, J. Atta-Politou, C.G. Georgakopoulos. Two-step silylation procedure for the unified analysis of 190 doping control substances in human urine samples by GC-MS. *Bioanalysis*. 2009, *1*, 1209–24.

[10] D. Van de Kerkhof. *Steroid Profiling in Doping Analysis [PhD Thesis]*. 2001, 237. http://igitur-archive.library.uu.nl/dissertations/1977561/full.pdf

[11] J. Munoz-Guerra, D. Carreras, C. Soriano, C. Rodriguez, A.F. Rodriguez. Use of ion trap gas chromatography-tandem mass spectrometry for detection and confirmation of anabolic substances at trace levels in doping analysis. *Journal of chromatography. B, Biomedical sciences and applications*. 1997, *704*, 129–41.

[12] WADA. WADA Technical Document - TD2004EAAS. 2004, 11. http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-IS-Laboratories/WADA_TD2004EAAS_EN.pdf

[13] J.F. Lévesque, C. Ayotte. The Oral Administration of DHEA□: The Efficiency of Steroid Profiling. in *Proceedings of the 7th Cologne Workshop on Dope analysis*. (W. Schänzer A. Gotmann, U. Mareck-Engelke, H. G.) 1999, *7*, 213–21.

[14] J.L.C. Ayotte. Criteria for the Detection of Androstenedione Oral Administration. *7th Cologne Workshop on Dope Analysis, Cologne. Sport und Buch Strauß,*. 1999, 169.

[15] A.T. Cawley, E.R. Hine, G.J. Trout, A. V George, R. Kazlauskas. Searching for new markers of endogenous steroid administration in athletes: “looking outside the metabolic box”. *Forensic science international*. 2004, *143*, 103–14.

[16] A.T. Cawley, G.J. Trout, R. Kaziauskas, A. V George. The detection of androstenedione abuse in sport: a mass spectrometry strategy to identify the 4-hydroxyandrostenedione metabolite. *Rapid Communications in Mass Spectrometry*. 2008, *22*, 4147–57.

[17] U. Mareck, H. Geyer, G. Opfermann, M. Thevis, W. Schanzer. Factors influencing the steroid profile in doping control analysis. *Journal of mass spectrometry□: JMS*. 2008, *43*, 877–91.

[18] WADA. Technical Document: Minimum Required Performance Limits for Detection of Prohibited Substances: TD2010MRPL. 2010, http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-IS-Laboratories/WADA_TD2010MRPLv1.0_Minimum Required Performance Levels_Sept 01 2010_EN.pdf

[19] R. de la Torre, X. de la Torre, C. Alia, J. Segura, T. Baro, J.M. Torres-Rodriguez. Changes in androgenic steroid profile due to urine contamination by microorganisms: a prospective study in the context of doping control. *Analytical Biochemistry*. 2001, *289*, 116–23.

[20] M. Tsivou, D. Livadara, D.G. Georgakopoulos, M.A. Koupparis, J. Atta-Politou, C.G. Georgakopoulos. Stabilization of human urine doping control samples: II. microbial degradation of steroids. *Analytical Biochemistry*. 2009, *388*, 146–54.

[21] M. Mazzarino, M.G. Abate, R. Alocci, F. Rossi, R. Stinchelli, F. Molaioni, X. de la Torre, F. Botre. Urine stability and steroid profile: towards a screening index of urine sample degradation for anti-doping purpose. *Analytica Chimica Acta*. 2011, *683*, 221–26.

[22] G.A.W.M. Association. Ethical Principles for Medical Research Involving Human Subjects. 2008, http://www.wma.net/en/30publications/10policies/b3/17c.pdf

[23] WADA. WADA Technical Document – TD2010IDCR, Identification criteria for qualitative assays incorporation column chromatography and mass spectometry. 2010, http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-IS-Laboratories/WADA_TD2010IDCRv1.0_Identification Criteria for Qualitative Assays_May 08 2010_EN.doc.pdf

[24] P. Van Eenoo, W. Van Gansbeke, N. De Brabanter, K. Deventer, F.T. Delbeke. A fast, comprehensive screening method for doping agents in urine by gas chromatography-triple quadrupole mass spectrometry. *Journal of chromatography. A*. 2011, *1218*, 3306–16.

[25] E. Hoffmann, V. Stroobant. *Mass spectrometry: principles and applications*. 2007, http://books.google.be/books?id=pqhr5XQZDtUC

[26] B. Castillo, V. Bansal, A. Ganesan, P. Halling, F. Secundo, A. Ferrer, K. Griebenow, G. Barletta. On the activity loss of hydrolases in organic solvents: II. a mechanistic study of subtilisin Carlsberg. *BMC biotechnology*. 2006, *6*, 51.

[27] M. Galesio, R. Rial-Otero, J. Simal-Gandara, X. de la Torre, F. Botre, J.L. Capelo-Martinez. Improved ultrasonic-based sample treatment for the screening of anabolic steroids by gas chromatography/mass spectrometry. *Rapid Communications in Mass Spectrometry*. 2010, *24*, 2375–85.

[28] N. Baume, G. Steel, T. Edwards, E. Thorstensen, B.F. Miller. No variation of physical performance and perceived exertion after adrenal gland stimulation by synthetic ACTH (Synacthen (R)) in cyclists. *European Journal of Applied Physiology*. 2008, *104*, 589–600.

[29] WADA. International Standard for Laboratories, version 7.0. 2012, http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-IS-Laboratories/ISL/WADA_Int_Standard_Laboratories_2012_EN.pdf

[30] P. Van Renterghem, P. Van Eenoo, H. Geyer, W. Schanzer, F.T. Delbeke. Reference ranges for urinary concentrations and ratios of endogenous steroids, which can be used as markers for steroid misuse, in a Caucasian population of athletes. *Steroids*. 2010, *75*, 154–63.

[31] I.L.A.C. (ILAC). Introducing the Concept of Uncertainty of Measurement in Testing in Association with the Application of the Standard ISO/IEC 17025. 2002, 7. http://www.ilac.org/documents/ILAC_G17-2002_intro_the_concept_of_uncert_meas_with_17025.pdf

[32] N.M. Faber. Regulations in the field of residue and doping analysis should ensure the risk of false positive declaration is well-defined. *Accreditation and Quality Assurance*. 2009, *14*, 111–15.

Chapter 5:

Confirmatory quantification of threshold substances: 11-nor-∆9-tetrahydrocannabinol-9-carboxylic acid (THCA).

Adapted from:

N. De Brabanter, W. Van Gansbeke, F. Hooghe, P. Van Eenoo.

Fast quantification of 11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid (THCA) using microwave-accelerated derivatisation and gas chromatography–triple quadrupole mass spectrometry.

Forensic Science International. 2012, doi:10.1016/j.forsciint.2012.11.004

Abstract

A rapid and sensitive determination of cannabinoids in urine is important in many fields, from workplace drug testing over toxicology to the fight against doping. The detection of cannabis abuse is normally based on the quantification of the most important metabolite 11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid (THCA) in urine. In most fields THCA needs to be present at a concentration exceeding 15 ng/mL before a positive result can be reported.

In this paper a fast confirmation method is described to quantify THCA in 1 mL of urine. This method combines a 4 min GC-QqQ-MS method with a fast sample preparation procedure using microwave assisted derivatisation in order to complete the quantification of THCA in urine in 30 min.

The method is selective, linear over the range 5 – 100 ng/mL and shows excellent precision and trueness and hence, the estimated measurement uncertainty at the threshold level is small. The method also complies with applicable criteria for mass spectrometry and chromatography. Therefore, the method can be used for rapid screening and confirmatory purposes.

1 Introduction

The psychoactive substances from *Cannabis sativa* are amongst the most widely used illicit drugs in the world. Considering the various effects of cannabis [1], there is a need for rapid and sensitive detection methods in many fields: workplace drug testing [2], clinical and forensic toxicology [3], and the fight against doping [4]. Although they do not improve athletic performance, cannabinoids allow the athlete to relax and escape from pressure. Additionally, the use of cannabis reduces alertness and quick reflexes, making it dangerous in motor and team sports [5,6]. Therefore, natural (e.g. cannabis, hashish, marijuana) or synthetic Δ9-tetrahydrocannabinol (THC) are also prohibited in competition by the World Anti-Doping Agency (WADA) [7].

According to the guidelines of various scientific organizations, a two-step approach is applied: after detecting the substance or its metabolites in a screening method, a confirmation procedure is performed in order to obtain additional information to support a possible positive result. This confirmation procedure needs to have equal (in case a hyphenated mass spectrometry method was used in the screening) or greater selectivity than the initial screening method [8,9].

In most fields, immunoassays are used as screening methods for the detection of cannabis metabolites in biological matrices. However, since these techniques measure the total amount of cannabis metabolites [10] and can eventually show cross-reactivity, any positive result must be confirmed by a chromatographic technique, combined with mass spectral identification [11,12]. In general chromatography-mass spectrometry needs to be used to comply with the stringent requirements to unequivocally identify a compound in most fields.

To differentiate active from passive users [13], a threshold concentration of 15 ng/mL for 11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid (THCA), the major metabolite of THC (Fig. 5.1), in urine is set by WADA [14]. Similar as for the WADA-regulations, a threshold of 15 ng/ml is also commonly used in the fields of toxicology and in drugs at the workplace testing [9,15]. Of course any conclusion, whether or not a threshold concentration is exceeded, should take into account the measurement uncertainty associated with the result.

Both the limited reporting period and the limited amount of urine available are important factors in the development of a confirmation procedure. Traditionally, confirmatory methods for THCA use multiple sample preparation steps and are time-consuming [16].

In this work a fast confirmation method is presented for the quantification of Δ9-tetrahydrocannabinol in urine that not only fulfills all legal requirements (MS criteria, uncertainty, etc.), but which by using an accelerated sample pretreatment and a fast chromatographic method allows to quantify this metabolite rapidly and accurately in a low volume of urine.

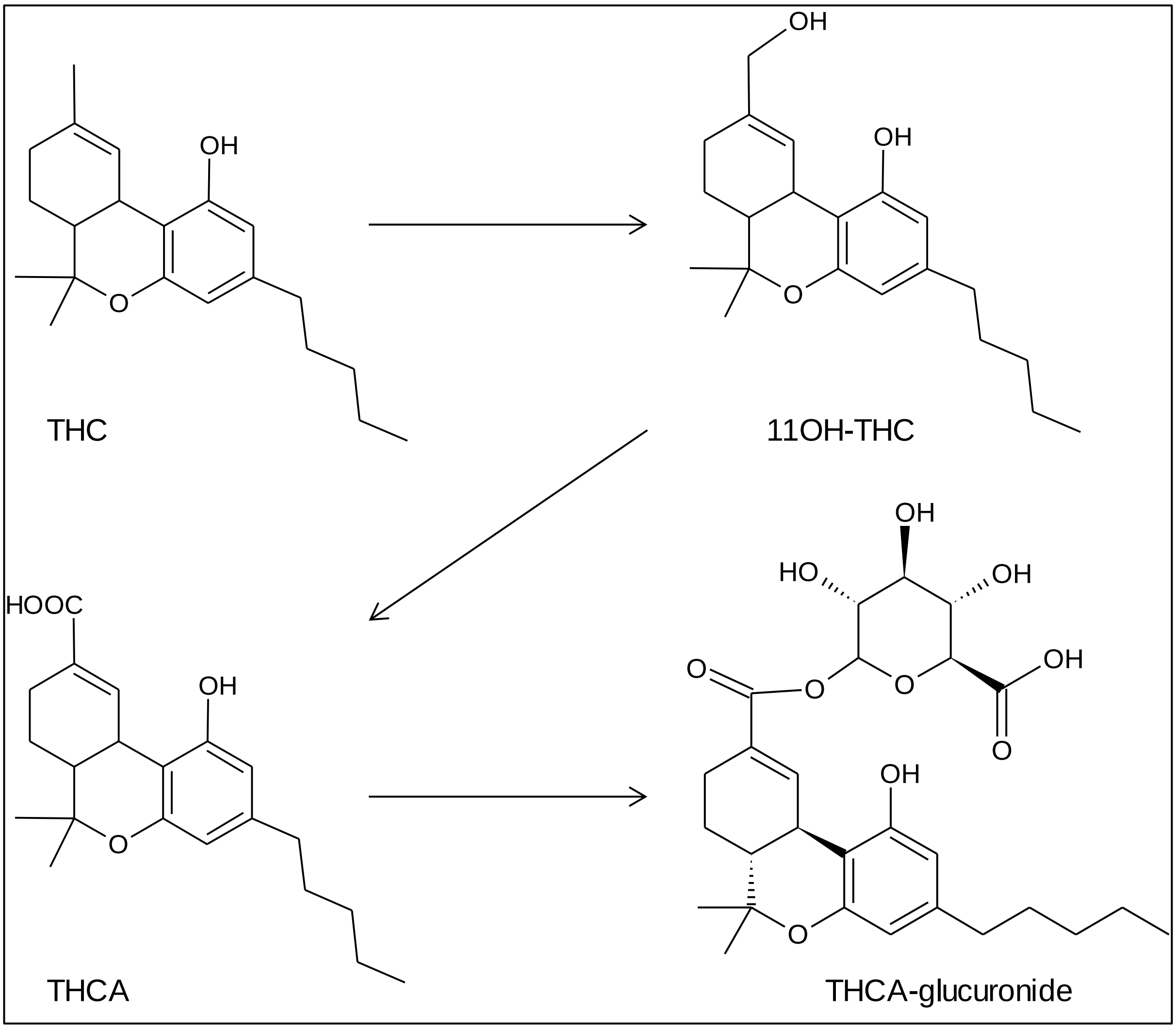


Fig. 5.1 Formation of THCA, the marker for detection of cannabis use in urine.

2 Experimental

2.1 Instrumentation

An Agilent (Agilent Technologies, Palo Alto, USA) GC 7890 gas chromatograph coupled to an Agilent 7000B triple quadrupole mass spectrometer (Agilent Technologies) and a MPS2 autosampler and PTV-injector from Gerstel (Mülheim and der Ruhr, Germany) were used.

The GC column – 12 m x 250 µm and 0.25 µm film thickness – was a HP-1MS from J&W Scientific (Agilent Technologies, USA). The temperature program was as follows: the initial temperature was 110°C (0.15min), increased at 70°C/min to 310°C (held for 1min). The transfer line was set at 310°C. Helium (Air Liquide, Desteldonk, Belgium) was used as carrier gas at a flow rate of 3 mL/min.

Injection was performed using the following PTV setting: in the solvent vent mode 10 µL was injected (0.5 µL/s injection speed) and the PTV-temperature settings were: 110°C (0.15 min), 12°C/min to 310°C (2 min) and 12°C/min to 380°C (1 min). The vent flow was 60 ml/min and the vent pressure 5 psi until 0.1 min.

In the QqQ collision cell He was used as a quench gas at 2.25 mL/min and N_2 as collision gas at 1.5 mL/min.

Quantification and confirmation were performed in selected reaction monitoring (SRM) mode. The monitored transitions and corresponding collision energies are given in Table 5.1.

For derivatisation a domestic microwave oven (Samsung M643) was used in this study, which has a total capacity of 750W. The effective capacity was verified with every batch.

Table 5.1. Monitored transitions for THCA and THCA-d9 (internal standard), corresponding collision energies and transition ratios (tested against WADA criteria for identification).

Compound name	Transition	Collision energy (eV)	Transition ratio (%)	WADA	Max. tolerance window	
				Identification criterium	Under limit	Upper limit
THCA	371 -> 305	10				
THCA	371 -> 289	10	98.8	10	88.8	108.8
THCA	371 -> 265	10	86.8	10	76.8	96.8
THCA	371 -> 95	10	78.4	10	68.4	88.4
THCA-d9	380 -> 67	25				
THCA-d9	380 -> 101	25	35.2	7.04	28.16	42.24
THCA-d9	380 -> 84	25	50.5	10	40.5	60.5
THCA-d9	380 -> 292	25	43.2	8.64	34.56	51.84

2.2 Reagents

11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid (THCA) and d9-11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid (THCA-d9) were purchased from Cerriliant (Round Rock, Texas, USA). NaOH and acetic acid were from Merck (Overijse, Belgium). N-methy-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) was obtained from Chem. Fabrik Karl Bucher (Waldstedt, Germany). Ethanethiol and ethylacetate were purchased from Acros (Geel, Belgium) and acetonitrile (ACN) was from Biosolve (The Netherlands). Ammoniumiodide (NH_4I) from Sigma Aldrich (Bornem, Belgium) and n-hexane from Biosolve (Valkenswaard, The Netherlands). All reagents were analytical grade.

2.3 Sample preparation

1 mL of urine was spiked with 50 µL of the internal standard containing 0.5 µg/mL THCA-d9. Hydrolysis was performed by incubation for 7 min in an oven at 56°C ± 5°C after addition of 100 µL of 6M NaOH solution. After briefly cooling 1.5 mL acetic acid and 3 mL n-hexane/ethyl acetate (9/1) were added and the mixture was extracted by vortexing for 1 min. The organic phase was evaporated under oxygen free nitrogen at 40°C ± 5°C. The dried residue was derivatised using 20 µL

acetonitrile, 50 µL MSTFA and 50 µL of MSTFA/ethanethiol/NH_4I (500:4:2) in a microwave reactor at 750 W during 1.5 min.

2.4 Validation

2.4.1 Microwave calibration

The microwave calibration procedure was based upon a calorimetric methodology. In a glass beaker, 1L distilled water was heated in the microwave for 90 s at 100% power. The microwave power absorbed by the water that was used for heating the volume was calculated on the measured temperature rise ΔT [17].

$$\Delta T = \frac{P\,.\,t}{V\,.\,C_P\,.\,\rho}$$

In this formula P is the microwave power in Watt (W), t the time of heating in seconds, V the volume of heated water (m^3), C_P the heat capacity (J/kg.K) and ρ the density (kg/m^3).

2.4.2 Quantitative

To construct calibration curves, blank urine samples were spiked at 6 concentration levels. The least squares method was used for fitting the calibration curves and controlling linearity of the method. At every level accuracy and precision were tested over 3 replicates. The tolerances for precision were calculated following the Horwitz equation (RSDmax= 2(1-0,5logC)) and should not exceed 2/3 RSDmax. The accuracy, expressed as bias, may not exceed 15%.

For correct identification of the compound, WADA criteria state that retention times (RT) of the analyt shall not differ more than ±0.1 min or 2% from that of the same substance in a spiked urine sample. Further, the transition ratios are calculated by dividing the area of the given qualifier by the quantifier. Maximum tolerance windows for these relative ion intensities are determined by WADA [18]. For the least intense diagnostic transition, signal-to-noise (S/N) ratio shall be greater than 3.

To verify to what extent the method can determine THCA and THCA-d9 in matrices without interferences from other compounds, several mixtures of possible interfering compounds (narcotics, anabolic steroids, beta-blockers, diuretics and stimulants) were analysed.

The limit of detection (LOD) and limit of quantification (LOQ) were determined by using the IUPAC standard approach SA1, which uses the mean blank signal as the basis for the calculation of the LOD and LOQ values [19].

The standard deviation, calculated in 40 control samples, was used to determine tolerance windows for quality control and measurement uncertainty for THCA.

2.4.3 Cross validation

To verify the applicability of the method for the analysis of routine samples, the method was compared to the GC-MS method which was formerly used in our lab [16]. Therefore, 20 urines, positive for THCA, were analysed with both methods and the obtained concentrations were compared.

3 Results and discussion

3.1 Sample preparation procedure

In previous described methods using gas chromatography-mass spectrometry, the quantity of urine used per analysis varied between 2 and 3 mL [10,11,16,20–23]. Since the amount of urine available is limited and for confirmation procedures usually 3 aliquots are analysed, it is important to lower the volume of urine consumed per analysis. The combination of large volume injection using a PTV injector and tandem mass spectrometry for higher selectivity and sensitivity (Fig. 5.2), allowed for using only 1 mL of urine.

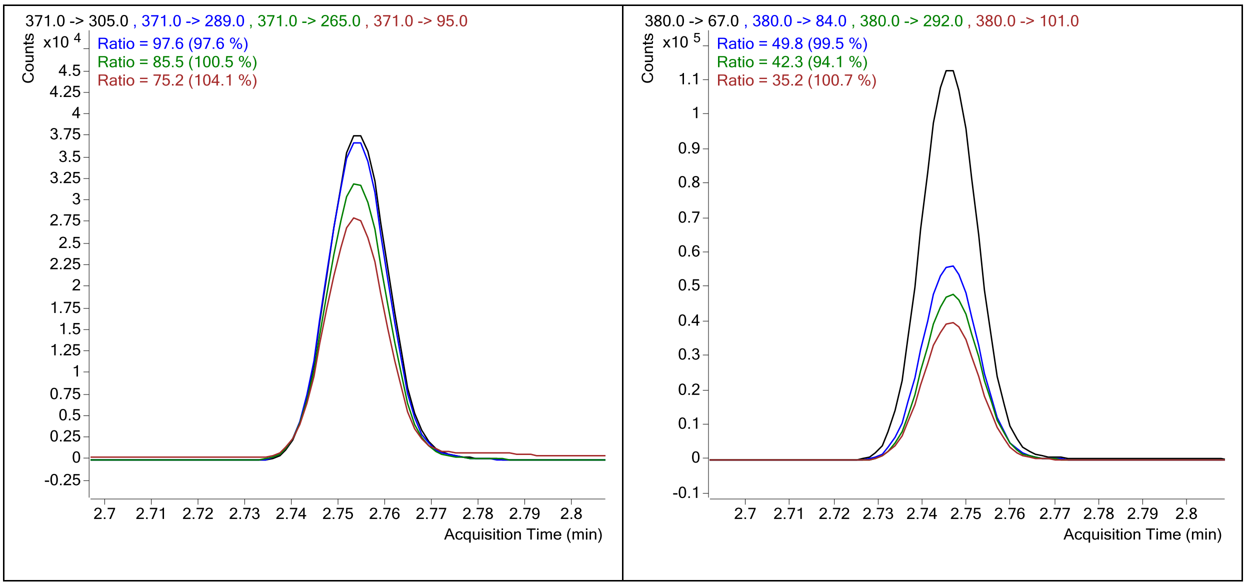


Fig. 5.2 THCA and THCA-d9 ion traces in a urine sample, spiked with THCA at 15 ng/ml (left) and THCA-d9 at 50 ng/ml (right).

Since THCA is mostly excreted in urine in a conjugated form (Fig. 5.1), first a hydrolysis step is performed. In previously described methods this is either done with an enzymatic hydrolysis using β-glucuronidase (incubation up to 60min) [23] or an alkaline hydrolysis using NaOH or KOH (usually between 15min and 30 min incubation) [10,11,20]. THCA however is connected to the glucuronide using an ester bond, which allows the use of a faster alkaline hydrolysis. Indeed, previous research showed that incubation for 7 min provides complete hydrolysis [24]. Hence, after verification of this procedure the hydrolysis time in this study was set to 7 min as well.

While in the past, when using single MS technology, a preliminary extraction was needed for sample clean-up in order to obtain sufficient selectivity [16], this is not necessary when using MS/MS. The elimination of this step reduces the sample preparation time. Hence, sample pretreatment for GC-MS/MS is less time-consuming and becomes comparable to the fastest recently published LC-MS procedures [24–27].

Subsequently, the free compound is usually isolated out of the urine matrix using labor-intensive solid-phase extraction (SPE) [21] or time consuming liquid-liquid extraction (up to 20min mixing/rolling) [16,22,23]. This step is optimized by analyzing blank urine samples spiked at 15 ng/mL THCA using different extraction times (3 replicates each). From the recoveries given in Fig. 5.3 it can be concluded that the extraction can be shortened to 1 min of vortexing without compromising on efficiency, resulting in a drastic reduction of analysis time. This can be explained by the addition of the deuterated internal standard THCA-d9 which compensates very well for any losses during this and other sample preparation steps. Further, the changes in peak area of THCA in this experiment are showing that the equilibrium is quickly set. Indeed, compared to the traditional 20 min of rolling where an average peak area of 30877 with a RSD of 4.33% was found (n = 3), all other peak areas did not differ more than 0.14% from this value.

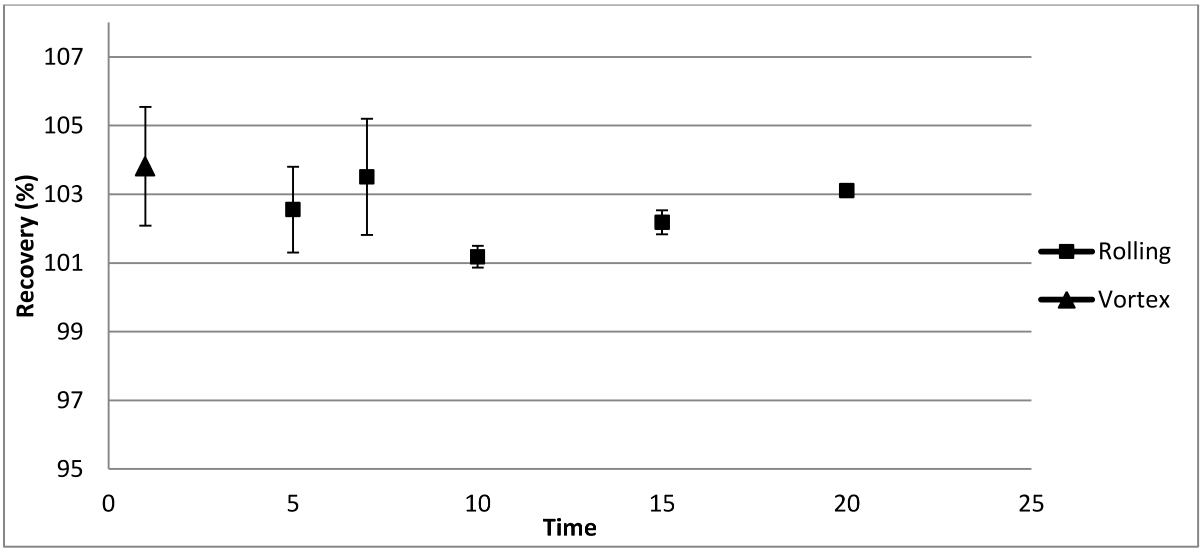


Fig. 5.3 Optimization extraction procedure: recoveries as a function of the mixing time for samples spiked at 15 ng/mL THCA (n=3).

Recently, most of the fast quantification methods for THCA have been based on LC-MS analysis. One of the primary reasons for this is that GC-MS requires a time-consuming derivatisation step to improve the compounds characteristics in terms of chromatography. This derivatisation is usually performed with silylation reagents and the energy needed to complete the silylation reaction is traditionally added by conventional heating, which requires long reactions times up to 1h [28,29]. Supplying the energy transfer by microwave irradiation instead of thermal heating can reduce this derivatisation time to a few minutes [30]. When using microwave-assisted derivatisation (MAD), the reaction mixture is heated rapidly from the inside, in contrast to conventional heating which is slow and enters the sample from the surface, heating also the reaction tube [31]. Reducing the time required for this and other sample preparation steps prior to GC analysis greatly improves the competitiveness of GC-MS. Indeed, GC-MS is less costly than LC-MS and shows better separation power.

The GC-MS method described here allows for a perfect separation of the target compound from any matrix interferences in less than 4 min. To the best of our knowledge, this is faster than previously described GC [16,22,23,32] and LC [24–26] method run times, greatly improving sample turn-around and throughput.

Hence, the optimized sample preparation procedure together with a GC run of only 4 min allows for a quantification of THCA in 1 mL of urine in less than 30 minutes.

3.2 Derivatisation procedure

The domestic microwave system used in this experiment has a nominal power of 750W. Since quality control is of great importance in doping control, a procedure is needed to monitor the performance of the microwave through time. When using the absorbed power as a parameter, the stability of the microwave can easily be monitored on a daily base [33]. Therefore, 1 L of distilled water is heated for 90 s at 100% nominal power. Using the abovementioned formula, one can calculate the effective absorbed power from the temperature rise of the water. From different measurements over a period of 2 months, an absorbed power of 576 ± 39 W was found.

When using microwave assisted derivatisation, reaction solvent, microwave power and reaction time are important parameters [34,35].

In contrast to conventional heating, the amount of heat transferred to the reaction mixture depends on the dielectric properties of the molecules [31]. Because polar molecules have a permanent dipole moment, they can absorb the microwave energy and convert it into heat. ACN has a high polarity and is therefore very suitable for absorbing electromagnetic energy [36]. This means that the derivatisation mixture for silylation commonly known in the doping control field [37] can still be used, only an easy addition of ACN is required.

To investigate the yield of the derivatisation reaction when using microwave energy, comparison was made with conventional heating in an oven. Therefore, 2 calibration curves were made by spiking 6 blank urines with THCA at different concentration levels (5, 10, 20, 50, 75 and 100 ng/mL). The samples of the first calibration curve were heated for 90 s in the microwave for derivatisation, while the samples of the second calibration curve were heated for 30 min at 80°C in a conventional oven. The equations of both calibration curves are showed in Table 5.2, coefficients of determination were both higher than 99%.

Table 5.2: Comparing microwave assisted derivatisation (90 s at 750 W) to derivatisation using conventional heating (30min at 80°C), control samples spiked at 25 ng/mL THCA.

	Oven	Microwave
Calibration curve eq.	$y = 0.128580\,x - 0.020451$ $R^2 = 0.99880$	$y = 0.129871\,x - 0.028104$ $R^2 = 0.99953$
Mean concentration control samples (ng/mL)	24.3	24.41
Standard deviation on concentration control samples	0.392	0.493
T-Test	0.7716	

Subsequently, 2 sets of 6 THCA control samples spiked at 25 ng/mL were prepared using both heating methods to compare their performance. A t-test was used to determine whether there is a significant difference between the means of both groups. The calculated P-value was 0.7716, which indicates no difference between both groups.

This proves that the derivatisation yield after 90 s of irradiation at 750 W is comparable to the yield of the derivatisation with 30 min of conventional heating at 80°C.

3.3 Validation

3.3.1 Quantitative

For the quantification of THCA, calibration curves over 6 concentration levels (5, 10, 20, 50, 75 and 100 ng/mL) were constructed, using the method of least squares. The coefficient of determination r^2 was above 99%.

At each level, precision and bias were determined in 3 replicates. The Horwitz equation was used to calculate the tolerance levels for precision. For every calibration level the obtained relative standard deviations (RSD) were lower than 2/3rd of the calculated RSDmax. The bias was always below 15% (Table 5.3).

Table 5.3: Relative standard deviation and bias for each calibration level.

Conc.(ng/mL)	5	10	20	50	75	100
2/3RSDmax	23.68	21.33	19.22	16.75	15.75	15.09
RSD %	11.21	6.59	1.32	1.3	5.19	3.75
Bias %	6.87	-1.73	-6.83	-3.93	0.60	0.92

Retention times did not differ more than 2% between all samples, thereby complying with WADA's criteria [18].

To ensure correct identification in tandem mass spectrometric detection, two or more precursor-product ion transitions are monitored. The relative abundance of each transition is calculated relative to the most abundant transition (quantifier). The allowed tolerance windows for the relative abundances are traditionally imposed by governing bodies in the given field. Here the tolerance levels were calculated according to the WADA technical document [18]. For THCA all calculated transition ratios are between the calculated lower and upper limits (Table 5.1). Additionally the S/N ratio of the least intense diagnostic transition is calculated. On each concentration level of the calibration curve, this ratio was higher than 3 to 1 for the least abundant transition.

Selectivity of the method was tested by analyzing ten blank urines and urines spiked with mixtures of other WADA prohibited drugs and/or metabolites (50 anabolic steroids, 15 beta-blockers, 30 diuretics, 17 narcotics and 35 stimulants). These results showed that there are no interferences of the urine matrix and spiked compounds with the target analytes.

The calculation of the LOD, described by IUPAC, is based on the mean value of the blank signal [19]. Therefore, 10 blank urine samples were analysed and the standard deviation on the blank signal was calculated as 0.0178. With a slope of the calibration curve of 0.9399 and a coverage factor k of 3 for a 99.9% probability, the calculated LOD was 0.1 ng/mL. For the quantification limit IUPAC uses a coverage factor of 10. Using the same calculation the LOQ was found at 0.2 ng/mL. Although this is a low detection limit, it is of little importance in doping control since THCA is a threshold substance, but it does show the possibility to detect trace amounts of this compound in a biological matrix with limited sample preparation using GC-MS/MS.

In an effort to harmonize the results amongst all laboratories world-wide, WADA has defined the maximum combined uncertainties ($u_{c\ max}$) for all threshold compounds and calculated a decision limit (DL) that needs to be used when reporting results. This DL is the sum of the original threshold value and a guard band. The guard band is based upon the maximum acceptable value of the combined standard uncertainty ($u_{c\ max}$) multiplied by an appropriate expansion factor k (1.645; representing a 95% one-sided confidence level). In the case of THCA, a urine sample can be reported as ‘positive’ if the concentration of THCA exceeds the DL of 18 ng/ml. Consequently, the standard uncertainty u_c of any methodology used should be less than the $u_{c\ max}$ of 1.5 ng/ml. This protocol is in agreement with the Eurachem guidelines on the use of uncertainty data in compliance testing [38].

Over 40 quality control samples, spiked at 15 ng/mL THCA, the RSD and bias were found to be 5.88% and 2.50% respectively. Using these values the calculation of the combined standard uncertainty u_c (by taking the square root out of the sum of squares of both parameters) resulted in 0.6 ng/mL. This is well below the maximal combined standard uncertainty $u_{c\ Max}$ of 1.5 ng/mL imposed by WADA. The expanded uncertainty for a 95% confidence level based on a 2-tailed distribution is calculated using a coverage factor k of 2.

3.3.2 Application to routine samples

20 urine samples, with concentrations of THCA above the WADA decision limit of 18 ng/mL, were analysed. The results were compared with concentrations obtained

when analyzing these urine samples with the GC-MS quantification method of De Cock et al [16]. To check the correlation between the 2 methods a Bland-Altman plot is used. As showed in Fig. 5.4 all values are in the 95% confidence interval, which proves the agreement between the GC-MS and the newly developed GC-QqQ-MS method.

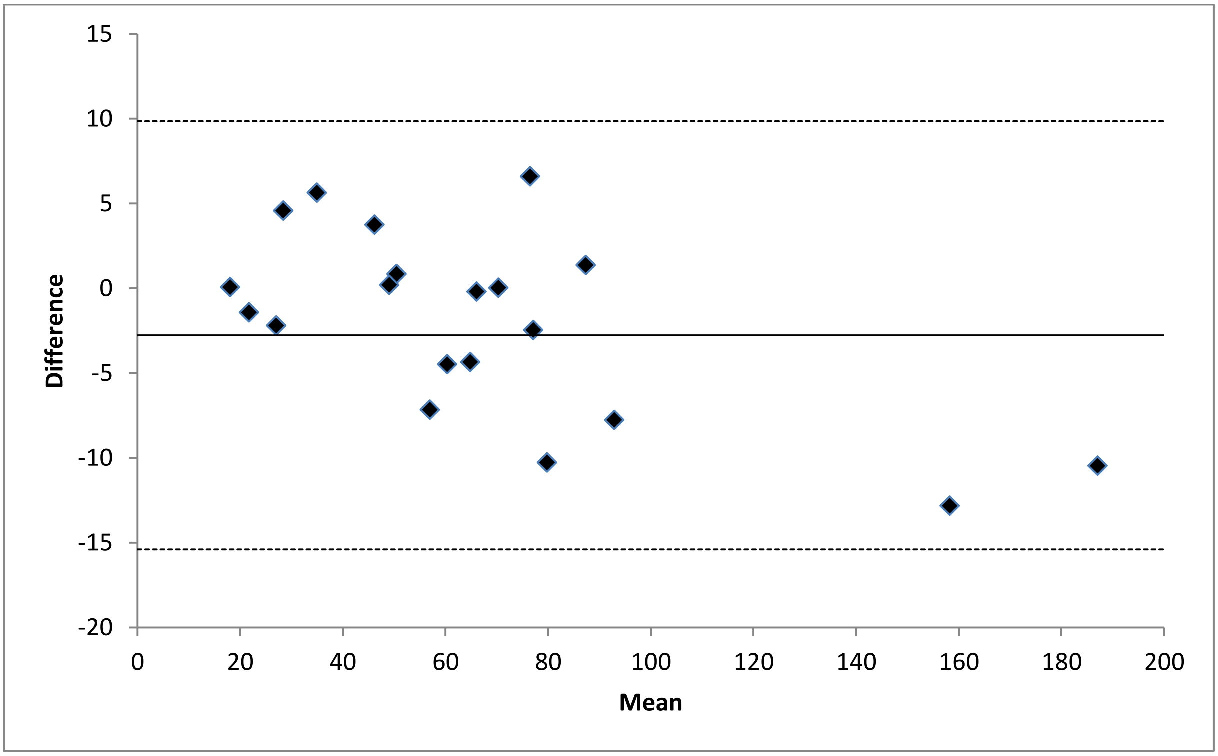


Fig. 5.4 Bland-Altman plot for the correlation between the GC-MS method of De Cock et al. [16] and the developed GC-QqQ-MS method for 20 urine samples positive for THCA. The 95% confidence interval (dotted lines) around the mean difference (solid line) is showed.

3.4 Additional considerations

Sample preparation is probably the most polluting part of an analytical method because of the high quantities of organic solvents used. Special attention was paid to the impact on the environment and the associated overall cost per analysis.

In order to speed up the sample preparation time, methods using (automated) SPE for the extraction of THCA in urine were developed [20,26]. The SPE protocol normally requires a conditioning step using 2 to 4 mL of solvent [25,32]. After loading the sample, unwanted interferences are removed by successive washes using several mL of solvent like methanol, acetic acid and ACN[20,25,26]. Finally more MeOH and ACN is added to elute the compounds of interest from the cartridge.

The LLE methodology proposed here only consumes 3 mL of organic solvents to extract the free THCA in urine and there is no need for SPE cartridges. Such procedure is environmentally friendlier and reduces the overall cost of the procedure.

4 Conclusions

A selective method for the quantification of THCA in urine was developed and validated. Special attention was paid to the limited volume of urine available and the total analysis time.

The use of microwave assisted derivatisation allows for a fast and complete silylation of THCA in 90 s, whereas this step would take at least 30 min using conventional heating in an oven. In order to monitor the performance of the domestic microwave a calorimetric methodology was used.

This method can be applied as a confirmation procedure after a positive finding of THCA in a screening method and allows for the quantification in less than 30 min.

5 Note: Implementation of the new Technical Document TD2013 DL

On 11 May 2013, WADA announced an update on the technical document on decision limits for the confirmatory quantification of threshold substances (TD2013 DL [39]). This document contains a new directive for the confirmation of THCA in urine, as an answer to the submissions received from stakeholders during the review process of the Code [40].

Concrete, this new document contains an increase of the threshold for THCA in urine from 15 ng/mL – as described in this chapter – to 150 ng/mL. This new threshold aims at reducing the chances for a positive test *in competition* after the (allowed) *out of competition* use of cannabis. Very likely, this will lead to a decrease in the number of Adverse Analytical Findings for THCA in the future (cfr. Statistics in Chapter 1, paragraph 4.1).

This new technical document necessitates a revision of the method described in this chapter. Indeed, both the range of the calibration curve (currently the highest level is 100 ng/mL) as the estimation of the measurement uncertainty need an update. However, since sample preparation and both chromatographic and mass spectrometric conditions are still applicable, the method in general, can be maintained.

References

[1] F. Grotenhermen. Pharmacokinetics and pharmacodynamics of cannabinoids. *Clin Pharmacokinet*. 2003, *42*, 327–60.

[2] H. Kalant. Adverse effects of cannabis on health: an update of the literature since 1996. *Progress in Neuro-Psychopharmacology & Biological Psychiatry*. 2004, *28*, 849–63.

[3] F. Musshoff, B. Madea. Review of biologic matrices (urine, blood, hair) as indicators of recent or ongoing cannabis use. *Therapeutic Drug Monitoring*. 2006, *28*, 155–63.

[4] M.A. Huestis, I. Mazzoni, O. Rabin. Cannabis in sport: anti-doping perspective. *Sports Medicine*. 2011, *41*, 949–66.

[5] M. Yonamine, D.R. Campos, R.L.D. Moreau. Marijuana as doping in sports. *Sports Medicine*. 2003, *33*, 395–99.

[6] M. Saugy, L. Avois, C. Saudan, N. Robinson, C. Giroud, P. Mangin, J. Dvorak. Cannabis and sport. *British Journal of Sports Medicine*. 2006, *40*, 13–15.

[7] WADA. The 2013 Prohibited List, International Standard. 2013, http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-Prohibited-list/2013/WADA-Prohibited-List-2013-EN.pdf

[8] WADA. International Standard for Laboratories, version 7.0. 2012, http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-IS-Laboratories/ISL/WADA_Int_Standard_Laboratories_2012_EN.pdf

[9] J. Penders, A. Verstraete. Laboratory guidelines and standards in clinical and forensic toxicology. *Accreditation and Quality Assurance*. 2006, *11*, 284–90.

[10] T.P. Lyons, C.K. Okano, J.A. Kuhnle, M.R. Bruins, W.D. Darwin, E.T. Moolchan, M.A. Huestis. A comparison of Roche Kinetic Interaction of Microparticles in Solution (KIMS) assay for cannabinoids and GC-MS analysis for 11-nor-9-carboxy-delta9-tetrahydrocannabinol. *Journal of Analytical Toxicology*. 2001, *25*, 559–64.

[11] S. Chericoni, I. Battistini, S. Dugheri, M. Pacenti, M. Giusiani. Novel method for simultaneous aqueous in situ derivatization of THC and THC-COOH in human urine samples: validation and application to real samples. *Journal of Analytical Toxicology*. 2011, *35*, 193–98.

[12] C. Moore. Oral fluid for workplace drug testing: Laboratory implementation. *Drug Testing and Analysis*. 2011, 89–93.

[13] J. Rohrich, I. Schimmel, S. Zorntlein, J. Becker, S. Drobnik, T. Kaufmann, V. Kuntz, R. Urban. Concentrations of Delta(9)-Tetrahydrocannabinol and 11-Nor-9-Carboxytetrahydrocannabinol in Blood and Urine After Passive Exposure to Cannabis Smoke in a Coffee Shop. *Journal of Analytical Toxicology*. 2010, *34*, 196–203.

[14] WADA. WADA Technical Document – TD2010DL, Decision Limits for the confirmatory quantification of threshold substances. 2010, http://www.wada-

ama.org/Documents/World_Anti-Doping_Program/WADP-IS-Laboratories/WADA_TD2010DLv1.0_Decision Limits for the Confirmatory Quantification of Threshold Substances_May 08 2010_EN.doc.pdf

[15] M. Felli, S. Martello, M. Chiarotti. LC-MS-MS method for simultaneous determination of THCCOOH and THCCOOH-glucuronide in urine: Application to workplace confirmation tests. *Forensic Science International*. 2011, *204*, 67–73.

[16] K.J.S. De Cock, F.T. Delbeke, D. De Boer, P. Van Eenoo, K. Roels. Quantitation of 11 -nor-delta9-tetrahydrocannabinol-9-carboxylic acid with GC-MS in urine collected for doping analysis. *Journal of Analytical Toxicology*. 2003, *27*, 106–09.

[17] J. Housova. Microwave Heating - the Influence of Oven and Load Parameters on the Power Absorbed in the Heated Load. *Czech J. Food Sci.* 2002, *20*, 117–24.

[18] WADA. WADA Technical Document – TD2010IDCR, Identification criteria for qualitative assays incorporation column chromatography and mass spectometry. 2010, http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-IS-Laboratories/WADA_TD2010IDCRv1.0_Identification Criteria for Qualitative Assays_May 08 2010_EN.doc.pdf

[19] J. Mocak, A.M. Bond, S. Mitchell, G. Scollary. A statistical overview of standard (IUPAC and ACS) and new procedures for determining the limits of detection and quantification: Application to voltammetric and stripping techniques (technical report). *Pure and Applied Chemistry*. 1997, *69*, 297–328.

[20] P.R. Stout, C.K. Horn, K.L. Klette. Solid-phase extraction and GC-MS analysis of THC-COOH method optimized for a high-throughput forensic drug-testing laboratory. *Journal of Analytical Toxicology*. 2001, *25*, 550–54.

[21] H. Madhavaram, R.A.F. Couch. Utilization of a detection level of 25 ng/mL for cannabinoids in urine using a CEDIA (R) THCPLUS immunoassay: Application of this cut-off to urines of school children. *Forensic Science International*. 2010, *198*, 28–30.

[22] S. Strano-Rossi, A.M. Bermejo, X. de la Torre, F. Botre. Fast GC-MS method for the simultaneous screening of THC-COOH, cocaine, opiates and analogues including buprenorphine and fentanyl, and their metabolites in urine. *Analytical and Bioanalytical Chemistry*. 2011, *399*, 1623–30.

[23] S. Strano-Rossi, F. Molaioni, F. Rossi, F. Botre. Rapid screening of drugs of abuse and their metabolites by gas chromatography/mass spectrometry: application to urinalysis. *Rapid Communications in Mass Spectrometry*. 2005, *19*, 1529–35.

[24] C. Chebbah, O.J. Pozo, K. Deventer, P. Van Eenoo, F.T. Delbeke. Direct quantification of 11-nor-Delta(9)-tetrahydrocannabinol-9-carboxylic acid in urine by liquid chromatography/tandem mass spectrometry in relation to doping control analysis. *Rapid Commun Mass Spectrom*. 2010, *24*, 1133–41.

[25] W. Weinmann, M. Goerner, S. Vogt, R. Goerke, S. Pollak. Fast confirmation of 11-nor-9-carboxy-Delta(9)-tetrahydrocannabinol (THC-COOH) in urine by LC/MS/MS using negative atmospheric-pressure chemical ionisation (APCI). *Forensic Science International*. 2001, *121*, 103–07.

[26] M. Fernandez Mdel, S.M. Wille, N. Samyn, M. Wood, M. Lopez-Rivadulla, G. De Boeck. On-line solid-phase extraction combined with liquid chromatography-tandem mass spectrometry for high throughput analysis of 11-nor-Delta9-tetrahydrocannabinol-9-carboxylic acid in urine. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2009, *877*, 2153–57.

[27] N. Stephanson, M. Josefsson, R. Kronstrand, O. Beck. Accurate identification and quantification of 11-nor-delta(9)-tetrahydrocannabinol-9-carboxylic acid in urine drug testing: evaluation of a direct high efficiency liquid chromatographic-mass spectrometric method. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2008, *871*, 101–08.

[28] P. Van Eenoo, W. Van Gansbeke, N. De Brabanter, K. Deventer, F.T. Delbeke. A fast, comprehensive screening method for doping agents in urine by gas chromatography-triple quadrupole mass spectrometry. *Journal of Chromatography A*. 2011, *1218*, 3306–16.

[29] C.G. Georgakopoulos, P. Kiousi, Y.S. Angelis, E. Lyris, M. Koupparis, A.C. Calokerinos, J. Atta-Politou. Two-step silylation procedure for the unified analysis of 190 doping control substances in human urine samples by GC-MS. *Bioanalysis*. 2009, *1*, 1209–24.

[30] C.O. Kappe, S.L. Soderholm, M. Damm. Microwave-assisted derivatization procedures for gas chromatography/mass spectrometry analysis. *Molecular Diversity*. 2010, *14*, 869–88.

[31] A. de la Hoz, A. Diaz-Ortiz, A. Moreno. Microwaves in organic synthesis. Thermal and non-thermal microwave effects. *Chemical Society Reviews*. 2005, *34*, 164–78.

[32] J.C. Cheong, S.I. Suh, B.J. Ko, J.Y. Kim, M.K. In, W.J. Cheong. Gas chromatography-mass spectrometric method for the screening and quantification of illicit drugs and their metabolites in human urine using solid-phase extraction and trimethylsilyl derivatization. *Journal of Separation Science*. 2010, *33*, 1767–78.

[33] F. Botre, L. Amendola, C. Colamonici, M. Mazzarino. Rapid determination of diuretics in human urine by gas chromatography-mass spectrometry following microwave assisted derivatization. *Analytica Chimica Acta*. 2003, *475*, 125–36.

[34] X.M. Zhang, C.H. Deng, X.Y. Yin, L.J. Zhang. Development of microwave-assisted derivatization followed by gas chromatography/mass spectrometry for fast determination of amino acids in neonatal blood samples. *Rapid Communications in Mass Spectrometry*. 2005, *19*, 2227–34.

[35] M.R. Lee, L.W. Chung, G.J. Liu, Z.G. Li, Y.Z. Chang. Solvent-enhanced microwave-assisted derivatization following solid-phase extraction combined with gas chromatography-mass spectrometry for determination of amphetamines in urine. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences*. 2008, *874*, 115–18.

[36] Z.M. Wang, X. Xu, X. Zhao, Y.P. Zhang, D. Li, R. Su, Q.L. Yang, X.Y. Li, H.H. Zhang, H.Q. Zhang. Microwave-accelerated derivatization prior to GC-MS determination of sex hormones. *Journal of Separation Science*. 2011, *34*, 1455–62.

[37] A.G. Fragkaki, Y.S. Angelis, A. Tsantili-Kakoulidou, M. Koupparis, C. Georgakopoulos. Statistical analysis of fragmentation patterns of electron ionization mass spectra of enolized-trimethylsilylated anabolic androgenic steroids. *International Journal of Mass Spectrometry*. 2009, *285*, 58–69.

[38] Eurachem. *The Fitness for Purpose of Analytical Methods A Laboratory Guide to Method Validation and Related Topics*. 1998,

[39] WADA. Decision limits for the confirmatory quantification of threshold substances. 2013, http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-IS-Laboratories/Technical_Documents/WADA-TD2013DL-Decision-Limits-for-the-Confirmatory-Quantification-Threshold-Substances-2.0-EN.pdf

[40] WADA. World Anti-Doping Code. 2009, http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-The-Code/WADA_Anti-Doping_CODE_2009_EN.pdf

PART III

Synthetic cannabinoids, a new class of designer drugs.

Chapter 6:

Synthetic cannabinoids: general considerations

Adapted from:

N. De Brabanter , K. Deventer , V. Stove, P. Van Eenoo

Synthetic cannabinoids: general considerations.

Proceedings of the Belgian Royal Academies of Medicine, *2013, 2, 209-225.*

Abstract

Around 2008, synthetic cannabinoids were found to be present in and responsible for the psychoactive effects of herbal mixtures with names like ‘Spice’ or ‘K2’. In response to the increased popularity of these products, (inter)national organizations and governments started banning these cannabimimetics gradually. However, the lack of a uniform and international regulation makes it hard to control this issue.

For the different types of synthetic cannabinoids the scientific knowledge in terms of pharmacokinetics and pharmacodynamics is limited. This also means that little is known on the health of users, both on short and long term.

In the last years effort has been made to make detection of these products possible in different biological matrices. However, since the number of cannabimimetic compounds on the market appears to grow every month, both scientist and legislators run after a moving target.

1 Introduction

Starting from 2004, a new generation of psychoactive substances appeared on the market. These products, with brand names like ‘Spice’ or ‘K2’, are sold as herbal mixtures and are available in many European countries [1]. Packed as ‘natural herbal incense’ or ‘room odorizers’, these products can be traded legally in head shops and online stores [2,3]. After smoking these mixtures, users reported cannabis-like effects on internet forums. These effects were first explained in 2008 by the detection of synthetic cannabinoids like JWH-018 as active ingredient [4], although not mentioned on the package.

Throughout the years, more of these products were identified as additives in these packages of herbal material. As a response to the rising popularity of these compounds, several countries started monitoring and even banning these products [5].

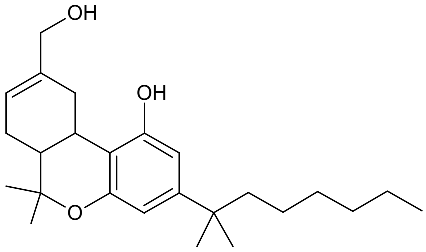
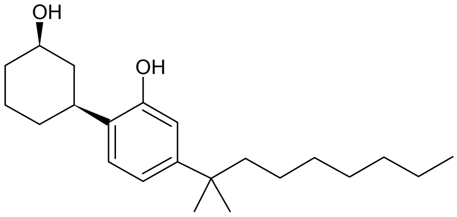
The search for compounds with THC-like properties in the human body, i.e. synthetic cannabinoid receptor agonists or briefly cannabimimetics, started in the pharmaceutical industry. In a way to separate the wanted pain-relieving effects from the unwanted psychotropic effects, several categories of products were synthesized and subjected to SAR (structure activity relationship) tests. The academic and/or pharmaceutical origin of these compounds is often reflected in the name of the product. In the best known class of JWH-compounds, these initials stands for the name of the organic chemistry professor John W. Huffman, who first synthesized these products in the 1990s. In a similar way AM (e.g. in AM-630) refers to professor Alexandros Makriyannis from Northeastern University and HU (e.g. from HU-210) to Hebrew University. Also, the pharmaceutical industry realized the potential value of these products, leading to the synthesis of the CP-family (e.g. CP-47,497) by Pfizer and the WIN-group (e.g. WIN 55,212-2) by the former Sterling Winthrop Pharmaceuticals.

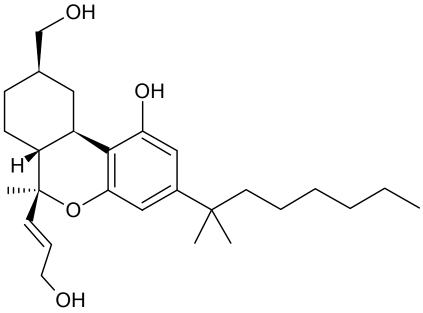
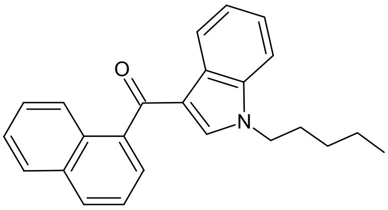
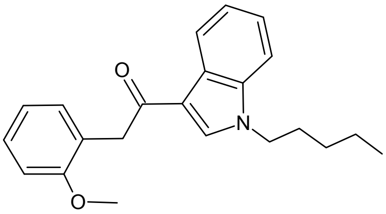
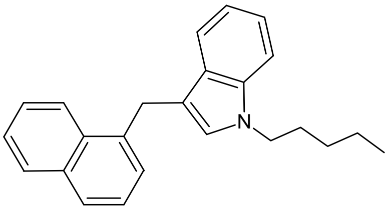
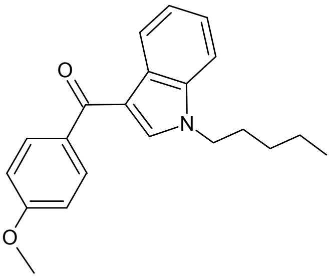
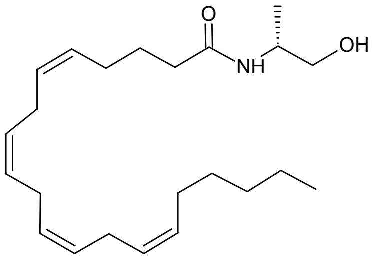
In general, they are lipid soluble, non-polar molecules, containing 20 to 26 carbon atoms [6]. Based upon this chemical structure, synthetic cannabinoids can be divided into different classes (Table 6.1) [1].

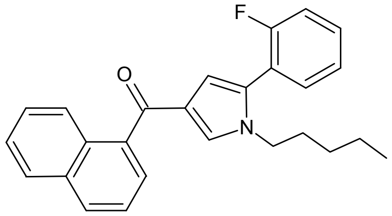
- Classical cannabinoids: structurally related to THC from *Cannabis sativa.*
- Non-classical cannabinoids: cyclohexylphenols or 3-arylcyclohexanols
- Hybrid cannabinoids: structural combinations of both classical and non-classical cannabinoids.
- Aminoalkylindoles;
  - Naphthoylindoles
  - Phenylacetylindoles
  - Naphthylmethylindoles
  - Benzoylindoles
- Eicosanoids: endocannabinoids and synthetic analogs
- Others: diarylpyrazoles, naphthoylpyrroles, etc.

It should be noted that, depending on the source, the classifications can vary. In the light of an internationally uniformed approach, referring to the (abovementioned) classification of a leading institution as the United Nations Office on Drugs and Crime (UNODC) is recommended.

Table 6.1. Classification of synthetic cannabinoids according to the UNODC [1], with some typical examples. The underlined compound is illustrated in the right column.

Classical cannabinoids e.g. THC, HU-210, AM906, …	
Non-classical cannabinoids e.g. CP-47,497-C8, CP-55,940, HU-308, …	

Hybrid cannabinoids e.g. AM-4030	
Naphthoylindoles e.g. JWH-018, JWH-073, JWH-122, JWH-200, …	
Phenylacetylindoles e.g. JWH-250, RCS-8, JWH-203, …	
Naphthylmethylindoles e.g. JWH-175, JWH-184, JWH-185, …	
Benzoylindoles e.g. AM-630, AM-2233, RCS-4, …	
Eicosanoids e.g. anandamide, methanandamide, …	

Others e.g. JWH-307, CRA-13, …	

2 Abuse of synthetic cannabinoids

Packages of 'Spice' usually contain approximately 3g of herbal material and are often sold in head shops, gas stations or via internet shops. The price varies around €10/g, which is considered expensive compared to traditional cannabis [4]. It is promised that the inhalation of the blends of psychoactive plants gives the user a similar experience as marijuana, only using legal alternatives. A survey in the US showed that 'Spice' products were primarily smoked, but also administration via vaporization, oral and rectal ingestion were reported [7].

Little is known on the exact composition and the properties of the used plants and in many cases the ingredients listed on the package do not cover the content either [8,9]. The manufacturers of these blends make users believe the effects are caused by the mix of plant material used. However, research on the botanical material showed that most of the plant species do not have psychoactive properties and are therefore only used to dilute the added cannabimimetics [9]. Moreover, the producers try to present their products as natural and safe in order to circumvent the marijuana policy of governments. The UNODC concluded that producers respond very fast to changes in legislations by making small modifications to the new products launched [1].

The success of this 'legal-highs' business is reflected in the increasing number of web shops selling these products online. In 2009, the European Monitoring Centre for Drugs and Drugs Addiction (EMCDDA) found 115 online shops offering psychoactive substances in Europe, in 48% of these 'Spice' products were offered. In a recent report mention is made of an increase to 314 online shops in 2011 and 690 in January 2012 [10]. Moreover, an investigation via Google® performed by the Belgian Monitoring Centre for Drugs and Drug Addiction (BMCDDA), showed that all new products reported in 2010 were already offered for sale online even before their existence was picked up by the Belgium Early Warning System for Drugs (BEWSD) [11]. Also the list of products with names like ‘Spice gold’, ‘Yucatan Fire’ and ‘Lava Red’ continued to rise [8]. Furthermore, recent investigation in Poland demonstrated that many herbal blends contained more than one psychoactive ingredient [12].

A survey of the member states of the European Union by the EMCDDA showed that in 2009, ‘Spice’ products were identified in 21 out of the 30 countries. At that time no products containing synthetic cannabinoids were found in Belgium. In 2011 however, Belgian laboratories reported 11 synthetic cannabinoids to the European Early Warning System (EWS) and for the first time a complete laboratory capable of producing and packing synthetic cannabinoids was dismantled in Belgium. Similar facilities were reported in Ireland and the Netherlands and are the link between the producers – mainly located in China and India – and the customers in Europe. Since these substances can be produced cheaply, it is clear that these businesses are very lucrative [10]. During the production process, the synthesized synthetic cannabinoids are distributed over the dried plant material. This is usually done by homogenization with cannabinoids in the crystalline form or by spraying the products dissolved in an organic solvent. However, recently also the starting materials are being sold as ‘research chemicals’ via online shops or traders [13].

The rate at which a specific product is spreading, is also noteworthy. In Europe, JWH-018 was first reported by Austria in December 2008. Only in the first year after this detection, eight more neighboring countries confirmed this finding, followed by ten more in the next months. Similar developments were reported for other compounds like JWH-073 (13 countries), CP47, 497 (10 countries), JWH-122 (14 countries), JWH-081 (10 countries) and AM-2201 (11 countries) [11]. In 2011, already 23 new synthetic cannabimimetics were reported through the European Early Warning System (EWS), in 2012 another 30 followed. With a current total of 84 compounds (May 2013), the synthetic cannabinoid receptor agonists are, despite their recent introduction, already the largest drug family monitored by the EMCDDA [14].

An internet search learned that mostly young people - especially men - aged between 25 and 40 are using Spice-like products. The reasons are various; ranging from previous cannabis users looking for a substitute over people in search of legal drugs to experimental users seeking sensation [8]. In 2008, German authorities found a strong increase in the interest for these products after a period of biased media attention in which their use as legal cannabis substitutes was announced. Once the presence of synthetic compounds was demonstrated and some of these products were banned, the opposite trend was observed, leaving only the users looking for a cannabis substitute to avoid positive testing [1].

Even in sport drug testing, the first cannabimimetics were reported in the statistics of the World Anti-Doping Agency (WADA). However, with only 3 positive cases in 2011, the number remains small compared to the traditional THC abuse in sport competitions (n = 442 in 2011) [15].

3 Legislation

Since synthetic cannabinoids are currently not controlled under the UN Drug control conventions, the legal status of these compounds depends on the drug laws of individual countries [1].

In Europe, the first actions by governments were taken in 2009. The first discovered compounds JWH-018, HU-210 and CP47,479 and its homologues were included in national drug laws in Austria, Germany, France, Luxembourg, Sweden, Estonia, Poland, Hungary and the United Kingdom. A recent report on the evolution of the situation in Poland over the last few years, showed that both the compounds and the way of trading changed in response to the successive actions of the Polish government [12]. As mentioned earlier, it is sufficient to make a small change in the drug structure to stay one step ahead of the legislator. Therefore, the UK and Ireland started using generic definitions to include products which will appear in the future [8]. Recently, other European countries also adopted this strategy in substitution for the earlier used approach of individual listing of already identified synthetic cannabinoids [10].

In Belgium the first legislative actions were taken in 2011, by adding the first seven compounds to the list of prohibited psychotropic substances. In 2013 six more cannabimimetics were listed (Table 6.2). Unfortunately, to this day, no generic definitions have come into force.

In the United States, the Synthetic Drug Abuse Prevention Act of 2012 placed cannabimimetic agents in Schedule I, making manufacturing, distributing, or possessing these products illegal. Besides this federal law, several states and even individual cities have taken additional measures to control 'Spice' abuse [16].

Since, similar to classical cannabis, the synthetic analogues are predominantly smoked, it is not inconceivable that passive inhalation of the smoke can result in positive testing. Once this was observed for cannabis [17], threshold concentrations were installed to distinguish active from passive use [18]. However, up to now, this possibility has not been studied for the cannabimimetics currently flooding the market.

In general, legislation, both within and outside Europe, is too diverse and therefore not efficient to tackle international issues as 'legal highs'.

Table 6.2. Synthetic cannabinoids present on the list of prohibited psychotropic substances in Belgium.

KB 2011-09-26/16, art. 1,011 *Effective date: 23/10/2011*	**KB 2013-03-20/08, art. 1,012** *Effective date: 22/04/2013*
JWH-018	AM-694
JWH-073	AM-2233
JWH-250	WIN 48,098
JWH-398	JWH-307
CP-47,497	A-796,260
HU-210	XLR-11 (5F-UR144)
JWH-210	

4 Pharmacokinetics

Contrary to the classical THC for which the pharmacokinetics have been investigated [19,20], no such data are available for the synthetic analogues.

As described in several reports on the experiences of ‘Spice’ users, the effect is quickly noticeable after smoking a few grams of herbal material [2,6,16]. These observations are supported by a recent study on the quantification of JWH-018 in blood after smoking the incense ‘Smoke’ [21]; the maximum concentrations were found 5 min post-smoking. This shows that after inhalation, the absorption via the lungs and the distribution over organs like the brain takes place in a few minutes [1]. It was found that the measured maximum blood concentrations of JWH-018 were already decimated after 3h and the parent compound was detectable until 48h after administration [21].

Investigation of the metabolisation of cannabimimetics is not as straightforward as for pharmaceutically approved agents. Since there are little or no pharmacological data available for these compounds, human administration in order to perform excretion studies is ethically questionable. Therefore, most studies use models to reveal the metabolic pathways in the human body. In one of the most common approaches human liver microsomes are used to investigate the metabolisation *in vitro* [22–25], in the search for a more complete model with higher complexity also *in vivo* mice experiments are performed [26]. In some cases, human urine samples are available from caught users [27–29] or conducted self-experiments [30].

In general, these compounds are excessively metabolized in the human body. In all metabolic studies on compounds of the aminoalkylindole family described up to now a similar series of modifications were found: single or multiple hydroxylations, carboxylation, dehydrogenation, dealkylation and dihydrodiol formation. From the data obtained using *in vivo* models or positive urine samples, it was found that these metabolites are mainly excreted as glucuronide and/or sulphate conjugates in urine [26]. The monohydroxylated [31] and carboxylated [28] metabolites are mainly found in the highest quantities in urine.

5 Pharmacodynamics

Although the effects of *Cannabis sativa* and derivates are known for centuries, it was only in the last twenty years that the interactions in the human body were revealed [6,32].

Today, two cannabinoid receptors are described in the human body. Both CB1 and CB2 are G-protein coupled receptors with an important function in intercellular signaling. The CB1 receptor is distributed in the brain and the central nervous system, mainly expressed presynaptically, and decreases the release of neurotransmitters like dopamine [33]. Activation of the CB1 receptor is responsible for the psychotropic effects assigned to cannabis use.

CB2 receptors are located in immune cells and interfere in the regulation of the inflammatory process [19]. Therefore in the medical field, research has focused on receptor agonists selective for this CB2 receptor aiming for the therapeutic effects and hereby avoiding the psychotropic effects induced by the interaction with the CB1 receptor.

Next to endocannabinoids [33], plant derived and other exogenous cannabinoids act as agonists of both receptors with varying affinity. Classical cannabinoids like THC have comparable affinity for both receptors, about 40 nM, without a major selectivity for a particular receptor [34]. As shown in Table 6.3, this is different for synthetic cannabinoids. The affinity of the most prevalent cannabimimetic compounds is significantly higher, especially towards the CB1 receptor. With this in mind, it can be expected that compounds with lower potency (i.e. lower affinity for the CB1 receptor) than classical THC will not be used in 'Spice'-like products. Nevertheless JWH-015 was recently detected in a herbal blend in Latvia [1].

When using data on receptor affinities, one should be careful when using exact numbers. Indeed, depending on the experimental set-up, variation in the values is possible [34]. In short, the receptor affinity (K_i) is determined as the ability of the given compound to displace a potent radio labeled cannabinoid (usually tritiated CP-55,940 or tritiated WIN-55,512-2) from their binding sites [35]. For a potent cannabinoid, low concentrations will be sufficient to achieve this. Since this concentration (IC50) is proportional to the receptor affinity K_i, the more potent the cannabinoid, the lower K_i (Table 6.3).

Table 6.3. Receptor affinities for both cannabinoid receptors for some common cannabimimetics.

Compound	K_i - CB1 (nM)	K_i - CB2 (nM)
HU-210	0.06 [34]	0.52 [34]
JWH-122	0.7 [36]	1.2 [36]
JWH-073	8.9 [35,37]	27 [35], 38 [37]
JWH-018	9 [35]	2.9 [35]
CP47,497	9.54 [38]	
JWH-250	11[39]	33[39]
Δ^9-THC	**39.5** [34]	**40** [34]
JWH-015	383 [34], 164[35]	13.8 [34,35]

6 Health risks

Although there are case reports describing the effects experienced immediately after the use of ‘Spice’ [2,3,16], little or no information is available on the long term effects or the consequences of regular use.

In 2009, Zimmermann et al. reported on a patient who showed symptoms of a physical withdrawal syndrome after using ‘Spice Gold’ on a daily basis for about 8 months. While the patient initially used only 1g of product every day, the decreasing effect experienced made him increase the dose up to 3g daily. Both physical (sweating, tremor, insomnia, nausea, etc.) as psychological (depression, desperation, desire for ‘Spice Gold’) effects were observed the first days of treatment in hospital [40]. In another paper, psychosis was diagnosed in ten patients after smoking herbal blends containing synthetic cannabinoids, which lasted months after the final use [41].

Recently, compound specific data related to harm assessment have been included in the European Database on New Drugs (EDND). For two compounds, chronic physical damage after use is mentioned. It is related to learning difficulties and cognitive ailment for HU-210 and JWH-018, respectively. Moreover, for both compounds physical dependence (withdrawal symptoms) was reported, together with psychological dependence for JWH-018 and JWH-122 [11].

Predicting the possible effects of a particular herbal blend is almost impossible. It was shown that the content of these packages varies significantly and is often not in accordance with the indications on the package. Toxicological data on the used plant material are not available and then again, mostly the indications on the packages with regard to the herbal material are not reliable [9]. Concerning the added synthetic cannabinoids, it was shown that concentrations can vary [5] and that some blends may contain two or more active compounds [42]. Although research showed that the used chemicals are of high purity [43], the presence of impurities with unknown toxicity cannot be ruled out. In that way, it is not possible to estimate the impact when smoking a few grams of a given mixture. In general, the observed effects are very diverse and highly dependent on the type of herbal blend or synthetic cannabinoid(s) used. Most described psychoactive effects are: alterations in mood (from euphoria to anxiety) [5], hallucinations, agitated behavior and hyperreflexia [16]. Medical investigation showed symptoms like increased pulse rates [6] and blood pressure, flushed skin, dilated pupils and nausea [16]. It was reported that the major psychotropic and physical effects disappear after 6h to 8h [5,6].

Together with the fact that these cannabimimetics have stronger affinities for the cannabinoid receptors compared to THC, it is not unlikely that overdosing would lead to life-threatening intoxications. This is confirmed in case studies [44] and reflected in the increasing statistics of Poison Control Centers in the US: in 2011 there were reports of over 4000 synthetic marijuana exposures in a period of 8 months, which is an increase of 52% compared to 2010 [45].

7 Detection of synthetic cannabinoids abuse

In the past, several screening procedures – both via immunological and chromatographic techniques – have been developed to screen for the use of products from *Cannabis sativa* in different matrices [18,46–49]. The abuse of the growing group of cannabimimetics, synthesized over the last years, however, cannot be detected with these methods.

For the identification of spiked substances in the herbal material in particular, a more or less standard strategy is used. The herbal material is extracted and subsequently analyzed by means of a chromatographic technique mostly combined with mass spectrometric detection [50]. Next, the outcome is compared with databases containing the already known synthetic cannabinoids [51]. If it turns out to be an unknown compound, the structure is elucidated by using high resolution mass spectrometry (HRMS) or NMR (Nuclear Magnetic Resonance) technology [52–54].

When it comes to detection in the human body, different approaches can be used depending on the type of biological sample available. In serum or whole blood, both the unchanged target compound and its metabolites are present and can be extracted and analyzed by means of liquid chromatography (LC) [55,56]. The methods developed up till now mostly target the parent compounds, since this eliminates the need for the time-consuming search for metabolites and allows the quick update of the method after the release of a new compound in the future [57,58]. For oral fluid testing, detection of this parent compound is possible, even via direct injection on the LC system [59]. However, it should be noted that detection in the latter matrix is limited to a few hours after consumption [60].

If urine is the matrix of choice, knowledge on the metabolisation is essential, since no unchanged parent compound is found to be excreted. To detect these metabolites, an enzymatic hydrolysis is usually performed, followed by an extraction and analysis by means of liquid chromatography coupled to mass spectrometry (LC-MS) [61]. For JWH-018 it was found that monohydroxylated [31] and carboxy metabolites [28,62] are excreted in the highest concentrations, which makes these the metabolites of choice to implement in routine screening methods. Similar results are found for other indole-based cannabimimetics [26,29,63,64]. Based upon these findings, chromatographic methods are developed and validated to screen for synthetic cannabinoid metabolites in urine [65,66]. Also commercial tests for synthetic cannabinoids became available, promising a detection window of 72 h after a single use. Peer-reviewed data on detection times - although rare - indicate similar ranges [30,67]. No information is available on the accumulation in the body of chronic users. However, in those cases detection in urine would be possible up to 3 weeks [67].

Immunochemical-based detection methods have the advantage of being cheaper and faster than the chromatographic procedures referred to above, but the development and implementation was long in coming. Indeed, developing such immunoassays is a challenging task given the great structural variety between the compounds of the cannabimimetics family. Only recently, the first screening method, using enzyme linked immunosorbent assays (ELISA) for the detection of metabolites of the naphthoylindole group in urine, has been described in literature [68]. Gradually, also commercial kits for high-throughput screening of synthetic cannabinoids have become available [69,70]. For any positive outcome however, a confirmatory analysis by means of the more selective chromatographic techniques remains essential.

It should be noted that correct identification of these products remains a difficult task since the availability of reference material is lagging behind on the rapid release of new products on the market [6]. The latter makes it also difficult to keep screening methods up to date, since the existing methods are not able to detect non-target (i.e. currently unknown) compounds. To close this gap, an open screening approach whereby the method is capable of detecting a class of cannabinoids in a non-targeted way could be a solution.

8 Perspectives - Conclusions

Despite the increasing number of actions taken by governments and other (inter)national institutions, the ‘Spice’ issue is still expanding. The list of synthetic cannabinoids detected continues to grow and the statistics on hospitalizations due to the use of these herbal blends do follow the same trend.

Although effective interventions of the authorities are necessary to tackle these problems, strict legislation also has a downside. The total ban on these products takes away the opportunity to investigate the therapeutic properties. Taking into account the successful use of plant derived cannabis in medicine, there is a demand to provide the possibility to do research that leads towards the medicinal use of these synthetic analogues [71].

It is clear that further research in this field is necessary. When it comes to pharmacodynamics, so far only the properties of the parent compound are investigated. However, recent data show that also the formed metabolites remain active in the human body by binding to both cannabinoid receptors [72].

For routine testing, methods should be developed to improve detection in different biological matrices. Given the rapidly growing number of products appearing on the market, an open-screening approach could be a big step forward. When routine screening becomes more common, there will be a need for uniform regulations taking also into account the problem of passive inhalation, as known for THC smoke.

References

[1] UNODC. Synthetic cannabinoids in herbal products. http://www.unodc.org/documents/scientific/Synthetic_Cannabinoids.pdf

[2] I. Vardakou, C. Pistos, C. Spiliopoulou. Spice drugs as a new trend: Mode of action, identification and legislation. *Toxicology Letters*. 2010, *197*, 157–62.

[3] E.W. Gunderson, H.M. Haughey, N. Ait-Daoud, A.S. Joshi, C.L. Hart. “Spice” and “K2” herbal highs: a case series and systematic review of the clinical effects and biopsychosocial implications of synthetic cannabinoid use in humans. *The American journal on addictions / American Academy of Psychiatrists in Alcoholism and Addictions*. *21*, 320–26.

[4] R. Lindigkeit, A. Boehme, I. Eiserloh, M. Luebbecke, M. Wiggermann, L. Ernst, T. Beuerle. Spice: a never ending story? *Forensic science international*. 2009, *191*, 58–63.

[5] K.A. Seely, J. Lapoint, J.H. Moran, L. Fattore. Spice drugs are more than harmless herbal blends: A review of the pharmacology and toxicology of synthetic cannabinoids. *Progress in neuro-psychopharmacology & biological psychiatry*. 2012, *39*, 234–43.

[6] S. Hudson, J. Ramsey. The emergence and analysis of synthetic cannabinoids. *Drug testing and analysis*. 2011, *3*, 466–78.

[7] R. Vandrey, K.E. Dunn, J.A. Fry, E.R. Girling. A survey study to characterize use of Spice products (synthetic cannabinoids). *Drug and alcohol dependence*. 2012, *120*, 238–41.

[8] EMCDDA. Understanding the “Spice” phenomenon. 2009, 1–34. http://www.emcdda.europa.eu/attachements.cfm/att_80086_EN_Spice Thematic paper□? final version.pdf

[9] J. Ogata, N. Uchiyama, R. Kikura-Hanajiri, Y. Goda. DNA sequence analyses of blended herbal products including synthetic cannabinoids as designer drugs. *Forensic science international*. 2013, *227*, 33–41.

[10] EMCDDA, Europol. EMCDDA-Europol 2011 Annual Report on the implementation of Council Decision 2005/387/JHA. 2012, http://www.emcdda.europa.eu/attachements.cfm/att_155113_EN_EMCDDA-Europol Annual Report 2011_2012_final.pdf

[11] Belgian monitoring centre for drugs and drug addiction. Nieuwe psychoactieve substanties in België (2005-2011). 2012, 59. http://ewsd.wiv-isp.be/Shared documents/Reports/Nieuwe psychoactieve substanties in Belgi? 2005-2011.pdf

[12] D. Zuba, B. Byrska. Analysis of the prevalence and coexistence of synthetic cannabinoids in “herbal high” products in Poland. *Forensic Toxicology*. 2012, *31*, 21–30.

[13] UNODC. Identification and Analysis of Synthetic Cannabinoid Receptor Agonists in Seized Materials. 2013,

http://www.unodc.org/documents/scientific/STNAR48_Synthetic_Cannabinoids_ENG.pdf

[14] EMCDDA. Synthetic cannabinoids in Europe. 2013, http://www.emcdda.europa.eu/topics/pods/synthetic-cannabinoids

[15] WADA. 2011 Laboratory Testing Figures. 2012, http://www.wada-ama.org/Documents/Resources/Testing-Figures/WADA-2011-Laboratory-Testing-Figures.pdf

[16] C.R. Harris, A. Brown. Synthetic Cannabinoid Intoxication: A Case Series and Review. *The Journal of Emergency Medicine*. 2013, *44***,** 360–66.

[17] B. Law, P.A. Mason, A.C. Moffat, L.J. King, V. Marks. Passive inhalation of cannabis smoke. *The Journal of pharmacy and pharmacology*. 1984, *36***,** 578–81.

[18] N. De Brabanter, W. Van Gansbeke, F. Hooghe, P. Van Eenoo. Fast quantification of 11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid (THCA) using microwave-accelerated derivatisation and gas chromatography–triple quadrupole mass spectrometry. *Forensic Science International*. 2012, 90–95.

[19] F. Grotenhermen. Pharmacokinetics and pharmacodynamics of cannabinoids. *Clin Pharmacokinet*. 2003, *42***,** 327–60.

[20] M.A. Huestis. Human cannabinoid pharmacokinetics. *Chemistry & biodiversity*. 2007, *4***,** 1770–804.

[21] J. Teske, J.-P. Weller, A. Fieguth, T. Rothämel, Y. Schulz, H.D. Tröger. Sensitive and rapid quantification of the cannabinoid receptor agonist naphthalen-1-yl-(1-pentylindol-3-yl)methanone (JWH-018) in human serum by liquid chromatography-tandem mass spectrometry. *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences*. 2010, *878***,** 2659–63.

[22] A. Wintermeyer, I. Moller, M. Thevis, M. Jubner, J. Beike, M.A. Rothschild, K. Bender. In vitro phase I metabolism of the synthetic cannabimimetic JWH-018. *Anal Bioanal Chem*. 2010, *398***,** 2141–53.

[23] T. Sobolevsky, I. Prasolov, G. Rodchenkov. Detection of urinary metabolites of AM-2201 and UR-144, two novel synthetic cannabinoids. *Drug testing and analysis*. 2012, doi:10.1002/dta.1418

[24] U. Kim, M.J. Jin, J. Lee, S.B. Han, M.K. In, H.H. Yoo. Tentative identification of phase I metabolites of HU-210, a classical synthetic cannabinoid, by LC-MS/MS. *J Pharm Biomed Anal*. 2012, *64-65***,** 26–34.

[25] Q. Zhang, P. Ma, R.B. Cole, G. Wang. Identification of in vitro metabolites of JWH-015, an aminoalkylindole agonist for the peripheral cannabinoid receptor (CB2) by HPLC-MS/MS. *Anal Bioanal Chem*. 2006, *386***,** 1345–55.

[26] N. De Brabanter, S. Esposito, L. Geldof, L. Lootens, P. Meuleman, G. Leroux-Roels, K. Deventer, P. Van Eenoo. In vitro and in vivo metabolisms of 1-pentyl-3-(4-methyl-1-naphthoyl)indole (JWH-122). *Forensic Toxicology*. 2013, *31***,** 212–22.

[27] C.L. Moran *et al.* Quantitative measurement of JWH-018 and JWH-073 metabolites excreted in human urine. *Analytical chemistry*. 2011, *83*, 4228–36.

[28] S. Beuck, I. Moller, A. Thomas, A. Klose, N. Schlorer, W. Schanzer, M. Thevis. Structure characterisation of urinary metabolites of the cannabimimetic JWH-018 using chemically synthesised reference material for the support of LC-MS/MS-based drug testing. *Anal Bioanal Chem*. 2011, *401*, 493–505.

[29] M. Hutter, S. Broecker, S. Kneisel, V. Auwärter. Identification of the major urinary metabolites in man of seven synthetic cannabinoids of the aminoalkylindole type present as adulterants in “herbal mixtures” using LC-MS/MS techniques. *Journal of mass spectrometry□: JMS*. 2012, *47*, 54–65.

[30] A. Grigoryev, P. Kavanagh, A. Melnik. The detection of the urinary metabolites of 1-[(5-fluoropentyl)-1H-indol-3-yl]-(2-iodophenyl)methanone (AM-694), a high affinity cannabimimetic, by gas chromatography - mass spectrometry. *Drug testing and analysis*. 2013, *5*, 110–15.

[31] T. Sobolevsky, I. Prasolov, G. Rodchenkov. Detection of JWH-018 metabolites in smoking mixture post-administration urine. *Forensic science international*. 2010, *200*, 141–47.

[32] G.C. Loewinger, E.B. Oleson, J.F. Cheer. Using dopamine research to generate rational cannabinoid drug policy. *Drug testing and analysis*. 2013, *5*, 22–26.

[33] A.C. Porter, C.C. Felder. The endocannabinoid nervous system: unique opportunities for therapeutic intervention. *Pharmacology & Therapeutics*. 2001, *90*, 45–60.

[34] A.C. Howlett. International Union of Pharmacology. XXVII. Classification of Cannabinoid Receptors. *Pharmacological Reviews*. 2002, *54*, 161–202.

[35] J.W. Huffman. Cannabimimetic indoles, pyrroles and indenes. *Current medicinal chemistry*. 1999, *6*, 705–20.

[36] J.W. Huffman, G. Zengin, M.-J. Wu, J. Lu, G. Hynd, K. Bushell, A.L.S. Thompson, S. Bushell, C. Tartal, D.P. Hurst, P.H. Reggio, D.E. Selley, M.P. Cassidy, J.L. Wiley, B.R. Martin. Structure-activity relationships for 1-alkyl-3-(1-naphthoyl)indoles at the cannabinoid CB(1) and CB(2) receptors: steric and electronic effects of naphthoyl substituents. New highly selective CB(2) receptor agonists. *Bioorganic & medicinal chemistry*. 2005, *13*, 89–112.

[37] M.M. Aung, G. Griffin, J.W. Huffman, M.-J. Wu, C. Keel, B. Yang, V.M. Showalter, M.E. Abood, B.R. Martin. Influence of the N-1 alkyl chain length of cannabimimetic indoles upon CB1 and CB2 receptor binding. *Drug and Alcohol Dependence*. 2000, *60*, 133–40.

[38] J.W. Huffman, S.A. Hepburn, P.H. Reggio, D.P. Hurst, J.L. Wiley, B.R. Martin. Synthesis and pharmacology of 1-methoxy analogs of CP-47,497. *Bioorganic & medicinal chemistry*. 2010, *18*, 5475–82.

[39] J.W. Huffman, P. V Szklennik, A. Almond, K. Bushell, D.E. Selley, H. He, M.P. Cassidy, J.L. Wiley, B.R. Martin. 1-Pentyl-3-phenylacetylindoles, a new class of cannabimimetic indoles. *Bioorg Med Chem Lett*. 2005, *15*, 4110–13.

[40] U.S. Zimmermann, P.R. Winkelmann, M. Pilhatsch, J. a Nees, R. Spanagel, K. Schulz. Withdrawal phenomena and dependence syndrome after the consumption of “spice gold”. *Deutsches Ärzteblatt international*. 2009, *106*, 464–67.

[41] D. Hurst, G. Loeffler, R. McLay. Psychosis associated with synthetic cannabinoid agonists: a case series. *The American journal of psychiatry*. 2011, *168*, 1119.

[42] D. Zuba, B. Byrska, M. Maciow. Comparison of “herbal highs” composition. *Analytical and bioanalytical chemistry*. 2011, *400*, 119–26.

[43] B.C. Ginsburg, L.R. McMahon, J.J. Sanchez, M.A. Javors. Purity of synthetic cannabinoids sold online for recreational use. *Journal of analytical toxicology*. *36*, 66–68.

[44] T.S. Heath, Z. Burroughs, A.J. Thompson, F.W. Tecklenburg. Acute Intoxication Caused by a Synthetic Cannabinoid in. 2012, *17*, 177–81.

[45] J.H. Moran. Smart resource allocation needed to study “legal highs”. *Nature medicine*. 2011, *17*, 1339.

[46] E. Han, Y. Park, E. Kim, S. In, W. Yang, S. Lee, H. Choi, S. Lee, H. Chung, J.M. Song. Simultaneous analysis of Δ(9)-tetrahydrocannabinol and 11-nor-9-carboxy-tetrahydrocannabinol in hair without different sample preparation and derivatization by gas chromatography-tandem mass spectrometry. *Journal of pharmaceutical and biomedical analysis*. 2011, *55*, 1096–103.

[47] A. Molnar, J. Lewis, P. Doble, G. Hansen, T. Prolov, S. Fu. A rapid and sensitive method for the identification of delta-9-tetrahydrocannabinol in oral fluid by liquid chromatography-tandem mass spectrometry. *Forensic science international*. 2012, *215*, 92–96.

[48] J. Rohrich, I. Schimmel, S. Zorntlein, J. Becker, S. Drobnik, T. Kaufmann, V. Kuntz, R. Urban. Concentrations of Delta(9)-Tetrahydrocannabinol and 11-Nor-9-Carboxytetrahydrocannabinol in Blood and Urine After Passive Exposure to Cannabis Smoke in a Coffee Shop. *Journal of Analytical Toxicology*. 2010, *34*, 196–203.

[49] C. Abbara, R. Galy, A. Benyamina, M. Reynaud, L. Bonhomme-Faivre. Development and validation of a method for the quantitation of Delta9 tetrahydrocannabinol in human plasma by high performance liquid chromatography after solid-phase extraction. *Journal of pharmaceutical and biomedical analysis*. 2006, *41*, 1011–16.

[50] H.J. Penn, L.J. Langman, D. Unold, J. Shields, J.H. Nichols. Detection of synthetic cannabinoids in herbal incense products. *Clinical biochemistry*. 2011, *44*, 1163–65.

[51] B.K. Logan, L.E. Reinhold, A. Xu, F.X. Diamond. Identification of synthetic cannabinoids in herbal incense blends in the United States. *Journal of forensic sciences*. 2012, *57*, 1168–80.

[52] R. Denooz, J.-C. Vanheugen, M. Frederich, P. de Tullio, C. Charlier. Identification and structural elucidation of four cannabimimetic compounds (RCS-4, AM-2201, JWH-203 and JWH-210) in seized products. *Journal of analytical toxicology*. 2013, *37*, 56–63.

[53] S. Kneisel, F. Westphal, P. Bisel, V. Brecht, S. Broecker, V. Auwärter. Identification and structural characterization of the synthetic cannabinoid 3-(1-adamantoyl)-1-pentylindole as an additive in “herbal incense”. *Journal of mass spectrometry□: JMS*. 2012, *47*, 195–200.

[54] N. Uchiyama, M. Kawamura, R. Kikura-Hanajiri, Y. Goda. Identification and quantitation of two cannabimimetic phenylacetylindoles JWH-251 and JWH-250, and four cannabimimetic naphthoylindoles JWH-081, JWH-015, JWH-200, and JWH-073 as designer drugs in illegal products. *Forensic toxicology*. 2010, *29*, 25–37.

[55] S. Dresen, S. Kneisel, W. Weinmann, R. Zimmermann, V. Auwarter. Development and validation of a liquid chromatography-tandem mass spectrometry method for the quantitation of synthetic cannabinoids of the aminoalkylindole type and methanandamide in serum and its application to forensic samples. *J Mass Spectrom*. 2011, *46*, 163–71.

[56] S.L. Kacinko, A. Xu, J.W. Homan, M.M. McMullin, D.M. Warrington, B.K. Logan. Development and validation of a liquid chromatography-tandem mass spectrometry method for the identification and quantification of JWH-018, JWH-073, JWH-019, and JWH-250 in human whole blood. *Journal of analytical toxicology*. 2011, *35*, 386–93.

[57] M. Dziadosz, J.-P. Weller, M. Klintschar, J. Teske. Scheduled multiple reaction monitoring algorithm as a way to analyse new designer drugs combined with synthetic cannabinoids in human serum with liquid chromatography-tandem mass spectrometry. *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences*. 2013, *929C*, 84–89.

[58] S. Kneisel, V. Auwärter. Analysis of 30 synthetic cannabinoids in serum by liquid chromatography-electrospray ionization tandem mass spectrometry after liquid-liquid extraction. *Journal of Mass Spectrometry*. 2012, *47*, 825–35.

[59] S. Strano-Rossi, L. Anzillotti, E. Castrignanò, F.S. Romolo, M. Chiarotti. Ultra high performance liquid chromatography-electrospray ionization-tandem mass spectrometry screening method for direct analysis of designer drugs, “spice” and stimulants in oral fluid. *Journal of chromatography. A*. 2012, *1258*, 37–42.

[60] S. Kneisel, M. Speck, B. Moosmann, T.M. Corneillie, N.G. Butlin, V. Auwärter. LC/ESI-MS/MS method for quantification of 28 synthetic cannabinoids in neat oral fluid and its application to preliminary studies on their detection windows. *Analytical and bioanalytical chemistry*. 2013, 24–26. doi:10.1007/s00216-013-6887-0

[61] D. Favretto, J.P. Pascali, F. Tagliaro. New challenges and innovation in forensic toxicology. Focus on the “New Psychoactive Substances”. *Journal of chromatography. A*. 2012, *1287*, 84–95.

[62] T.G. Sobolevskii, I.S. Prasolov, G.M. Rodchenkov. Application of mass spectrometry to the structural identification of the metabolites of the synthetic cannabinoid JWH-018 and the determination of them in human urine. *Journal of Analytical Chemistry*. 2011, *66*, 1314–23.

[63] A. Grigoryev, P. Kavanagh, A. Melnik. The detection of the urinary metabolites of 1-[(5-fluoropentyl)-1H-indol-3-yl]-(2-iodophenyl)methanone (AM-694), a high affinity cannabimimetic, by gas chromatography - mass spectrometry. *Drug testing and analysis*. 2013, *5*, 110–15.

[64] N. De Brabanter, S. Esposito, E. Tudela, L. Lootens, P. Meuleman, G. Leroux-Roels, K. Deventer, P. Van Eenoo, L.-R. Geert, V.E. Peter. In vivo and in vitro metabolism of the synthetic cannabinoid JWH-200. *Rapid Communications in Mass Spectrometry*. 2013, *27*, 1–12.

[65] E.G. Yanes, D.P. Lovett. High-throughput bioanalytical method for analysis of synthetic cannabinoid metabolites in urine using salting-out sample preparation and LC-MS/MS. *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences*. 2012, *909*, 42–50.

[66] A. Wohlfarth, K.B. Scheidweiler, X. Chen, H.-F. Liu, M.A. Huestis. Qualitative Confirmation of 9 Synthetic Cannabinoids and 20 Metabolites in Human Urine Using LC-MS/MS and Library Search. *Analytical chemistry*. 2013, *85*, 3730–38.

[67] A.D. de Jager, J. V Warner, M. Henman, W. Ferguson, A. Hall. LC-MS/MS method for the quantitation of metabolites of eight commonly-used synthetic cannabinoids in human urine--an Australian perspective. *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences*. 2012, *897*, 22–31.

[68] A. Arntson, B. Ofsa, D. Lancaster, J.R. Simon, M. McMullin, B. Logan. Validation of a novel immunoassay for the detection of synthetic cannabinoids and metabolites in urine specimens. *Journal of analytical toxicology*. 2013, *37*, 284–90.

[69] Cayman Chemical. JWH Metabolite ELISA. https://www.caymanchem.com/app/template/Product.vm/catalog/580210

[70] NMS Labs. Synthetic Cannabinoid Metabolites Screen - Expanded, Urine (Forensic) Test (9562U). http://www.nmslabs.com/tests/Synthetic-Cannabinoid-Metabolites-Screen---Expanded--Urine--Forensic-/9562U

[71] J. Kerwin. Cannabinoids Part I--legal policies and physiological effects. *Drug testing and analysis*. 2013, *5*, 20–21.

[72] M. Rajasekaran, L.K. Brents, L.N. Franks, J.H. Moran, P.L. Prather. Human metabolites of synthetic cannabinoids JWH-018 and JWH-073 bind with high affinity and act as potent agonists at cannabinoid type-2 receptors. *Toxicology and applied pharmacology*. 2013, *269*, 100–08.

Chapter 7:

Metabolomics as a tool to enable routine screening of JWH-122

Adapted from:

N. De Brabanter, S. Esposito, L. Geldof, L. Lootens, P. Meuleman, G. Leroux-Roels, K. Deventer, P. Van Eenoo.

In vitro and *in vivo* metabolisms of 1-pentyl-3-(4-methyl-1-naphthoyl)indole (JWH-122).

Forensic Toxicology. 2013, 31, 212–222.

Abstract

1-Pentyl-3-(4-methyl-1-naphthoyl)indole (JWH-122) is an agonist of the cannabinoid receptors CB 1 and CB 2. In this study, the phase I and phase II metabolism of JWH-122 were investigated using two models. *In vitro* studies using incubations of JWH-122 with human liver microsomes were performed to obtain metabolites of the drug at the first step; 11 classes of metabolites were found and analysed by liquid chromatography-mass spectrometry (LC-MS) and liquid chromatography-tandem mass spectrometry (LC-MS-MS). Hydroxylation(s) on the naphthalene moiety and/or the indole moiety of the molecule took place as such or in combination with dehydrogenation or cleavage of the *N*-pentyl side chain. Furthermore also dihydrodiol metabolites are probably formed via epoxide formation on the naphthalene moiety, irrespective of the combination with hydroxylation(s). A metabolite carrying a carboxylgroup on the *N*-pentyl side chain was also detected. In the second step of the study, *in vivo* experiments, using chimeric mice were performed; the mice were orally administered JWH-122, and the urine samples were collected to be subjected to enzymatic hydrolysis, followed by LC-MS and LC-MS/MS. The urine samples without hydrolysis were also analysed for their molecular formulae in the conjugated forms by LC-high resolution MS. The *in vivo* model using chimeric mice could confirm most metabolite classes and clarify the phase II metabolism of JWH-122. From these results, it can be concluded that all metabolites formed *in vivo* are excreted conjugated as glucuronide or sulphate, with conjugation rates above 50%.

1 Introduction

With the discovery of the cannabinoid receptors CB 1 and CB 2 in humans in the 1980s, the search for synthetic cannabinoid receptor agonists started. CB 1 is mainly located in the brain and is responsible for the psychoactive effects, while CB 2, found in the immune cells, is involved in pain perception. Therefore, pharmaceutical research has been directed to novel compounds with high-affinity binding for CB 2 and low-affinity for CB 1 to separate the desired properties from the unwanted psychoactive effects [1,2]. This has led to the synthesis and the study of structure-activity relationships of a wide range of synthetic cannabinoids receptor agonists, including a series of aminoalkylindoles known as JWH-compounds [3]. However, unfortunately, these JWH-cannabinoids have found their way to the black market, and many of these have been found in herbal smoking mixtures [4]. Products like Spice Gold, Spice Silver and Spice Diamond are abused like cannabis and produce similar effects [5].

To cope with this problem, the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) and Europol organized a conference on the ‘spice’ topic in 2009. Based upon this report [2], most European countries started banning the use of some of these compounds. This report also pointed out the problem on the screening for these JWH-compounds: the variety of these products is large and still growing in the world [6–10]. Moreover little is known on the metabolism of these substances, making an adequate detection in biological specimens difficult. In a recent briefing paper [11] the EMCDDA describes a strong increase in the number of shops selling these products online. Beside the well-known JWH-018, of which the metabolism has already been investigated [12,13], 1-pentyl-3-(4-methyl-1-naphthoyl)indole (JWH-122) appeared also high on the list. This JWH-class compound was reported as an active ingredient in illegal products in Germany and Japan [14,15].

To allow for the development of detection methods for this compound, the metabolism of JWH-122 have been investigated in this study. *In vitro* studies using human liver microsomes were initially conducted to identify possible metabolites. Although *in vitro* metabolism via human liver microsomes has shown to be a valuable tool for the elucidation of drug metabolism [16], the exact mimicking of human metabolism is not always true [17,18]. Therefore, in the second step, the urinary detectability of the metabolites was investigated *in vivo*. Urine allows long term detection of cannabinoids [19] and is the preferred matrix in drugs of abuse and anti-doping analysis. Since administration to humans is ethically questionable, a more complex model, namely the chimeric mouse model was applied. This has shown to be an excellent model for the study of the metabolism of several substances, including steroids [20,21], where a wide variety of human metabolic pathways were shown to be present, including phase II metabolic pathways [22]. The parent compound was administered to both chimeric and non-chimeric mice, to verify the metabolism of JWH-122.

2 Materials and methods

2.1 Reagents

The enzyme β-glucuronidase (140 U/mg at 37°C) from *E. coli*, used for hydrolysis, was purchased from Roche Diagnostics GmbH (Mannheim, Germany); methanol from Fisher Scientific (Loughborough, UK); disodium hydrogen phosphate (Na_2HPO_4) and sodium dihydrogen phosphate (NaH_2PO_4) from Merck (Darmstadt, Germany); phosphate buffer solution, pH 7.2 from Invitrogen (Merelbeke, Belgium); the internal standard (IS), deuterated 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (THCA-*d*9) was from Cerriliant (Round Rock, TX, USA); JWH-122 from Bio-Connect (Te Huissen, Netherlands). All reagents were of the analytical grade. For microsomal incubations, all reagents such as NADPH generating system, phosphate buffer (pH 7.4) and pooled human liver microsomes were from BD Bioscience (Erembodegem, Belgium). The gas used for the mass spectrometer was nitrogen (Alphagaz2-grade), from Air Liquide (Desteldonk, Belgium).

2.2 *In vivo* model with chimeric mice

The project was approved by the Animal Ethics Committee of the Faculty of Medicine of the Ghent University (ECD 06/09). The production and maintenance of the chimeric uPA$^{+/+}$-SCID mice were done according to a previously described protocol [23].

The administration experiments were performed in metabolic cages (Tecniplast, Casale Litta, Italy), allowing unlimited access to water and food for the mice and easy collection of the urine, separated from the faeces. The excretion studies were performed by oral administration of 50 µl of PBS containing the synthetic cannabinoid JWH-122 (0.1 mg drug dissolved in 2 ml of ethanol/PBS (20:80, v/v)) to both chimeric (human albumin 3.8 mg/ml) and non-chimeric mice.

Non-transplanted uPA$^{+/-}$-SCID mice, i. e. without human hepatocytes, were included in the administration study as a control group for the interspecies differences. Urine was collected prior to and 24 h after a single dose administration (2.5 µg). All samples were stored at -20ºC until analysis.

A 100 µl aliquot of mouse urine was spiked with 50 µl of IS solution containing 0.5 µg/mL THCA-*d*9. For hydrolysis, samples were incubated in an oven at 56°C for 1.5 h after adding 1 ml of 0.1 M phosphate buffer solution (pH = 7) and 50 µl of β-glucuronidase. Solid-phase extraction (SPE) was used for further purification and concentration of the compounds of interest; Oasis® HLB 3cc (60 mg) extraction cartridges from Waters (Milford, MA, USA) were used. The cartridge was first activated with 2 ml of methanol and was subsequently rinsed with 2 ml of water. Before loading, the sample was first diluted up to 2 ml with water. After loading, the column was washed twice with 2 ml of water. Then 2 ml of methanol was used to elute the compounds from the column. After evaporation of the methanol under a stream of oxygen-free nitrogen, the residue was redissolved in 100 µl of water/methanol (95:5, V/V) for liquid chromatography-mass spectrometry (LC-MS) analysis.

A part of each urine sample collected from the chimeric and non-chimeric mice after administration of JWH-122 was purified by the SPE without the enzymatic hydrolysis for detection of metabolites in their conjugated forms by LC-high resolution (HR)-MS.

2.3 *In vitro* model with human liver microsomes

For microsomal incubation, a solution of 4 mg/ml JWH-122 in dimethyl sulfoxide was prepared. A volume of 250 µl reaction medium was prepared containing 226 µl 0.1 M phosphate buffer (pH 7.4) and 15 µl of a 10 mM NADPH generating system. This contained 12.5 µl of a mixture of $NADP^+$ (1.3 mM), glucose-6-phosphate (3.3 mM) and $MgCl_2$ (3.3 mM) and 2.5 µl glucose-6-phosphate dehydrogenase (0.4 U/ml) in sodium citrate solution (0.05 mM). To this, 2.5 µl of the JWH-122 solution was added, and the mixture was incubated at 37ºC. The reaction was initiated by adding 6.5 µl microsomal protein (20 mg/ml). The mixture was vortexed briefly and incubated at 37ºC for 4 h. To stop the incubation, 250 µl of ice-cold methanol was added, and the samples were centrifuged for 5 min at 12,000g at 4ºC. Then the mixture was evaporated under oxygen-free nitrogen at room temperature. The dry residue was redissolved in 2 ml of distilled water prior to further purification by SPE with the Oasis® HLB (60 mg) cartridges. The procedure used was the same as described before for the *in vivo* experiments.

2.4 Instrumentation

All experiments were performed under the same LC conditions, using a MS Pump Plus coupled with a Surveyor Plus autosampler (Thermo Scientific, Bremen, Germany). Separation was performed with a Sunfire™ C18 column (50 x 2.1 mm i.d., particle size 3.5 µm) from Waters (AH Etten-Leur, Netherlands). The volume for injection was 20 µl. The mobile phase A was 0.1% acetic acid plus 1 mM ammonium acetate in water, mobile phase B was 0.1% acetic acid plus 1 mM ammonium acetate in methanol. Gradient elution was: 95% A / 5% B for 1.5 min, then linearly changed to 0% A / 100% B in 25 min and held for 3 min. This was followed by the return to the initial conditions in 0.1 min and an equilibration for 2.9 min before the next injection (total run time 31 min). The flow rate was constant at 250 µl/min.

Both low-resolution and HR-MS(-MS)-based strategies were used to detect and characterize JWH-122 metabolites. Low resolution MS experiments were performed on a Finnigan TSQ Quantum mass spectrometer (Thermo Scientific). The MS conditions were: interface, electrospray ionization (ESI); capillary voltage, 3.5 kV; source temperature, 350 °C; sheath gas pressure, 50 (arbitrary units); auxiliary gas pressure, 20 (arbitrary units); tube lens offset, 100 V; collision energy 25 to 40 eV; scan range, *m/z* 50 – 800; scan time, 0.5 s.

For HRMS experiments, an Exactive Mass spectrometer (Orbitrap-based mass spectrometer) from Thermo Scientific was operated in both positive and negative polarity modes. The conditions were: sheath gas, 60 (arbitrary units); the auxiliary gas, 30 (arbitrary units); capillary temperature, 350 °C; capillary voltage, 30V; spray voltage, 4 kV; full-scan range, *m/z* 50-1,000; high-collision dissociation energy, 30 eV; resolving power, 100,000 at *m/z* 200; data acquisition rate, 1 Hz.

3 Results and discussion

3.1 Product ion scanning for precursor JWH-122 to obtain clues for estimation of its metabolite structures.

Identification and revealing of unknown metabolites for a given parent substance are often a challenging task. On the basis of the knowledge of microsomal transformations [16] and the metabolism of synthetic cannabinoids of the aminoalkylindole type investigated earlier [1,12,24], a list of theoretically possible metabolites can be composed. To check their presence and study their fragmentation, LC-MS-MS is the technique of choice [13]. Due to the presence of an amino group in the molecule, abundant protonated molecular ions were observed in the positive ionization mode. Full scans were performed on the protonated molecular ions of each of the 12 predicted categories (M1 – M12, Table 7.1) of metabolites using collision energies at 25 and 40 eV. Initially, the product ion formation pattern of JWH-122 was investigated. As shown in Fig. 7.1, the protonated molecular ion at *m/z* 356 (M+H) fragmented into 2 main parts; the complete naphthalene moiety (*m/z* 169) and the indole moiety containing the *N*-pentyl side chain (*m/z* 214), both containing the central carbonyl functional group. The observed fragmentation was in agreement with the previous described work on naphthoylindoles [1,12,25]. The former ion was further fragmented to form an ion at *m/z* 141, which is 28 amu less than that at *m/z* 169 (loss of CO). For the latter ion, the alkyl side chain was cleaved from the indole moiety, leaving a fragment at *m/z* 144. The origin of the fragment at *m/z* 115, formed at higher collision energies, was investigated using HRMS. From the accurate mass number of 115.0543 found by the high collision dissociation experiment, the structural formula of $C_9H_7^+$ (theoretic mass number of 115.0542 with Δ=0.78 ppm) was obtained. This is in accordance with a fragmentation pathway suggested earlier for the naphthalene moiety of naphthoylindoles [25].

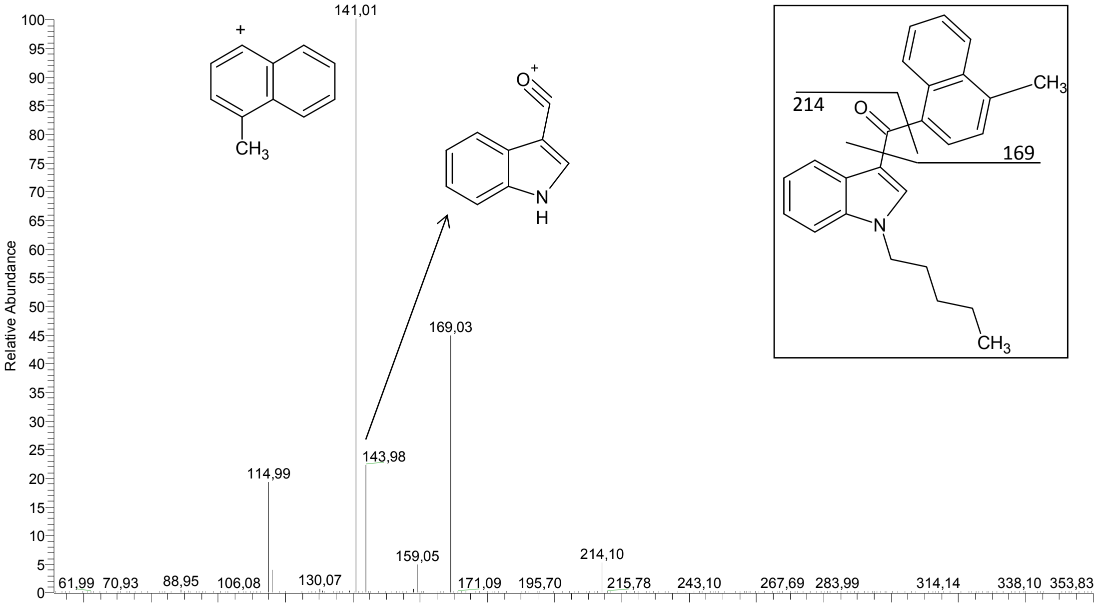


Fig. 7.1 Product ion mass spectrum obtained from the protonated precursor JWH-122 molecular ion at *m/z* 356.5 using collision energy at 40 eV, and the probable fragmentation pathways.

Modifications in one part of the molecule (the indole or naphthalene side) without simultaneous changes in the other moiety should leave detectable ions at *m/z* 144 and 214 or ions at *m/z* 115 and 141 respectively. Therefore, these product ions will be used to estimate the structure of metabolites produced by *in vitro* incubation of JWH-122 with human liver microsomes.

3.1.1 Monohydroxylation (M1)

The protonated molecular ions of the monohydroxylated JWH-122, if present, should show peaks on the channel at *m/z* 372 of the mass chromatograms. As shown on the chromatogram at *m/z* 372 (Fig. 7.2 M1), at least 7 monohydroxylated metabolites could be identified after the *in vitro* incubation of JWH-122 with human liver microsomes. The location of the hydroxylation can be estimated from each product ion mass spectrum. For metabolites A, B and C, the presence of peaks at *m/z* 141, 144 and 169 (same as the parent compound, Fig. 7.1) showed that neither the naphthalene nor the indole moiety was modified. Therefore, the hydroxylation should be situated on the alkyl side chain of the indole moiety. Attribution of the exact position of the hydroxylation on the alkyl side chain, however, was not possible for these metabolites.

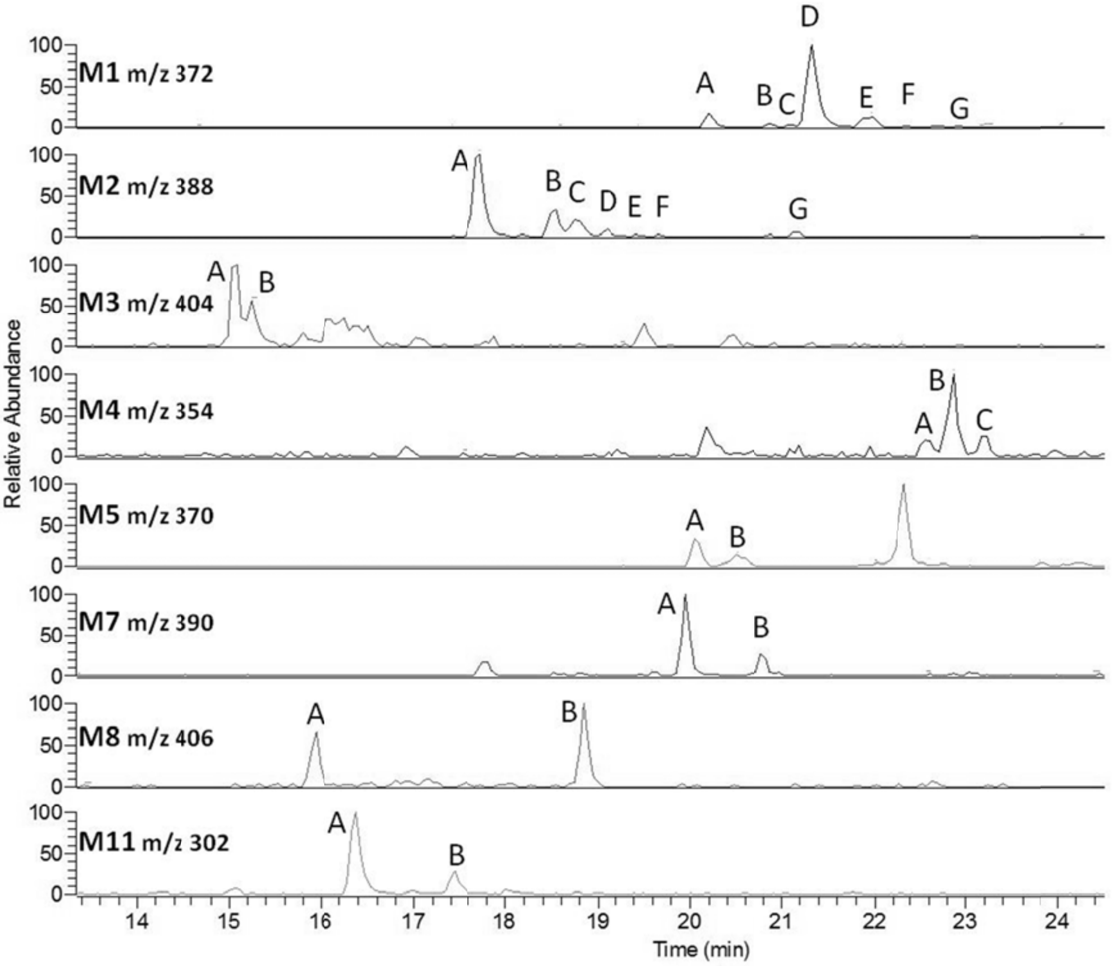


Fig. 7.2 Mass chromatograms for the protonated molecular ions of the metabolites in the free forms obtained after *in vitro* incubation of JWH-122 with human liver microsomes. For the metabolic modification, see Table 7.1.

In metabolites D, F and G, a fragment ion at *m/z* 214 appeared, indicating that the part containing the indole moiety and alkyl chain was unchanged (cf. Fig. 7.1). The proof of the hydroxylation on the naphthalene moiety was given by the appearance of an ion at *m/z* 185 (Fig. 7.3).

For metabolite E, the fragment peak at *m/z* 169 appeared, showing the presence of an unchanged naphthalene moiety. In addition, a fragment peak appeared at *m/z* 230 showing the presence of hydroxylation on the indole or the alkyl side chain. On which of the 2 moieties the hydroxylation is located, could not be clarified by the product ion mass spectrum obtained.

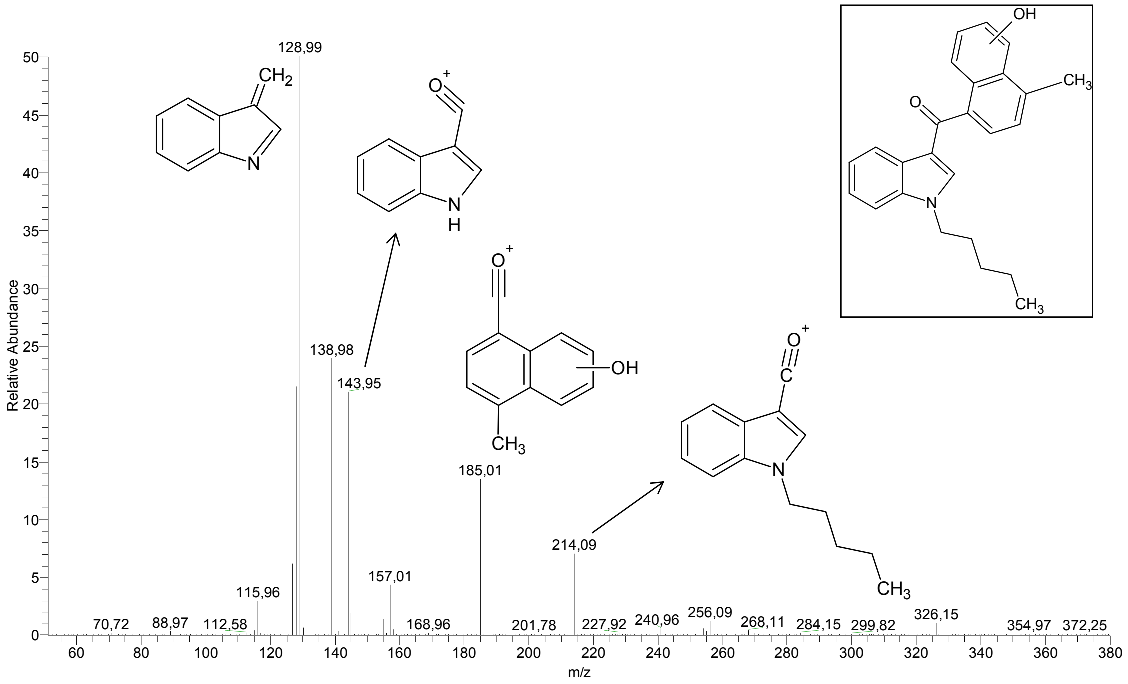


Fig. 7.3 Product ion mass spectrum obtained from the monohydroxylated metabolite M1 D shown in Table 7.1 and Fig. 7.2 using collision energy at 40 eV, and the probable fragmentation pathways.

3.1.2 Dihydroxylation (M2)

The protonated molecular ions of the dihydroxylated JWH-122 metabolites should show peaks at *m/z* 388 of the mass chromatograms. As shown in Fig. 7.2 M2, 7 peaks appeared for these metabolites. At least one hydroxyl group can be found on the naphthalene moiety, because of the appearance of a product ion at *m/z* 185 ($[169\text{-H+OH}]^+$) in the product ion mass spectra for all peaks (Table 7.1, M2). The second hydroxyl-group was placed on the indole moiety of the molecule including the alkyl side chain, yielding a product ion at *m/z* 230, which could be explained as the sum of fragment at *m/z* 214 (cf. Fig. 7.1) and a hydroxyl group. To allow unequivocal distinction between hydroxylation at the indole ring and the *N*-pentyl chain, the interpretation of the origin of additional fragments was necessary. For metabolites A and C, the fragment ion appearing at *m/z* 144, representing an unchanged indole ring (cf. Fig. 7.1), indicated that the hydroxylation was located on the alkyl side chain.

More accurate location of the hydroxylation in the side chain could be found for metabolites B and G. Fragments at *m/z* 314 and 342 (cfr. molecular weight of JWH-122 = 355) appeared showing product ions including the monohydroxylated naphthalene moiety, which lacked only a part of the *N*-pentyl side chain. For the ion at *m/z* 314, there was a loss of 73 amu from the molecular ion at *m/z* 388, according to the loss of a hydroxyl group together with an *n*-butyl group. Similarly, the fragment ion at *m/z* 342 originated from a loss of 45 amu; i.e., a hydroxyl group and the peripheral 2 carbons of the side chain. Therefore, it can be concluded that a hydroxyl group is located at the end part of the *N*-pentyl side chain, probably on the 4th or 5th carbon. The same ion at *m/z* 342 appeared in metabolite D, leading to the same conclusion. However, distinction between the indole ring and the alkyl chain could not be made for metabolite F; it was possible for metabolite E, where the ion at *m/z* 160 proved the hydroxylation on the indole ring (Table 7.1, M2).

3.1.3 Trihydroxylation (M3)

In the mass chromatogram at *m/z* 404, evidence for two kinds of trihydroxylated metabolites was found (Fig. 7.2, M3). For both metabolites, the appearance of a product ion at *m/z* 185 indicated a single hydroxylation on the naphthalene moiety. Therefore the other two hydroxyl groups were probably located on the *N*-alkylated indole. However, evidence for this assumption could not be found in the product ions. The difficulty to detect these trihydroxylated metabolites, produced in low amounts *in vitro*, has already been encountered in another study on a similar compound [12].

3.1.4 Dehydrogenation (M4)

In the mass chromatogram at *m/z* 354, 3 different metabolites appeared (Fig. 7.2 M4), which were deprotonated molecular ions of JWH-122. Although the exact position of the double bond in the *N*-pentyl chain could not be located using the MS-MS experiments, the product ions at *m/z* 141 and 144 indicated an unchanged naphthalene moiety and an indole moiety, respectively. The appearance of the product ion at *m/z* 212 – i.e. the ion 2 amu less than the corresponding product ion at *m/z* 214 of JWH-122 (Fig. 7.1) - provides further evidence that the dehydrogenation is located in the alkyl part of the molecule.

3.1.5 Dehydrogenation + hydroxylation(s) (M5 and M6)

The above described modification in the *N*-pentyl chain was also found together with hydroxylation(s). Two monohydroxylated variants were found (Fig. 7.2 M5, Table 7.1 M5). In metabolite M5A, the product ion at *m/z* 212 was absent. However, a product ion at *m/z* 228 appeared, which can be explained by the addition of a hydroxyl group to the dehydrogenated *N*-alkylated indole (*m/z* 212). Because the product ion at *m/z* 144, showing the unchanged indole ring (cf. Fig. 7.1) was still present, it can be concluded that the hydroxylation took place on the alkyl side chain. This was not the case for metabolite M5B (Fig. 7.2 M5, Table 7.1 M5), where the product ion at *m/z* 212 was still present. This, together with the appearance of a product ion at *m/z* 185 (cf. Fig. 7.3), indicates a single hydroxylation on the naphthalene part of the structure.

Additionally, one metabolite bearing 2 hydroxyl groups (Table 7.1, M6) was found in combination with dehydrogenation. The simultaneous occurrence of both *m/z* 185 and 228 indicates that one hydroxyl group is located on the naphthalene moiety and the other on the dehydrogenated *N*-pentyl side chain.

3.1.6 Dihydrodiol formation (M7)

As shown in the previous studies on the *in vitro* metabolism of similar compounds [1,12], some metabolites can be formed via epoxide formation on the naphthalene moiety. This epoxide then undergoes enzymatic hydrolysis to give dihydrodiol metabolites. For JWH-122, two such metabolites were found in the mass chromatogram at *m/z* 390 (Fig. 7.2 M7). Because in the fragmentation pattern for both substances a product ion at *m/z* 214 was found (Fig. 7.4), the *N*-alkylated indole part seems to be intact. The presence of a product ion at *m/z* 203 proved that a modification took place at the naphthalene ring structure. This is confirmed by the presence of product ions at *m/z* 157 and 185, both originating from a monohydroxylated naphthalene moiety formed by a loss of H_2O (Fig. 7.4). Although the exact locations of these diols on the ring cannot be clarified by the MS-MS experiments, only 2 possible locations were found and characterized in the previous study using NMR technology [26]. In comparison with the unmodified naphthalene ring of the investigated WIN55212-2 [26], JWH-122 bears an extra methyl group on the naphthalene ring, which limits the possible hydroxylation positions. Therefore, it can be assumed that the dihydrodiol group for JWH-122 is estimated to be located in positions 1 and 2, 3 and 4, respectively (Fig. 7.4).

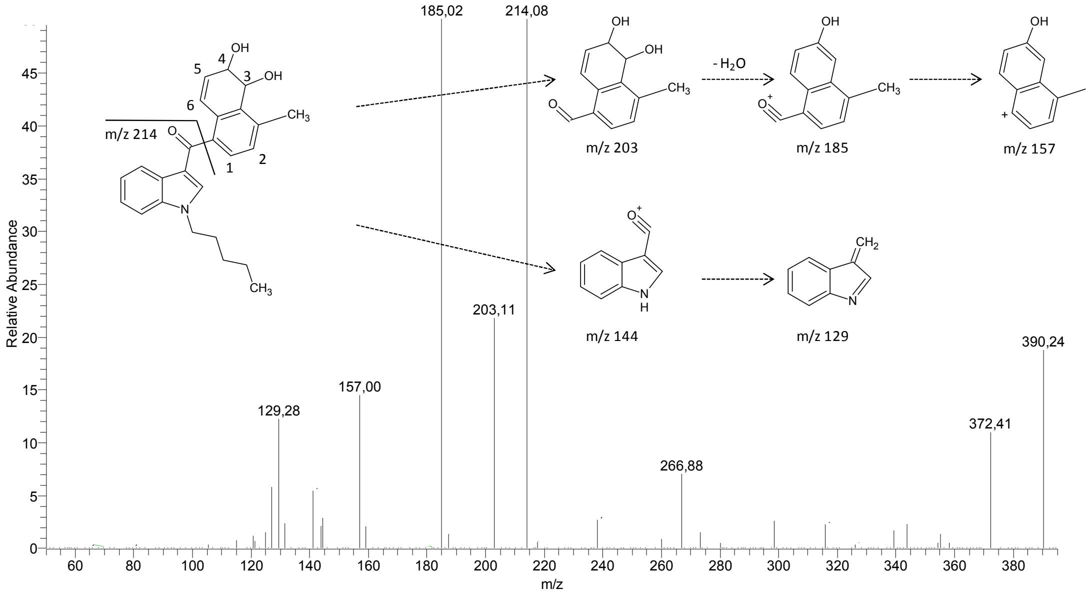


Fig. 7.4 Product ion mass spectrum obtained from the peak M7 B shown in Fig. 7.2 using collision energy at 25 eV. It was estimated to be a dihydrodiol metabolite of JWH-122 showing its protonated molecular ion at *m/z* 390. Only one possible structure with two hydroxyl groups on the naphthalene moiety is shown. The probable fragmentation pathways are also shown.

3.1.7 Dihydrodiol formation + hydroxylations (M8)

Some metabolites were found combining the dihydrodiol formation with one hydroxylation. In metabolite A (Fig. 7.2 M8), the proof for the dihydoxylation via epoxide hydrolysis on the naphthalene moiety was again given by ions at *m/z* 203, 185 and 157, as described for M7 (Fig. 7.4). The product ion at *m/z* 214, however, was replaced by the ion at *m/z* 230, which is the indication of hydroxylation on the N-alkylated indole.

In metabolite B, the product ion at *m/z* 203 was not observed, but an ion at *m/z* 219 appeared. Because the ion at *m/z* 214 was also present, it can be concluded that the extra hydroxylation took place on the naphthalene part of the molecule.

3.1.8 *N*-Dealkylation(s) (M10 and M11)

The M10 metabolite, with protonated molecular ion at *m/z* 286, was formed by the cleavage of the *N*-pentyl side chain (Table 7.1 M10). In the product ion spectra, both unchanged naphthalene (*m/z* 169 and 141) and indole moiety (*m/z* 144) were present. The product ion at *m/z* 214 for the *N*-alkylated indole did not appear.

Additionally, two monohydroxylated plus *N*-dealkylated metabolites were detected (Fig.7.2 M11). In metabolite A, the hydroxylation occurred on the naphthalene moiety, because product ions at *m/z* 185 and 157 were present (cf. Fig. 7.3), and the ion at *m/z* 144 shows that the indole moiety remained unchanged. For metabolite B, the product ions at *m/z* 169, 160 and 141 appeared. The ions at *m/z* 169 and 141 (cf. Fig. 7.1) indicate that the naphthalene structure was not modified, while the ion at *m/z* 160 was formed by hydroxylation on the indole ring.

3.1.9 Carboxylation (M12)

In the positive ionization mode, a metabolite with a carboxyl group could be detected (Table 7.1 M12). The fragmentation pattern of the molecular ion at *m/z* 386 shows product ions similar to those of the parent drug with respect to both the naphthalene moiety (*m/z* 169, 141 and 115) as well as the indole ring (*m/z* 144). However, the product ion corresponding to the indole moiety containing the alkyl side chain showed a change from *m/z* 214 for the parent drug to *m/z* 244 for the metabolite. This may indicate that the carboxyl-group was formed on the *N*-pentyl side chain. Because compounds containing a carboxyl group can easily be deprotonated using ESI [13], additional evidence for the presence of this metabolite is found when analyzing the sample in the negative ionization mode.

Table 7.1 JWH-122 metabolites found in the *in vitro* model experiments.

Class		Nr.	Location of modification
M1	Monohydroxylation	A	N-Pentyl side chain
		B	N-Pentyl side chain
		C	N-Pentyl side chain
		D	Naphthalene moiety
		E	Indole/alkyl moiety
		F	Naphthalene moiety
		G	Naphthalene moiety
M2	Dihydroxylation	A	Naphthalene moiety + *N*-pentyl side chain
		B	Naphthalene moiety + *N*-pentyl side chain
		C	Naphthalene moiety + *N*-pentyl side chain
		D	Naphthalene moiety + *N*-pentyl side chain
		E	Naphthalene moiety + indole ring
		F	Naphthalene moiety + indole/alkyl moiety
		G	Naphthalene moiety + *N*-pentyl side chain
M3	Trihydroxylation	A	1 x Naphthalene moiety, 2x indole/alkyl moiety
		B	1 x Naphthalene moiety, 2x indole/alkyl moiety
M4	Dehydrogenation	A	N-Pentyl side chain
		B	N-Pentyl side chain
		C	N-Pentyl side chain
M5	Dehydrogenation + monohydroxylation	A	N-Pentyl side chain
		B	Naphthalene moiety
M6	Dehydrogenation + dihydroxylation	A	N-Pentyl side chain + naphthalene moiety
M7	Dihydrodiol formation	A	Naphthalene moiety
		B	Naphthalene moiety
M8	Dihydrodiol formation + Monohydroxylation	A	Naphthalene moiety (dihydrodiol) + indole moiety (hydroxyl)
		B	Naphthalene moiety (dihydrodiol + hydroxyl)
M10	N-Dealkylation	A	N-Pentyl side chain
M11	N-Dealkylation + monohydroxylation	A	Naphthalene moiety
		B	Indole ring
M12	Carboxylation	A	N-Pentyl side chain

3.2 *In vivo* metabolism using the chimeric mouse model

For detection of the metabolites, an LC-MS-MS method was used. On the basis of the results from the *in vitro* experiments, two transitions per compound were selected and programmed in the selected reaction monitoring (SRM) mode for every metabolite. In the first experiments, all mouse urine samples were hydrolyzed prior to analysis. In this way all metabolites could be investigated, originating from both free and conjugated excreted metabolites.

Then the samples without the hydrolysis step were investigated for the phase II metabolism, using LC-HRMS, which could check the presence of both glucuronide and sulphate conjugates for all metabolites already discovered in the *in vitro* study. Finally these samples were also analysed using the SRM method to check the compounds excreted free into urine.

Twenty-four hours after the administration, no parent compound was found for all mouse urine samples. Hence, it can be concluded that there was a complete biotransformation and excretion within this period. This result was supported by similar findings for JWH-018 [27]. However, faecal excretion cannot be excluded.

Among the 7 monohydroxylated metabolites identified by the *in vitro* study (Table 7.1 M1), only 3 could be detected in the mouse model (Fig. 7.5, Fig. 7.2 M1 A, D, E). One extra peak named H, was detected at 23.3 min, which showed the presence of an additional monohydroxylated metabolite (Fig. 7.5 H). These 4 metabolites were produced in both the chimeric mouse and the non-chimeric control group. As shown in Fig. 7.5, production of metabolites M1A, D and E in the chimeric mice was much higher than that in the non-chimeric mice which can only originate from the contribution of the humanized liver. This is in contrast to metabolite H, for which the intensities of the peaks were similar for both mouse types, suggesting it is a murine-specific metabolite.

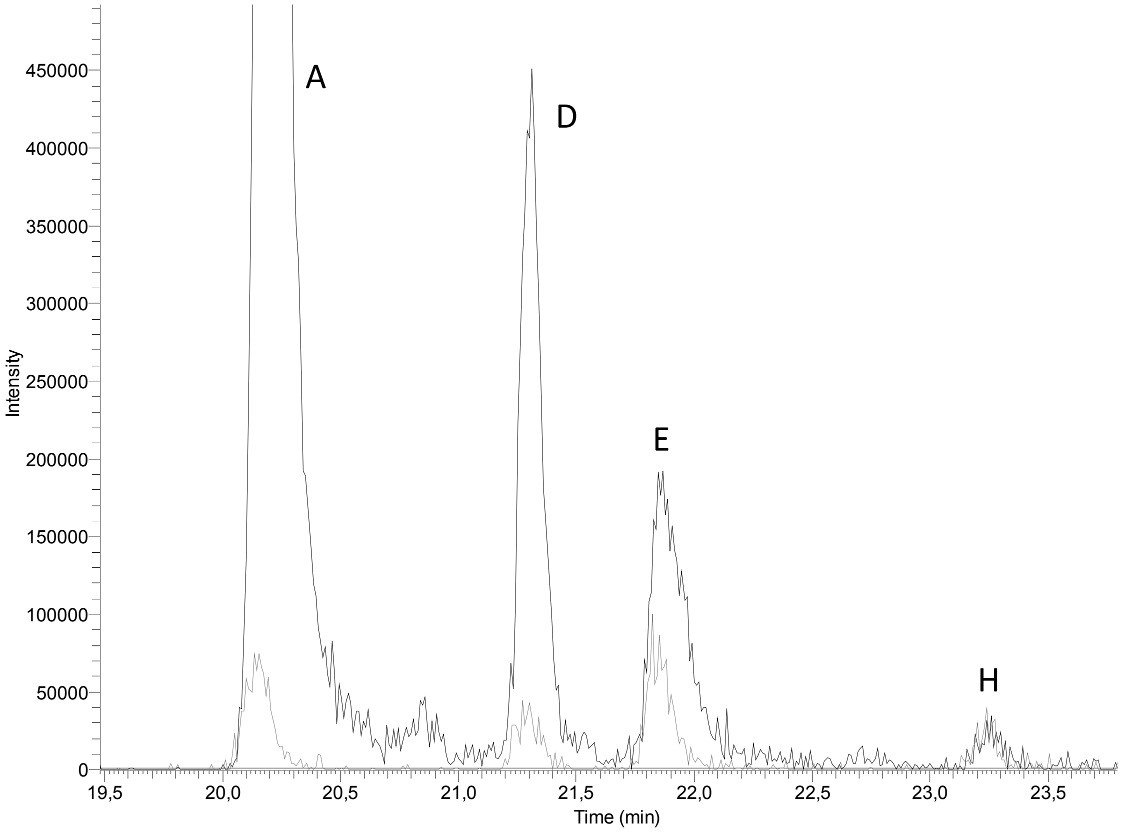


Fig. 7.5 Selected reaction monitoring (SRM) chromatogram for monohydroxylated metabolites of JWH-122 obtained from urine extracts of chimeric (top) and non-chimeric mice after hydrolysis. The used transitions for SRM were *m/z* 372 → 214, 372 → 185, 372 → 169, 372 → 141.

For these M1-class substances, their conjugated forms were carefully analysed by LC-HRMS as shown in Table 7.2; three glucuronides and one sulphate conjugate could be detected. Because almost no free monohydroxylated metabolites could be detected without enzymatic hydrolysis, it can be concluded that these metabolites are excreted almost exclusively as conjugates. Similar results could be obtained for the multiple hydoxylated metabolites. For the class of M2, only three metabolites could be detected (Fig. 7.2, M2 A, B, D) after hydrolysis in the *in vivo* experiments. However, four conjugates were found in the LC-HRMS experiments without hydrolysis: three glucuronides and one sulphate (Table 7.2). Also for the M2 metabolites no significant amounts of compounds were detected in the free forms. Therefore it can be assumed that one metabolite partially appeared as a glucuronide and partially as a sulphate, while the others appeared only as glucuronides. Only one metabolite containing three hydroxyl groups was found in the hydrolyzed mouse urine (Fig.7.2 M3 B), originating from a sulphate conjugate (Table 7.2). From the comparison of the M3 peak areas, the amount excreted in the free form in the non-hydrolyzed urine was about 50%; the comparable ratio was found to be excreted in the conjugated form. In the chimeric mouse urine, no metabolites with dehydrogenation (M4) or the combination of dehydrogenation and monohydroxylation (M5) were detected. However, only the dihydroxylated metabolite M6 was found in combination with the dehydrogenation in the *N*-pentyl side chain. Without hydrolysis for M6, one glucuronide and one sulphate metabolite could be detected in the LC-HRMS experiments. The conjugation reaction was complete for the M6 metabolites. In terms of the peak areas, the M6 glucuronide conjugate concentration was higher than the sulphate concentration. Only one metabolite carrying a dihydrodiol group on the naphthalene moiety could be found *in vivo* for M7 metabolite (Table 7.2 M7), which was identified as metabolite B (Fig. 7.2 M7) formed by microsomal incubation. This metabolite was present in urine as a glucuronide conjugate. The M7 B is only found in the chimeric mice and not in the non-chimeric control mice, indicating that the M7 metabolite is of human origin. A few combinations of this dihydrodiol group with other modifications were found. One metabolite with the dihydrodiol and monohydroxyl groups (M8 A) was excreted both in the free and glucuronidated forms in approximately equal amounts. One metabolite with the dihydrodiol and dihydroxyl groups, which was completely sulphated (Table 7.2 M9), was found in the *in vivo* study. The *N*-dealkylation was only seen in combination with monohydroxylation in the mouse model. After enzymatic hydrolysis both metabolites A and B (M11) could be identified by LC-MS-MS, one of which is excreted as sulphate and the other as glucuronide (Table 7.2 M11).

Table 7.2 JWH-122 conjugated metabolites found with the *in vivo* model experiments analysed by liquid chromatography-high resolution mass spectrometry. The deviation of theoretical and experimental mass number is calculated (RT = retention time).

Class	Metabolite	Conjugation	Structure formula	RT (min)	Mode	Theoretic mass (*m/z*)	Experimental mass (*m/z*)	Δ ppm
M1	Monohydroxylation	Glucuronide	C31H33NO8	21.7	+	548.2279	548.2272	1.295
M1	Monohydroxylation	Glucuronide	C31H33NO8	23.0	+	548.2279	548.2273	1.113
M1	Monohydroxylation	Glucuronide	C31H33NO8	23.4	+	548.2279	548.2271	1.496
M1	Monohydroxylation	Sulphate	C25H25NO5S	24.4	-	450.1381	450.1390	2.155
M2	Dihydroxylation	Glucuronide	C31H33NO9	19.8	+	564.2228	564.2222	1.028
M2	Dihydroxylation	Glucuronide	C31H33NO9	20.2	+	564.2228	564.2222	1.134
M2	Dihydroxylation	Glucuronide	C31H33NO9	21.0	+	564.2228	564.2225	0.638
M2	Dihydroxylation	Sulphate	C25H25NO6S	21.0	-	466.1330	466.1334	0.901
M3	Trihydroxylation	Sulphate	C25H25NO7S	19.5	-	482.1279	482.1281	0.436
M6	Dehydrogenation + dihydroxylation	Glucuronide	C31H31NO9	19.6	+	562.2072	562.2063	1.511
M6	Dehydrogenation + dihydroxylation	Sulphate	C25H25NO6S	21.0	-	466.1330	466.1335	1.094
M7	Dihydrodiol formation	Glucuronide	C31H35NO9	21,8	+	566.2385	566.2378	1.236
M8	Dihydrodiol formation + monohydroxylation	Glucuronide	C31H35NO10	17.1	+	582.2334	582.2326	1.305
M9	Dihydrodiol formation + dihydroxylation	Sulphate	C25H27NO8S	9.6	+	502.1530	502.1546	3.067
M11	N-Dealkylation + monohydroxylation	Glucuronide	C26H23NO8	19.6	+	478.1496	478.1493	0.648
M11	N-Dealkylation + monohydroxylation	Sulphate	C20H15NO5S	20.2	-	380.0598	380.0603	1.210

4 Conclusions

In this study, the metabolism of a synthetic cannabinoid JWH-122 was investigated using two experimental models; *in vitro* incubation of JWH-122 with human liver microsomes, and *in vivo* chimeric mouse experiments collecting their urine. Eleven classes of metabolites were detected *in vitro* by LC-MS and each chemical structure was estimated by LC-MS-MS. Secondly, the urine samples obtained by the *in vivo* experiments were subjected to enzymatic hydrolysis to clarify their structure by LC-MS and LC-MS-MS; the urine samples without hydrolysis were also analysed for their molecular formulae in the conjugated forms by LC-HRMS.

Comparison of the metabolism of both models showed that the classes found in the *in vitro* model are mostly present in the *in vivo* model as well. However, there are a few exceptions. Both dehydrogenation (M4) and dehydrogenation plus monohydroxylation (M5) were not detected in the chimeric mouse (Tables 7.1, 7.2). Nevertheless these modifications seem possible *in vivo* because the dehydrogenation was present in combination with dihydroxylation (Table 7.2 M6). This is not the case for carboxylation (M12), which was not detected at all in the *in vivo* model. The opposite is true for the combination of dihydrodiol formation and dihydroxylation (M9), because this is only observed in the *in vivo* mouse model (Table 7.2 M9).

Even though there was a high similarity between the classes of metabolites detected in both models, it is clear that the number of metabolites produced in a given class was limited in *in vivo* experiments compared to *in vitro* experiments. This is due to the higher complexity of the *in vivo* model, where a number of factors influencing the metabolic clearance exist. One of these factors is phase II metabolism, which is present in the chimeric mouse model. All metabolites detected in the mouse urine are at least partially excreted as conjugates (both glucuronide and sulphate), with conjugation rates between 50 and 100%.

The results seem to have great consequences for detection of JWH-122 abuse using urine samples. Because no parent compound was excreted, it seems advisable to look for the best detectable metabolites. From the results of the SRM method applied to the mouse urine samples, it was clear that hydroxylated metabolites are excreted in the highest amounts. This amount decreased from monohydroxylated over dihydroxylated to trihydroxylated compounds. Moreover, all these metabolites are excreted exclusively as conjugates. This means that screening of urine samples for the phase II metabolites is necessary for adequate detection of JWH-122 abuse.

References

[1] Q. Zhang, P. Ma, R.B. Cole, G. Wang. Identification of in vitro metabolites of JWH-015, an aminoalkylindole agonist for the peripheral cannabinoid receptor (CB2) by HPLC-MS/MS. *Anal Bioanal Chem*. 2006, *386*, 1345–55.

[2] EMCDDA. Understanding the “Spice” phenomenon. 2009, 1–34. http://www.emcdda.europa.eu/attachements.cfm/att_80086_EN_Spice Thematic paper□? final version.pdf

[3] J.W. Huffman, P. V. Szklennik, A. Almond, K. Bushell, D.E. Selley, H. He, M.P. Cassidy, J.L. Wiley, B.R. Martin. 1-Pentyl-3-phenylacetylindoles, a new class of cannabimimetic indoles. *Bioorganic & Medicinal Chemistry Letters*. 2005, *15*, 4110–13.

[4] R. Lindigkeit, A. Boehme, I. Eiserloh, M. Luebbecke, M. Wiggermann, L. Ernst, T. Beuerle. Spice: a never ending story? *Forensic science international*. 2009, *191*, 58–63.

[5] I. Vardakou, C. Pistos, C. Spiliopoulou. Spice drugs as a new trend: Mode of action, identification and legislation. *Toxicology Letters*. 2010, *197*, 157–62.

[6] J. Nakajima, M. Takahashi, T. Seto, M. Yoshida, C. Kanai, J. Suzuki, T. Hamano. Identification and quantitation of two new naphthoylindole drugs-of-abuse, (1-(5-hydroxypentyl)-1H-indol-3-yl)(naphthalen-1-yl)methanone (AM-2202) and (1-(4-pentenyl)-1H-indol-3-yl)(naphthalen-1-yl)methanone, with other synthetic cannabinoids in unregulat. *Forensic Toxicology*. 2012, *30*, 33–44.

[7] N. Uchiyama, M. Kawamura, R. Kikura-Hanajiri, Y. Goda. Identification of two new-type synthetic cannabinoids, N-(1-adamantyl)-1-pentyl-1H-indole-3-carboxamide (APICA) and N-(1-adamantyl)-1-pentyl-1H-indazole-3-carboxamide (APINACA), and detection of five synthetic cannabinoids, AM-1220, AM-2233, AM-1241, CB-1. *Forensic Toxicology*. 2012, *30*, 114–25.

[8] S. Kneisel, P. Bisel, V. Brecht, S. Broecker, M. Müller, V. Auwärter. Identification of the cannabimimetic AM-1220 and its azepane isomer (N-methylazepan-3-yl)-3-(1-naphthoyl)indole in a research chemical and several herbal mixtures. *Forensic Toxicology*. 2012, *30*, 126–34.

[9] P. Jankovics, A. Váradi, L. Tölgyesi, S. Lohner, J. Németh-Palotás, J. Balla. Detection and identification of the new potential synthetic cannabinoids 1-pentyl-3-(2-iodobenzoyl)indole and 1-pentyl-3-(1-adamantoyl)indole in seized bulk powders in Hungary. *Forensic science international*. 2012, *214*, 27–32.

[10] F. Westphal, F.D. Sonnichsen, S. Thiemt. Identification of 1-butyl-3-(1-(4-methyl)naphthoyl)indole in a herbal mixture. *Forensic Sci Int*. 2012, *215*, 8–13.

[11] EMCDDA. Online sales of new psychoactive substances / “Legal highs”:Summary of results from the 2011 multilingual snapshots. *EMCDDA*. 2011,

http://www.emcdda.europa.eu/attachements.cfm/att_143801_EN_SnapshotSummary.pdf

[12] A. Wintermeyer, I. Moller, M. Thevis, M. Jubner, J. Beike, M.A. Rothschild, K. Bender. In vitro phase I metabolism of the synthetic cannabimimetic JWH-018. *Anal Bioanal Chem*. 2010, *398***,** 2141–53.

[13] T.G. Sobolevsky, I.S. Prasolov, G.M. Rodchenkov. Application of mass spectrometry to the structural identification of the metabolites of the synthetic cannabinoid JWH-018 and the determination of them in human urine. *Journal of Analytical Chemistry*. 2011, *66***,** 1314–23.

[14] L. Ernst, H.M. Schiebel, C. Theuring, R. Lindigkeit, T. Beuerle. Identification and characterization of JWH-122 used as new ingredient in “Spice-like” herbal incenses. *Forensic Sci Int*. 2011, *208***,** 31–35.

[15] J. Nakajima, M. Takahashi, T. Seto, C. Kanai, J. Suzuki, M. Yoshida, T. Hamano. Identification and quantitation of two benzoylindoles AM-694 and (4-methoxyphenyl)(1-pentyl-1H-indol-3-yl)methanone, and three cannabimimetic naphthoylindoles JWH-210, JWH-122, and JWH-019 as adulterants in illegal products obtained via the Internet. *Forensic Toxicology*. 2011, *29***,** 95–110.

[16] F.T. Peters, M.R. Meyer. In vitro approaches to studying the metabolism of new psychoactive compounds. *Drug Test Anal*. 2011, *3***,** 483–95.

[17] E.F. Brandon, C.D. Raap, I. Meijerman, J.H. Beijnen, J.H. Schellens. An update on in vitro test methods in human hepatic drug biotransformation research: pros and cons. *Toxicol Appl Pharmacol*. 2003, *189***,** 233–46.

[18] M.D. Tingle, N.A. Helsby. Can in vitro drug metabolism studies with human tissue replace in vivo animal studies? *Environmental Toxicology and Pharmacology*. 2006, *21***,** 184–90.

[19] A. Verstraete. Detection Times of Drugs of Abuse in Blood, Urine, and Oral Fluid. *Therapeutic Drug Monitoring*. 2004, *26***,** 200–05.

[20] L. Lootens, P. Meuleman, O.J. Pozo, P. Van Eenoo, G. Leroux-Roels, F.T. Delbeke. uPA(+/+)-SCID Mouse with Humanized Liver as a Model for In Vivo Metabolism of Exogenous Steroids: Methandienone as a Case Study. *Clinical Chemistry*. 2009, *55***,** 1783–93.

[21] L. Lootens, P. Van Eenoo, P. Meuleman, O.J. Pozo, P. Van Renterghem, G. Leroux-Roels, F.T. Delbeke. Steroid metabolism in chimeric mice with humanized liver. *Drug Testing and Analysis*. 2009, *1***,** 531–37.

[22] S.C. Strom, J. Davila, M. Grompe. Chimeric mice with humanized liver: tools for the study of drug metabolism, excretion, and toxicity. *Methods Mol Biol*. 2010, *640***,** 491–509.

[23] P. Meuleman, L. Libbrecht, R. De Vos, B. de Hemptinne, K. Gevaert, J. Vandekerckhove, T. Roskams, G. Leroux-Roels. Morphological and biochemical

characterization of a human liver in a uPA-SCID mouse chimera. *Hepatology*. 2005, *41*, 847–56.

[24] A. Grigoryev, P. Kavanagh, A. Melnik. The detection of the urinary metabolites of 3-[(adamantan-1-yl)carbonyl]-1-pentylindole (AB-001), a novel cannabimimetic, by gas chromatography-mass spectrometry. *Drug testing and analysis*. 2012, *4*, 519–24.

[25] K. Sekula, D. Zuba, R. Stanaszek. Identification of naphthoylindoles acting on cannabinoid receptors based on their fragmentation patterns under ESI-QTOFMS. *Journal of Mass Spectrometry*. 2012, *47*, 632–43.

[26] Q. Zhang, P. Ma, M. Iszard, R.B. Cole, W. Wang, G. Wang, W.E.T. Al. In vitro metabolism of R(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo [1,2,3-de]1,4-benzoxazinyl]-(1-naphthalenyl) methanone mesylate, a cannabinoid receptor agonist. *Drug Metab Dispos*. 2002, *30*, 1077–86.

[27] A.D. de Jager, J. V Warner, M. Henman, W. Ferguson, A. Hall. LC-MS/MS method for the quantitation of metabolites of eight commonly-used synthetic cannabinoids in human urine--an Australian perspective. *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences*. 2012, *897*, 22–31.

Chapter 8:

Towards routine screening of the synthetic cannabinoid JWH-200

Adapted from:

N. De Brabanter, S. Esposito, E. Tudela, L. Lootens , P. Meuleman, G. Leroux-Roels, K. Deventer , P. Van Eenoo.

In vivo and in vitro metabolism of the synthetic cannabinoid JWH-200.

Rapid communications in mass spectrometry. 2013, 27, 1-12

Abstract

The synthetic cannabinoid JWH-200 (1-[2-(4-morpholinyl)ethyl]-3-(1-naphthoyl)-indole) appeared on the market around 2009. In order to identify markers for misuse of this compound and allow for the development of adequate routine methods, the metabolism of this compound was investigated using two models.

In vitro and *in vivo* (both with and without enzymatic hydrolysis) samples were purified by solid-phase extraction and analyzed using liquid chromatography. For the identification of the metabolites electrospray ionization was used in combination with high resolution mass spectrometry detection powered by Orbitrap™ technology. To confirm the results *in vivo*, triple-quadrupole mass spectrometry was applied.

In the *in vitro* model, using human liver microsomes, 22 metabolites were detected which could be divided into 11 metabolite classes.

By using the chimeric mouse model with humanized liver, most of these metabolites were confirmed *in vivo*. It was found that all metabolites are excreted in urine as conjugates, mostly as glucuronides with varying conjugation rates.

The metabolite formed by consecutive morpholine cleavage and oxidation of the remaining side chain to a carboxylic group, was detected in the highest amounts with the longest detection time. Therefore it is the best candidate metabolite to detect JWH-200 abuse in urine.

1 Introduction

The products of *Cannabis sativa* and corresponding derivates are considered the most frequently used illicit drugs for several hundreds of years. [1] While between 1990 and 2000 cannabis use increased in many countries, European statistics show a stabilization and even a small decrease in these numbers in the last years [2]. This can be seen as a positive trend in the traditional cannabis abuse. However the opposite evolution is observed for synthetic analogues of Δ9-tetrahydrocannabinol (THC) [3]. According to the annual report of Europol and the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), 23 new synthetic cannabinoids were reported via the EU early-warning system (EWS) in 2011, making these synthetic analogues of THC one of the largest drug groups monitored by the EWS [4].

These new psychoactive substances – also named ‘’legal highs’’ – are usually sold via internet, presented as room odors, herbal incenses or other deceptive names [5]. However, forensic investigation by German and Austrian authorities demonstrated in 2008 that synthetic cannabinoids were present in these blends of herbal material, causing the cannabis-like effects experienced by users [6]. From that moment on, the list of these compounds detected in products with names like ‘Spice’ [7], continued to grow. It started with JWH-018 and CP 47,497 [6],[8], but until now more variants are found almost every month [9,10], which can be divided in different classes [5].

The reasons for the growing popularity of these drugs vary. The synthetic analogues mimic the effect of cannabis and avoid a positive outcome on the current screening tests for THC [7]. In many countries legislation is based upon individual named substances, therefore these laws are quickly outdated when a new product is brought on the market. Indeed, previous research showed that as soon as the product appears on a prohibited list, a new generation is created and launched on the market in a few weeks. This clearly shows that the producers of these drugs have both legal and scientific background [11].

Further these products are readily available, they are - often falsely - sold as ‘legal’ alternatives for cannabis via internet shops and this online selling is still expanding at high speed [3]. The frequently seen indication ‘not for human consumption’ can be taken seriously, since little is known on the toxicology of the synthetic cannabinoids which has already resulted in some cases of severe overdosing [8,12].

In 2009 a new compound, 1-[2-(4-morpholinyl)ethyl]-3-(1-naphthoyl)-indole (JWH-200) was detected by the EWS [13] and was at the end of 2010 scheduled by the American Drug Enforcement Agency (DEA) [14] and several European countries [15]. This has led to the development of screening methods to detect JWH-200 in herbal mixtures [16,17], oral fluids [18], serum [19,20] and recently urine [21]. However, due to the lack of data on the metabolism of this new compound, optimized detection in urine was up to now not possible. Indeed, as described for other naphthoylindoles, the unchanged parent compound is not excreted in urine. [15,22] With the knowledge of the metabolism, long term detection via routine screening in urine becomes possible [23].

In this work both the *in vitro* and *in vivo* metabolism of JWH-200 is described.

2 Experimental

2.1 Reagents

The enzyme β-glucuronidase from E. coli, used for hydrolysis, was purchased from Roche Diagnostics GmbH (Mannheim, Germany).

Methanol was obtained from Fisher Scientific (Loughborough, UK). Disodium hydrogen phosphate (Na_2HPO_4) and sodium dihydrogen phosphate (NaH_2PO_4) were purchased from Merck (Darmstadt, Germany). Phosphate-buffered saline (PBS) solution, pH 7.2, was purchased from Invitrogen (Merelbeke, Belgium). The internal standard, deuterated 11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid (THCA-d9) was from Cerilliant (Texas, USA). JWH-200, JWH 200 5-hydroxyindole metabolite and JWH 200 6-hydroxyindole metabolite were purchased from Bio-Connect (Te Huissen, Netherlands). All reagents were of analytical grade.

The phosphate buffer solution used for hydrolysis was prepared by dissolving 7.1 g $Na_2HPO_4 \cdot 2H_2O$ and 1.4 g $NaH_2PO_4 \cdot H_2O$ in 100 mL of water.

For microsomal incubations, all reagents were purchased from BD Bioscience (Erembodegem, Belgium): NAPDH generating system, phosphate buffer (pH 7.4) and pooled Human Liver Microsomes.

LC–MS grade methanol and LC–MS grade water were purchased from Biosolve (Valkenswaard, Netherlands), respectively. Ammonium acetate (NH_4OAc) was obtained from Sigma (St. Louis, MO, USA). Acetic acid (HOAc) was purchased from Merck (Darmstadt, Germany).

The gases used for the mass spectrometer were nitrogen (Alphagaz2-grade) and argon (Alphagaz1-grade), from Air Liquide (Desteldonk, Belgium).

2.2 Chimeric mice – *in vivo* model

The project was approved by the Animal Ethics Committee of the Faculty of Medicine of Ghent University (ECD 06/09). The production and maintenance of the chimeric $uPA^{+/+}$-SCID mouse was done according to a previously described protocol [24].

For the administration studies metabolic cages (Tecniplast, Varese, Italy) were used, designed for giving the mice unlimited access to water and food and making an easy collection of the urine, separated from the faeces, possible.

Via oral gavation 50 µL of a PBS solution containing the synthetic cannabinoid JWH-200 (0.1 mg JWH-200 dissolved in 2 mL of ethanol/PBS 20/80 (v/v)) was administered to both chimeric (HuAlb 2.6 mg/mL, 2.1 mg/mL and 2.4 mg/mL) and non-chimeric mice.

Non-transplanted $uPA^{+/-}$-SCID mice, i.e. without human hepatocytes, were included in the administration study as a control group for the interspecies differences. Urine was collected prior to administration and 24 h and 48 h after a single dose of 2.5 µg. All samples were stored at -20°C until analysis.

100 µL of the mouse urine was spiked with 25 µL of the internal standard containing 0.5 µg/mL THCA-d9. For hydrolysis 1 mL of 0.1 M phosphate

buffer solution (pH = 7) and 50 µL of β-glucuronidase were added and the samples were incubated at 56°C for 1.5 h.

For purification solid-phase extraction (SPE) was applied, using Oasis® HLB 3cc (60 mg) extraction cartridges from Waters (Milford, Massachusetts, USA). The following protocol was used: 2 mL of MeOH and 2 mL of water were used for activating and rinsing the column, respectively. Then the sample was diluted with 2 mL of water prior to loading. The column was washed twice with 2 mL of water and eluted with 2 mL of MeOH. After evaporation of the MeOH, the residue was redissolved in 100 µL of H_2O/MeOH (95/5) for LC-MS analysis.

2.3 Human liver microsomes – *in vitro* model

To study the *in vitro* metabolism of JWH-200, microsomal incubations were performed using a stock solution of the parent compound in DMSO (4 mg/mL). The reaction medium (250 µL in total) contains 226 µL phosphate buffer (pH 7.4) and 15 µL of a 10 mM NADPH generating system. The latter consists of 12.5 µL of a mixture of $NADP^+$ (1.3 mM), glucose-6-phosphate (3.3 mM) and $MgCl_2$ (3.3 mM) and 2.5 µL glucose-6-phosphate dehydrogenase (0.4 U/mL) in sodium citrate (0.05 mM). After adding 2.5 µL of the JWH-200 stock solution, the reaction was initiated by adding 6.5 µL protein (20 mg/mL). The mixture was vortexed briefly and incubated at 37°C. After 4 h, the incubation was stopped by adding 250 µL of ice-cold MeOH and samples were centrifuged for 5 min at 12.000 g at 4°C. Subsequently the mixture was evaporated under oxygen free nitrogen (OFN) at room temperature. The dry residue was redissolved in 2 mL of distilled water prior to further purification by solid-phase extraction (SPE) with Oasis® HLB (60 mg) cartridges. The procedure is the same as described earlier for the *in vivo* experiments.

2.4 Instrumentation

The same chromatographic conditions were used for all experiments. A MS Pump Plus was coupled with a Surveyor Plus autosampler (both from Thermo Scientific, Bremen, Germany). 20 µL was injected using a Sunfire™ C18 3.5

µm, 2.1 x 50 mm column from Waters (AH Etten-Leur, Netherlands). The mobile phase A consisted of 1mM NH_4OAc in H_2O, 0.1% HOAc, mobile phase B consisted of 1mM NH_4OAc in MeOH, 0.1% HOAc.

The gradient used was as follows: 95% mobile phase A for 1.5 min, then decreased linearly to 0% mobile phase A in 25 min, hold for 3 min. This was followed by an increase to initial conditions in 0.1 min and an equilibration for 2.9 min before the next injection (total run time 31 min). A constant flow rate of 250 µL/min was used.

Both low-resolution and high-resolution mass spectrometry-based strategies were used to detect and characterize JWH-200 metabolites.

For high-resolution experiments, an Exactive mass spectrometer powered by Orbitrap™ technology from Thermo Scientific was operated in both positive and negative polarity switching modes and was equipped with an electrospray ionization (ESI) source. The sheath gas (nitrogen) flow rate was set at 60 (arbitrary units) and for the auxiliary gas (nitrogen) at 30 (arbitrary units). The temperature of the capillary was 350 °C. The capillary voltage and spray voltage were set to 30 V and 4 kV, respectively. The instrument was operated in full-scan mode from *m/z* 50 to *m/z* 1,000 and high-collision energy dissociation (HCD) mode at 10, 20, 50 and 100 eV from *m/z* 50 to *m/z* 1,000, at 100,000 resolving power (at *m/z* 200). The data acquisition rate was 1 Hz. The Orbitrap™ performance in both positive and negative ionization modes was evaluated daily and when failed, external calibration was realized with Exactive Calibration Kit solutions (Sigma-Aldrich, St. Louis, USA and ABCR GmbH & Co. KG, Karlsruhe, Germany).

Low resolution product ion scan experiments were performed on a Finnigan TSQ Quantum triple quadrupole mass spectrometer, from Thermo Scientific, using the electrospray source. The following ESI–MS parameters were used: capillary voltage 3.5 kV; source temperature 350 °C; sheath gas pressure, 50 (arbitrary units); auxiliary gas pressure, 20 (arbitrary units); tube lens offset, 100 V.

3 Results and discussion

In analogy with the metabolic pathways reported for similar compounds like JWH-015 [25], JWH-018 [22,26] and JWH-122 [27], the *in vitro* metabolism of JWH-200 was investigated. Therefore HRMS experiments were performed. Identification and structure elucidation of the metabolites was based on the occurrence of diagnostic product ions, related to the parent compound. Using all ion fragmentation in the HCD-cell [28], the fragmentation pattern of JWH-200 was investigated. As shown in Fig. 8.1 the protonated molecule generates two main product ions. The first ion with an accurate mass of *m/z* 155.0489 was found to be the naphthalene moiety containing the carbonyl function (mass error: 1.2 ppm). The second originates from the morpholine ring containing the alkyl side-chain with *m/z* 114.0912 (mass error: 1.7 ppm). The presence of both ions, called F1 and F2 respectively (Table 8.1), was used to prove the unchanged appearance of this part of the molecule in the metabolites. Also a product ion with *m/z* 145.0645 is found (F3), however with a lower abundance. This is formed from F1 after the loss of CO and the addition of water as previously described [29] and shown in Fig. 8.1 (mass error: 1.7 ppm). Product ions proving the presence of the indole ring however, were not found.

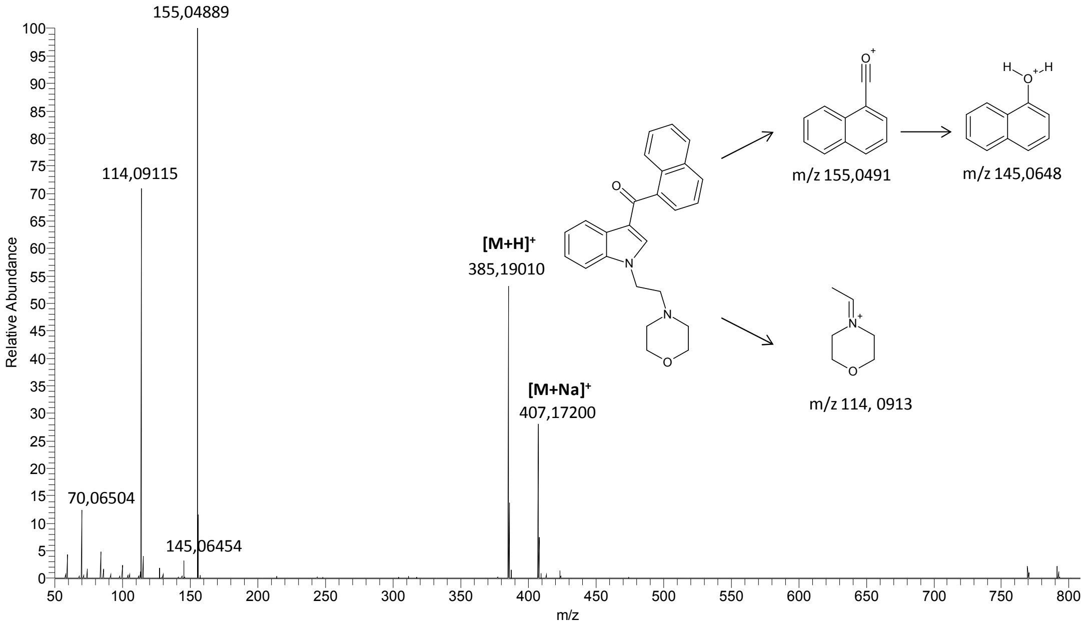
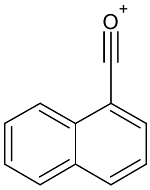
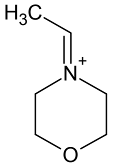
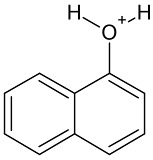
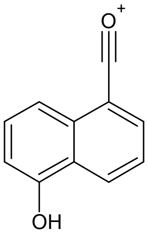
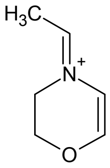
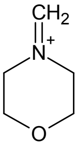
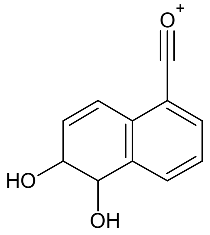
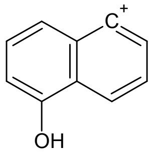
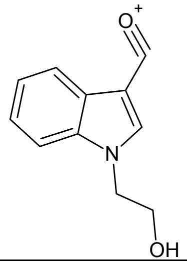
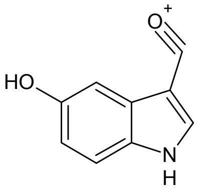


Fig. 8.1 The mass spectrum of the protonated JWH-200 with proposed structures for the formed product ions.

Table 8.1. Common product ions of the protonated JWH-200 and its metabolites formed after HCD fragmentation.

Fragment Nr.	Structure	Theoretical *m/z*	detected in metabolites
F1		155.0491	P, M1A, M1C, M2A, M2B, M2C, M3, M7, M8 M9A, M9B, M9D, M10A, M10B, M11
F2		114.0913	P, M1A, M1B, M4A, M4B
F3		145.0648	P, M7, M10A, M10B
F4		171.0441	M1B, M4A, M4B, M5A, M5B, M6A, M6B
F5		112.0757	M1C, M2A, M3
F6		100.0752	M2B, M2C

F7		189.0544	M4A, M5A, M6A
F8		143.0489	M4A, M4B, M5A, M5B, M6A, M6B
F9		188.0706	M6A, M6B, M7
F10		160.0393	M9A, M9B

3.1 Phase I metabolism of JWH-200

For the detection of metabolites in the *in vitro* experiments, accurate masses were calculated for theoretically possible metabolites. A mass tolerance window of 5 ppm was applied for identification of the metabolites. An overview of the metabolites described below is shown in Table 8.2.

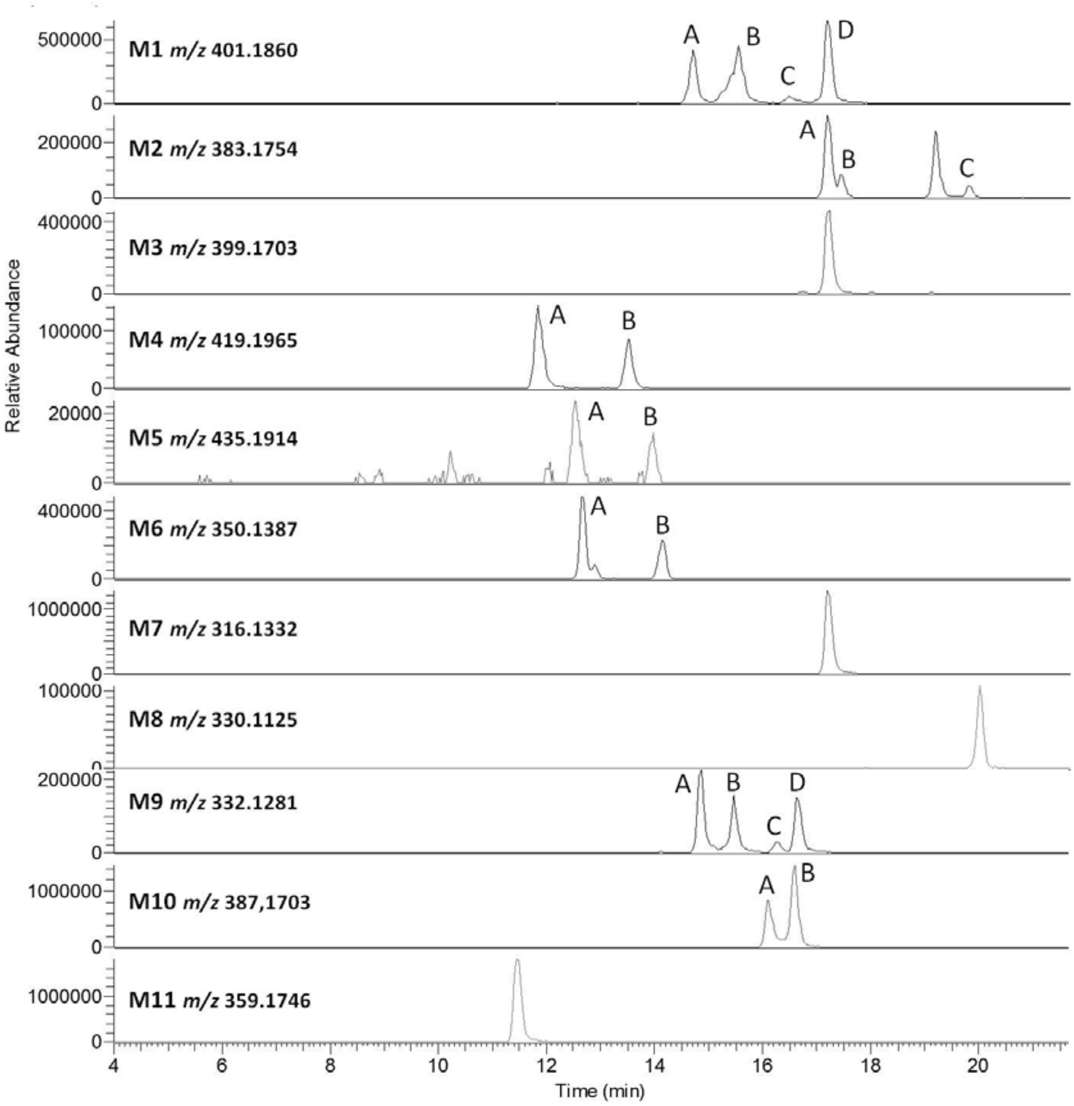


Fig. 8.2 HRMS chromatograms for the protonated molecules of the metabolites of JWH-200 obtained using the in vitro model with human liver microsomes.

3.2 Hydroxylation and dehydrogenation

Four monohydroxylated metabolites were detected as protonated molecules $[M+H]^+$ *m/z* 401.1860 (Fig. 8.2 M1). After HCD experiments, both ions F1 and F2 (Table 8.1) were found in metabolites M1A and M1B. This indicates an unchanged naphthalene and morpholine moiety, respectively (cf. JWH-200 in Fig. 8.1). Consequently it can be assumed that the hydroxylation is located on the indole ring for both metabolites. Even though a product ion proving this assumption could not be found, both metabolites could be identified by means of reference standards. As shown in Fig. 8.3, M1A corresponds to the 5-hydroxyindole metabolite, while M1B turns out to be the 6-hydroxyindole metabolite of JWH-200. While ion F2 is also detected in metabolite C, F1 is missing. This points towards a modification in the naphthalene moiety. Indeed, as shown by the presence of a product ion with *m/z* 171.0441 (F4 in Table 8.1) the hydroxylation is located on the naphthalene ring. For metabolite D, the opposite is true: ion F1 is still present, but F2 was not observed. Instead, two other ions were found proving the hydroxylation on the morpholine ring: *m/z* 130.0863 (i.e. F2 + OH) and *m/z* 112.0757. The latter is formed via the loss of water from the original ion *m/z* 130.0863 (F5, Table 8.1).

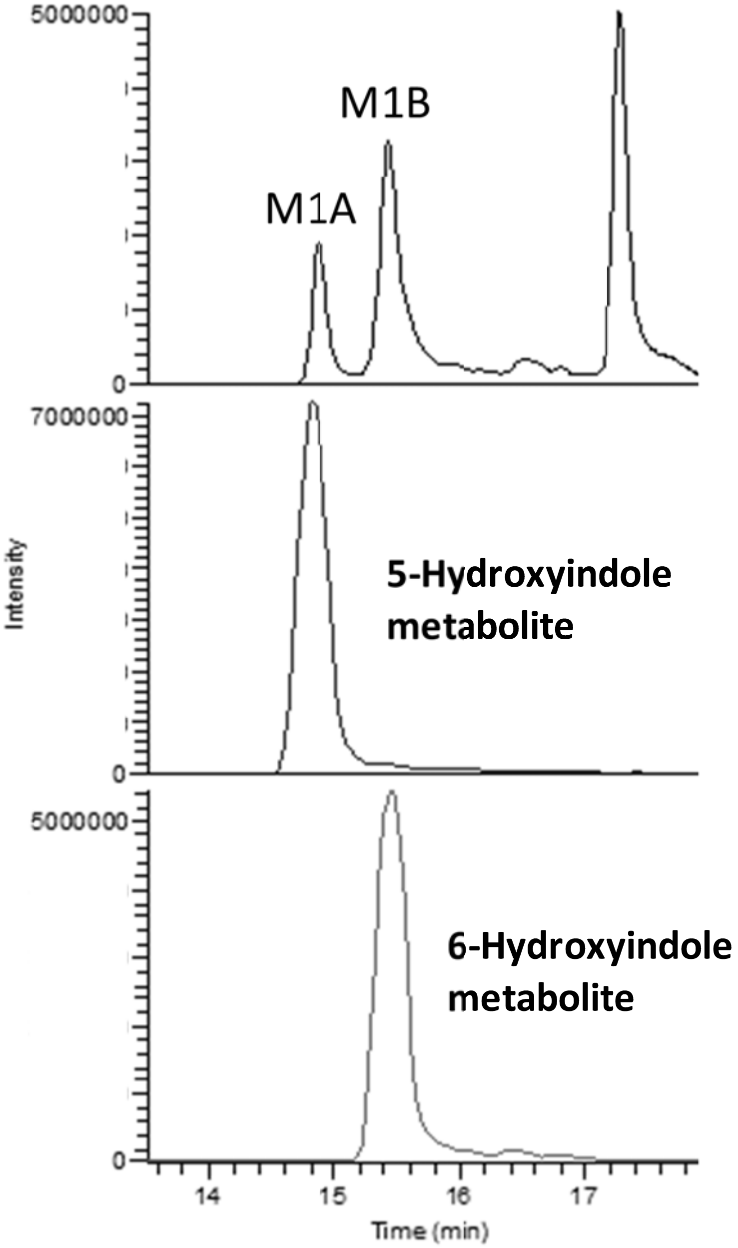


Fig. 8.3 Identification of the monohydroxylated metabolites (M1A and M1B) with reference standards of JWH-200 metabolites.

Three metabolites appeared, of which the protonated molecule has an accurate mass of *m/z* 383.1754 (M2 Fig. 8.2). Compared to the JWH-200 parent, this corresponds to a dehydrogenation. Theoretically, this is only possible in the short alkyl chain connecting the indole moiety and the morpholine ring, or in the morpholine ring itself. The latter is true for metabolite M2 A, since ion F5 (Table 8.1) is present. For the other metabolites B and C, this ion is missing. Instead, a product ion with *m/z* 100.0752 (F6,

Table 8.1) proves the intact morpholine ring. So the only remaining location for the dehydrogenation is the alkyl chain. Because the same product ions are observed for metabolites B and C, it can be assumed that the difference in retention time is caused by the formation of 2 isomers, i.e. *E* and *Z* configuration. The difference between the 3 ions can be visualized by means of product ions scans (Fig. 8.4).

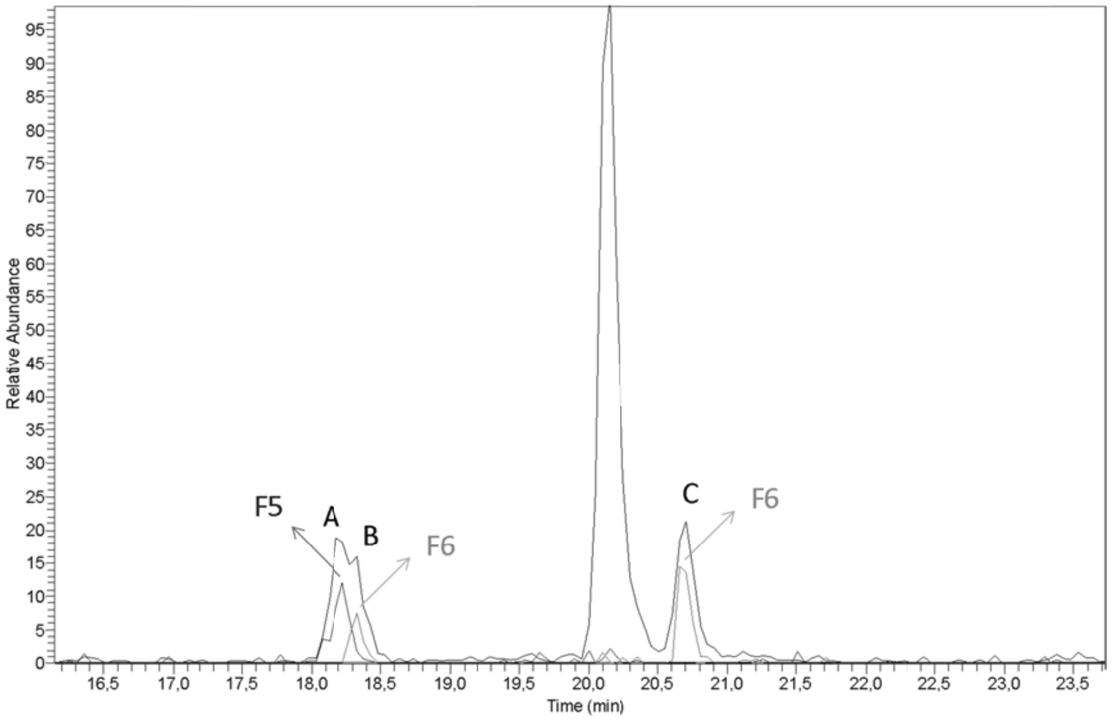


Fig. 8.4 Product ion scan experiments on the protonated metabolites M2, showing the difference in location of the dehydrogenation.

The combination of both abovementioned metabolic modifications is found in metabolite M3 detected with a retention time of 17.3 min and an accurate mass *m/z* 399.1703 for the corresponding protonated molecule. As shown in Fig. 8.5, the dehydrogenation is located in the morpholine ring because of the presence of ion F5. The presence of both F1, the unchanged naphthalene moiety, and a product ion with *m/z* 188.0704 allows locating the hydroxylation on the alkyl chain connected to the indole moiety.

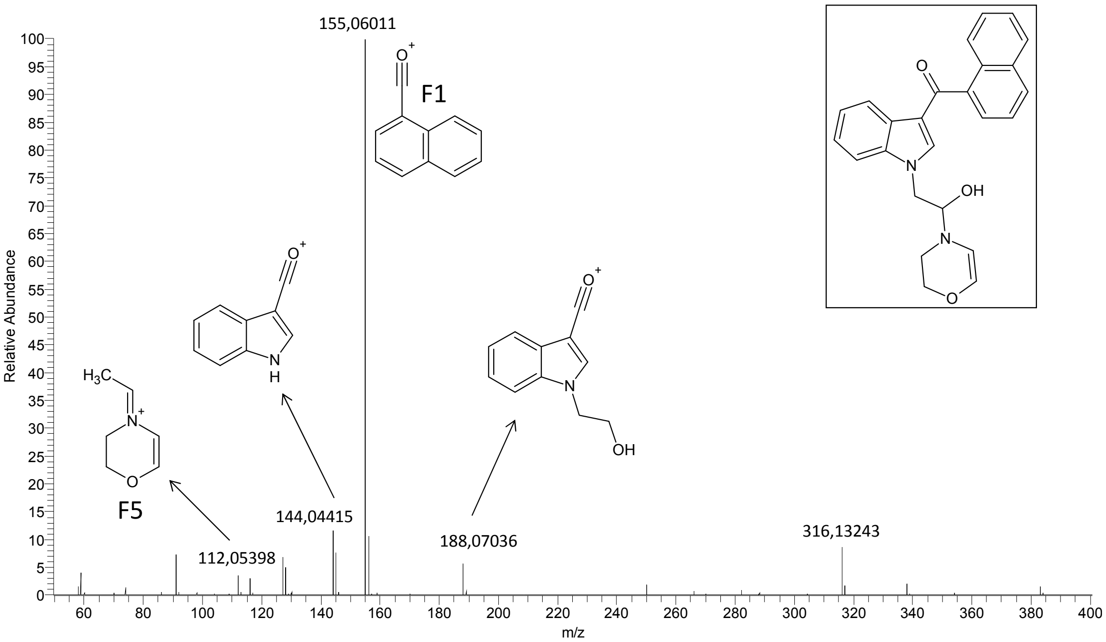


Fig. 8.5 HRMS mass spectrum of the protonated metabolite M3, using HCD at 50 eV.

3.3 Dihydrodiol metabolites

As known for naphthoylindoles, dihydrodiol metabolites are formed [22,25]. This loss of aromaticity in combination with two hydroxylations on vicinal positions, is most likely initiated through epoxidation and consecutive hydrolysis on the naphthalene ring [30]. As shown in Fig. 8.6 for metabolite M4 A, the unaltered morpholine ring is represented by ion F2 (Table 8.1). The modifications on the other part of the molecule are reflected in three product ions: *m/z* 189.0544 (Table 8.1, F7) corresponds to the dihydrodiol present on the naphthalene ring; *m/z* 171.0438 (Table 8.1, F4) and *m/z* 143.0489 (Table 8.1, F8) are formed after the loss of water and CO, respectively. The second metabolite with protonated mass *m/z* 419.1965 (M4 B, Fig.8.2) shows a similar fragmentation pattern, although the ion F7 is missing. The explanation for this can be found in the different location of the dihydrodiol in both isomeric metabolites as observed by Zhang et al. [30]. Because of the similar properties in terms of metabolism for both compounds, it can be assumed that also for JWH-200 the dihydrodiol functionalities are located on the same positions. For the metabolite carrying this functionality in ring *a* of the naphthalene, the aromaticity of the indole-naphthalene system is lost. Therefore the loss of water is favorable to restore the aromaticity, meaning that ion *m/z* 189.0544 (F7) is not formed. This would mean that the other metabolite carries the dihydrodiol functionality in the *b* ring.

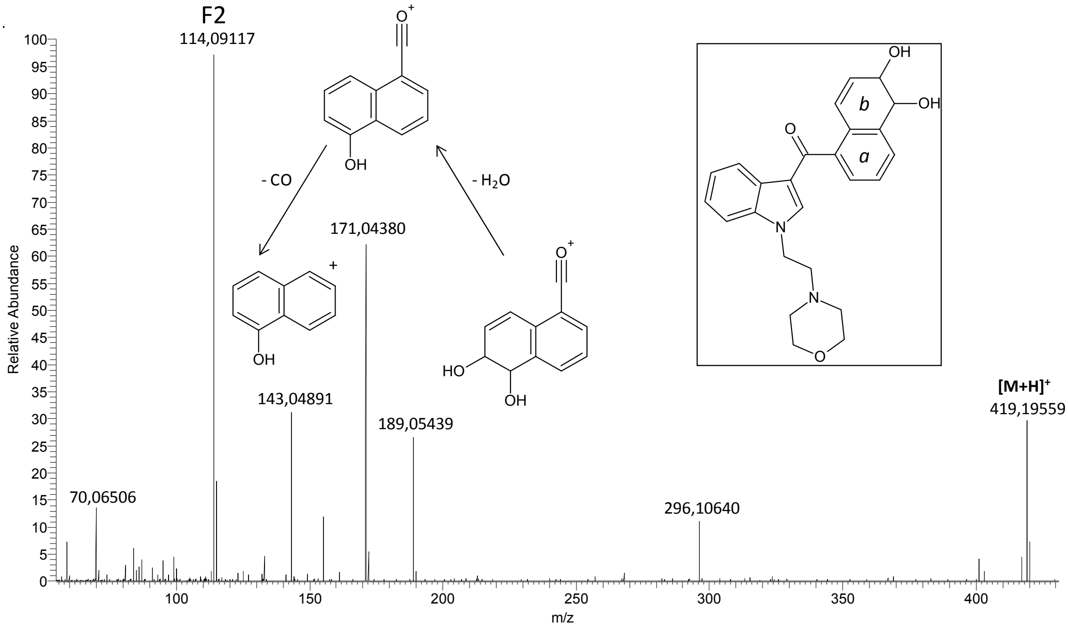


Fig. 8.6 Proposed fragmentation pattern (HCD, 50 eV) of the protonated metabolite M4 A. Only one possible configuration is shown.

Two peaks were detected with accurate masses *m/z* 435.1914 for the $[M+H]^+$ ion, i.e. the addition of an extra hydroxylation after the dihydrodiol formation. Unfortunately due to the low concentrations of these metabolites, efficient fragmentation using HRMS was not possible. Therefore more information was obtained using product ion scans on the LC-MS/MS system. The abovementioned fragmentation pattern proving the dihydrodiol on the naphthalene ring is also present in both these metabolites. Since in the first eluting substance (M5 A, Fig. 8.2) the product ions *m/z* 189, 171 and 143 are present and in the second (M5 B) *m/z* 189 is missing, this corresponds to the earlier proposed locations: in ring *b* of the naphthalene for M5 A and ring *a* for M5 B. From the presence of *m/z* 332 in the spectra of both molecules it can be derived that the hydroxylation is not located on the indole ring, since this ion corresponds to the complete metabolite after cleavage of the morpholine ring. Therefore it can be assumed that the latter was hydroxylated, however no additional proof in terms of product ions was found.

3.4 Morpholine ring cleavage

The dihydrodiol functionality is also detected together with the loss of the morpholine ring (M6, Fig. 8.2). After cleavage of the C-N bond, the alkyl side chain is hydroxylated at the end position [31]. This is the case for both metabolites M6 A and M6 B, since a product ion with *m/z* 188.0706 is detected (Table 8.1, F9). Since this morpholine-cleavage is identical, the difference between both metabolites is found in the location of the dihydrodiol on the naphthalene ring, similar as was proposed earlier: ring *b* for M6 A (F7, F4, F8) and ring *a* for M6 B (F4, F8).

Two metabolites are the result of a morpholine cleavage from the parent compound. In the first (M7) - with *m/z* 316.1332 and a retention time of 17.2 min – the end position of the alkyl side chain is again hydroxylated as for the M6 metabolites. Indeed, while the presence of F1 and F3 proved that the naphthalene moiety remained unchanged, the presence of F9 showed the modifications on the alkyl chain. In the second metabolite (M8 with *m/z* 330.1125 for the $[M+H]^+$ ion and retention time 20.0 min), the hydroxylation is replaced by a carboxylic group at the end of the alkyl chain. This is reflected in the mass spectrum by the presence of a product ion with *m/z* 202.0499, replacing F9. As a confirmation, this metabolite was also detected in negative mode with *m/z* 328.0979 for the $[M-H]^-$ ion. This metabolic transformation can be explained using the successive hydroxylation-hydrolysis pathway described by Denissen et al. [32].

As shown in Fig. 8.2, four metabolites were detected with *m/z* 332.1281 (M9). This corresponds to an increase of *m/z* 15.9944 compared to the abovementioned metabolite M7 (*m/z* 316.1332), pointing towards one extra hydroxylation in addition to the morpholine cleavage. For metabolites M9 A and D, the fragmentation is comparable to that of M7 but ion F9 is missing. This is replaced by a product ion with *m/z* 204.0655 (i.e. 15.9944 higher) in both metabolites. Upon further fragmentation the alkyl side chain is also cleaved, leading to a product ion with *m/z* 160.0393 (Table 8.2, F10). This allows locating the hydroxylation on the indole ring. Metabolite B differs from the previous two by the absence of ion F10, so it can be assumed the hydroxylation is located on the alkyl side chain instead of the indole ring. The low abundance of metabolite M9 C makes correct identification of the product ions difficult, therefore again low resolution MS/MS experiments were used. After conducting product ion scans at *m/z* 332, ions *m/z* 171 (cf. F4) and *m/z* 143 (cfr. F8) were found, proving the hydroxylation on the naphthalene ring.

3.5 Morpholine ring opening

The different metabolic pathways for the opening of the morpholine ring are well known. One of the described options is the cleavage of the C-O bond yielding a metabolite with a mass increased by 2 Da [31]. This transformation explained two metabolites for JWH-200 using liquid chromatography low resolution mass spectrometry by the presence of 2 peaks with *m/z* 387 for the $[M+H]^+$ ion (i.e. formula $C_{25}H_{26}N_2O_2$, compared to $C_{25}H_{24}N_2O_2$ for the parent molecule). However, in order to confirm this finding using HRMS technology, the accurate masses measured for both peaks (Fig. 8.2, M10) were found not to correspond to the proposed chemical formula. Indeed, when using a 5 ppm tolerance window the experimental protonated mass of *m/z* 387.1694 appeared to be the protonated form of $C_{24}H_{22}N_2O_3$. To obtain a metabolite with this formula, other modifications than cleaving the ether bond should occur. By performing experiments using all ion fragmentation in the HCD cell diagnostic ions were detected (Fig. 8.6): ion F1 showed the unaltered naphthalene ring, while a product ion with *m/z* 116.0704 could explain the modifications in the morpholine ring. To the best of our knowledge this pathway has not been previously described but can be explained by the two earlier proposed pathways for N-substituted morpholine rings. [32] In the first Denissen et al. propose an intermediate formed after a hydroxylation on the morpholine ring vicinal to the nitrogen, a ring opening via hydrolysis and a consecutive hydroxylation. When this product is further oxidized, a decarboxylation would lead to the ion proposed in Fig. 8.7. The second pathway is similar, although the initial hydroxylation is located next to the oxygen of the morpholine ring, leading to a different metabolite. This may explain the presence of 2 metabolites with identical fragmentation patterns but different retention times (Fig 2, M10). It should be noted that by only using low resolution techniques no distinction can be made between these metabolites and the metabolite as described by Zhang et al. [31].

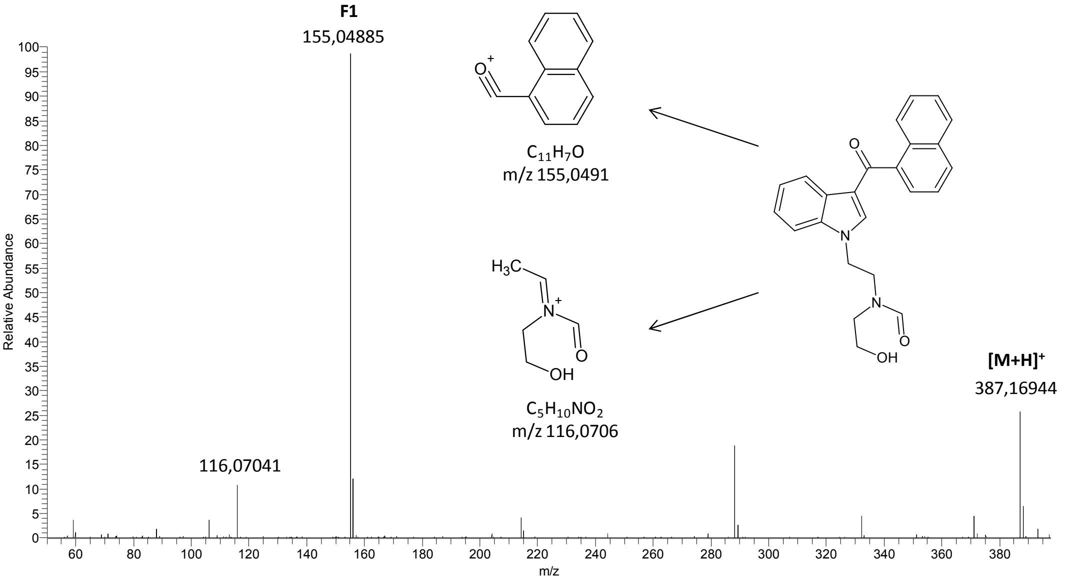
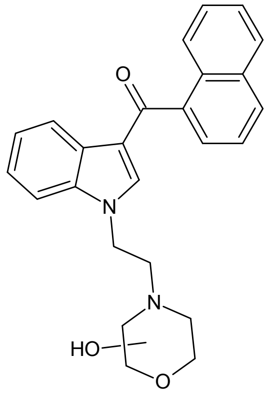
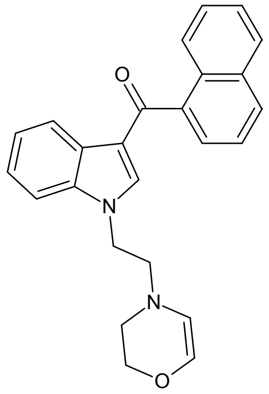
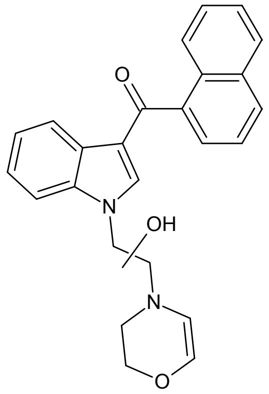
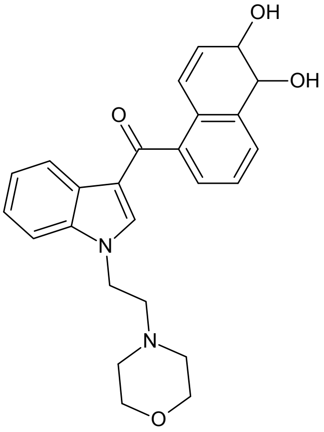
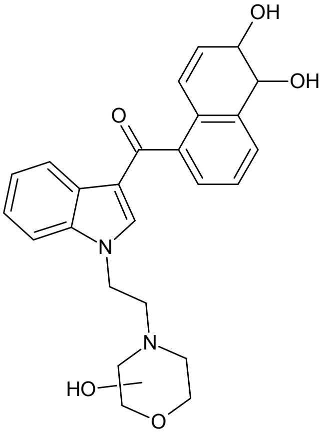
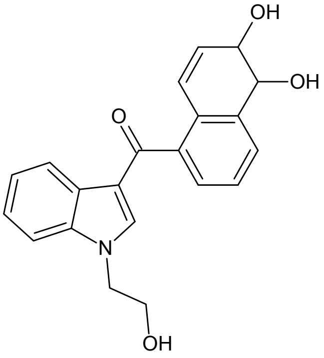
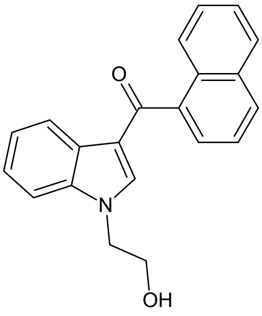


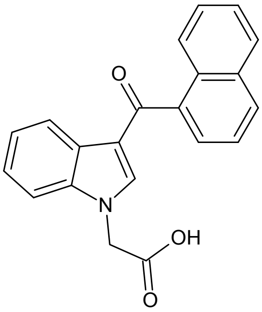
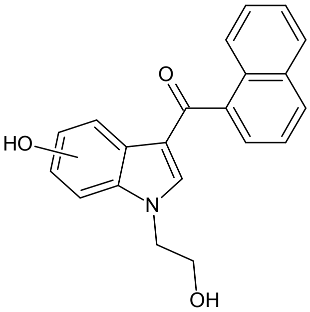
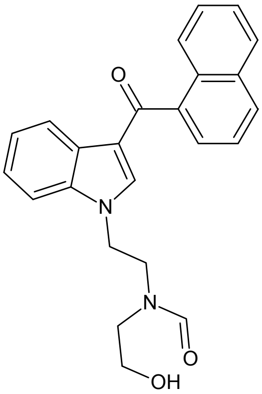
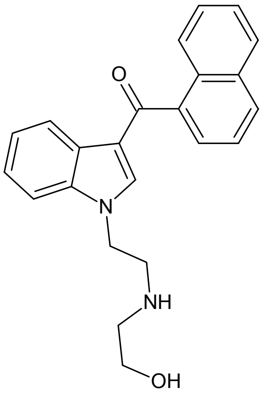
Fig. 8.7 Proposed fragmentation pathway (HCD, 20 eV) of the protonated ring opening metabolite M10. Only one possible configuration is shown.

One step further in the metabolic process the original morpholine ring is transformed to an ethanolamine moiety, via hydrolysis of the abovementioned intermediates [32]. In the metabolite formed via this pathway, a loss of C_2H_2 is seen compared to the parent molecule. Accordingly, one metabolite (M11) was detected at a retention time of 11.1 min with protonated mass *m/z* 359.1746. After HCD fragmentation, a product ion with *m/z* 88.0755 was detected. This corresponds to the loss of ethylene from ion F2 in the parent molecule. When applying low energies (10 to 20 eV) in the HCD cell, a product ion with *m/z* 315.1486 is also found. This is the cleavage of ethanol from the M11 metabolite, proving again the morpholine ring opening with the loss of ethylene.

Table 8.2. Proposed metabolite structures for JWH-200. Only one possible configuration is shown.

Category	**Modification**	**Example**
M1	Monohydroxylation *m/z* 401.1860	
M2	Dehydrogenation *m/z* 383.1754	
M3	Monohydroxylation + Dehydrogenation *m/z* 399.1703	

M4	Dihydrodiol functionality *m/z* 419.1965	
M5	Dihydrodiol functionality + monohydroxylation *m/z* 435.1914	
M6	Dihydrodiol functionality + cleavage morpholine ring *m/z* 350.1387	
M7	Cleavage morpholine ring (OH) *m/z* 316.1332	

M8	Cleavage morpholine ring (COOH) *m/z* 330.1125	
M9	Cleavage morpholine ring (OH) + monohydroxylation *m/z* 332.1281	
M10	Morpholine ring opening *m/z* 387.1703	
M11	Morpholine ring opening – loss of ethylene *m/z* 359.1746	

3.6 Analysis of mouse urine samples – chimeric mouse model

In the second part of this study uPA$^{+/+}$-SCID mice with humanized liver were used as a model for the *in vivo* metabolism of JWH-200. This model proved to be an efficient alternative for human administration studies for the investigation of steroid metabolism [33] and was recently used to reveal both phase I and phase II metabolism of synthetic cannabinoids [27].

Briefly this chimeric mouse model is based on the transplantation of human hepatocytes to uPA$^{+/+}$-SCID mice. Therefore mice suffering from a chronic liver disease caused by overexpression of the urokinase plasminogen activator (uPA) were used. This situation is beneficial for liver regeneration by transplanted hepatocytes. However, to avoid graft rejection when transplanting human hepatocytes, these mice were backcrossed with genetically immunodefficient (i.e. carrying a severe combined immune deficiency syndrome or SCID) mice. In the obtained uPA$^{+/+}$-SCID successful transplantation of human hepatocytes is possible [24,33].

For the following discussion a two-step approach was used. The phase I metabolism was investigated after performing a hydrolysis step on the mouse urine samples. Therefore the LC-MS/MS was operated in selected reaction monitoring mode (SRM). This method, containing two transitions for every metabolite detected *in vitro*, was used to confirm the previously described metabolites *in vivo*. Then the non-hydrolyzed urine was screened for glucuronide and sulphate conjugates by LC-HRMS and subsequently analyzed using the SRM method for the detection of potential free excreted metabolites.

Besides a small amount in one chimeric mouse urine, no parent compound was detected in the urines collected 24h after administration. The four *in vitro* monohydroxylated metabolites, M1A and M1C are also present in the mouse urine. Although metabolite M1D is not detected, one extra metabolite was found where the hydroxylation is also located on the naphthalene ring (M1E in Fig. 8.8). This finding was also reflected in the phase II metabolism, since three glucuronide conjugates were detected. When comparing the peak areas of the metabolites found in the hydrolyzed urine to those detected free in the non-hydrolyzed urine, it was possible to estimate the conjugation rate. Metabolite M1A was not detected free, while for both other monohydroxylated metabolites the conjugation rates were estimated around 50%.

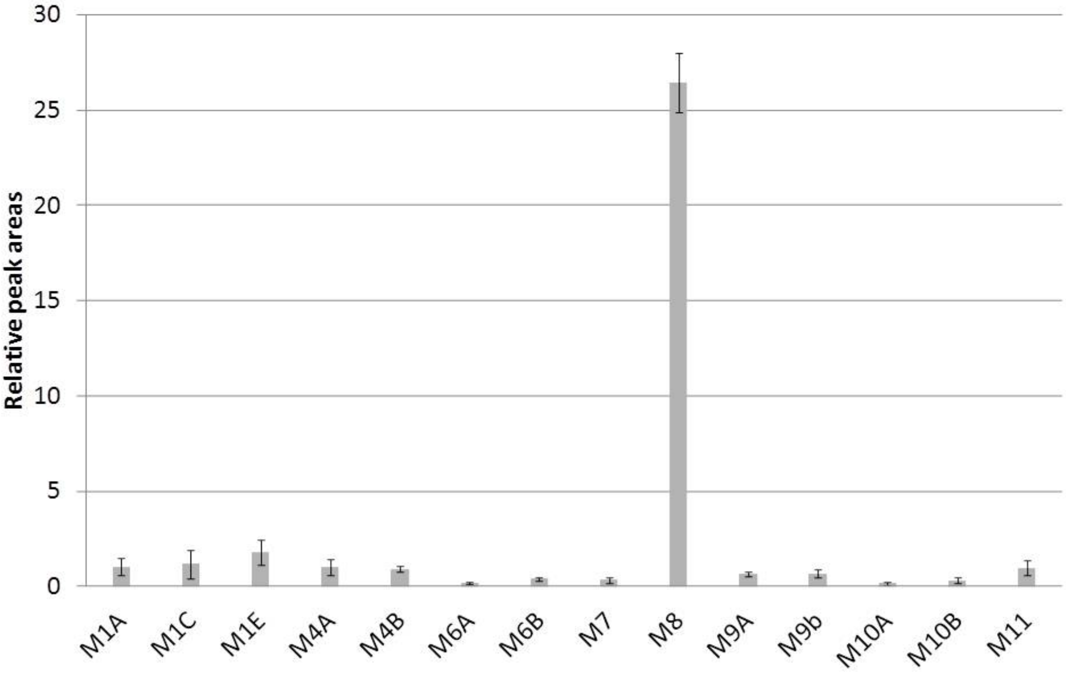


Fig. 8.8 Relative peak areas (corrected by the internal standard THCA-d9) of the metabolites detected using 3 chimeric mice for hydrolyzed urines collected 24h after administration.

In the chimeric mice, both metabolites with a dihydrodiol functionality (M4) were detected. From the HRMS experiments, two corresponding glucuronide conjugates were found. While for metabolite M4A the conjugation rate was estimated 80%, free M4B was not detected. It should be noted that only M4A was detected in the non-chimeric mouse (Fig. 8.9). Since metabolite M4B is absent in normal mice and only one glucuronide conjugate was detected in this control group, it can be considered an exclusive human metabolite.

Also the metabolites carrying a combination of the dihydrodiol and a morpholine cleavage (M6) seemed to be of human origin, since those were not detected in the non-chimeric control group either. In the hydrolyzed urine of the mice with humanized liver both metabolites M6A and M6B were detected, together with two glucuronide conjugates in the non-hydrolyzed urine. Since no free excreted metabolites were detected, the conjugation reaction was considered complete. A similar result was found for metabolite M7. This metabolite, with the alkyl side chain hydroxylated after the cleavage of the morpholine ring, is only found in the chimeric mouse urine and is completely excreted as glucuronide conjugate.

Not all metabolites are that extensively conjugated. Indeed, although metabolite M8 is detected in large amounts in the hydrolyzed urine (Fig. 8.8), only a small amount of the M8-glucuronide is detected. Taking into account the amount detected in the free fraction, the conjugation rate is estimated to be below 30%. However being excreted mostly non-conjugated, this metabolite could be detected in all hydrolyzed urines collected 48 h after administration using the SRM method.

Two metabolites from the M9-class could be confirmed *in vivo*, being M9A and M9B. Although only low amounts of these metabolites were present, two corresponding glucuronides were found but no significant amount could be detected in the free fraction.

Both types of morpholine ring opening detected in the *in vitro* model are confirmed in the chimeric mouse model. For the M10-class, were the ring opening is presumably followed by a decarboxylation, both metabolites M10A and M10B are detected in the hydrolyzed urine using the SRM method. In contrast to all other metabolites, no glucuronide but two sulphate conjugates were formed *in vivo*. Since sulphates easily undergo deprotonation, this finding could be confirmed by operating the HRMS in negative ionization mode. The metabolite formed after the loss of ethylene from the morpholine ring (M11) was also detected in the total fraction, excreted almost completely as glucuronide conjugate.

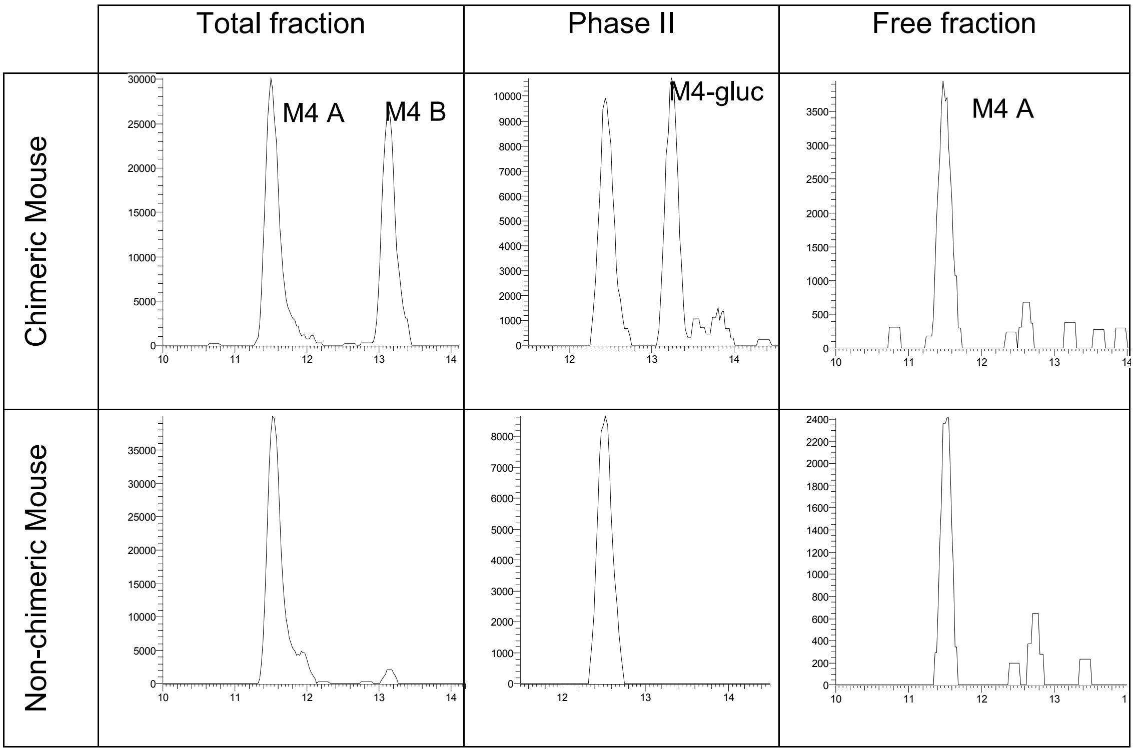


Fig. 8.9 *In vivo* metabolism of JWH-200: HRMS chromatograms for the protonated dihydrodiol metabolites (M4 – *m/z* 419.1965) and corresponding glucuronide conjugates (M4-gluc – *m/z* 595.2256) for mouse urine samples analyzed both with (total fraction) and without hydrolysis (phase II and free fraction).

4 Conclusion

Although the structure of JWH-200 shows similarities to other earlier described naphthoylindoles, the addition of the morpholine ring to the structure has a large impact on the metabolism. As shown in the *in vitro* research, this ring structure was modified in different ways. After cleavage of the complete ring, the remaining alkyl chain was hydroxylated (M7 and M9) or further oxidized towards a carboxylic acid on the end position (M8). In addition, the ring can also be opened in two different ways: via a new proposed pathway including a decarboxylation (M10) or via the known loss of ethylene (M11).

The chimeric mouse model allowed to confirm most of the metabolites discovered *in vitro*, including the mentioned modifications at the level of the morpholine ring. However the dehydrogenation pathway – as seen in metabolites M2 and M3 – could not be confirmed *in vivo*. Also the metabolites with the combination of the dihydrodiol functionality and a single hydroxylation (M5) were not found in the mouse urine.

By analyzing the non-hydrolyzed urine using HRMS, information was obtained on the phase II metabolism of JWH-200. Even though varied conjugation rates were observed, most metabolites were excreted as glucuronide conjugates. Only for the metabolites of the M10-class, two sulphate conjugates could be detected.

Using the information obtained from the *in vivo* model, it was possible to make conclusions towards routine screening of JWH-200 in urine. The relative abundances of the excreted metabolites in Fig. 8.8 clearly indicate that metabolite M8 is detected in the highest amounts in the *in vivo* model. Moreover, this metabolite was still detectable 48 hours after the dose was administered, which indicates that it is the metabolite of choice to monitor JWH-200 abuse. This can be done both on the free metabolite, after performing hydrolysis, and on the glucuronide conjugate, using non-hydrolyzed urine.

References

[1] M. Yonamine, D.R. Campos, R.L.D. Moreau. Marijuana as doping in sports. *Sports Medicine*. 2003, *33*, 395–99.

[2] European Monitoring Centre for Drugs and Drug Addiction. *Annual Report 2012: The state of the drug problem in Europe*.

[3] EMCDDA. Online sales of new psychoactive substances / “Legal highs”:Summary of results from the 2011 multilingual snapshots. *EMCDDA*. 2011, http://www.emcdda.europa.eu/attachements.cfm/att_143801_EN_SnapshotSummary.pdf

[4] EMCDDA, Europol. EMCDDA–Europol 2011 Annual Report. 2011, https://www.europol.europa.eu/sites/default/files/publications/emcdda-europol_annual_report_2011_2012_final.pdf

[5] S. Hudson, J. Ramsey. The emergence and analysis of synthetic cannabinoids. *Drug testing and analysis*. 2011, *3*, 466–78.

[6] EMCDDA. Understanding the “Spice” phenomenon. 2009, 1–34. http://www.emcdda.europa.eu/attachements.cfm/att_80086_EN_Spice Thematic paper□? final version.pdf

[7] I. Vardakou, C. Pistos, C. Spiliopoulou. Spice drugs as a new trend: Mode of action, identification and legislation. *Toxicology Letters*. 2010, *197*, 157–62.

[8] V. Auwärter, S. Dresen, W. Weinmann, M. Müller, M. Pütz, N. Ferreirós. “Spice” and other herbal blends: harmless incense or cannabinoid designer drugs? *Journal of mass spectrometry□: JMS*. 2009, *44*, 832–37.

[9] S. Kneisel, F. Westphal, B. Moosmann, V. Brecht, P. Bisel, C. Vidal, A. Jacobsen-Bauer, W.-R. Bork, V. Auwärter. Cannabimimetics II: Mass spectra and ATR-IR spectra of new compounds between the end of 2010 and late 2011. *Tiaft Bulletin*. 2011, *41*, 29–39.

[10] R. Kikura-Hanajiri, N. Uchiyama, M. Kawamura, Y. Goda. Changes in the prevalence of synthetic cannabinoids and cathinone derivatives in Japan until early 2012. *Forensic Toxicology*. 2012, 44–53. doi:10.1007/s11419-012-0165-2

[11] R. Lindigkeit, A. Boehme, I. Eiserloh, M. Luebbecke, M. Wiggermann, L. Ernst, T. Beuerle. Spice: a never ending story? *Forensic science international*. 2009, *191*, 58–63.

[12] L. Ernst, H.M. Schiebel, C. Theuring, R. Lindigkeit, T. Beuerle. Identification and characterization of JWH-122 used as new ingredient in “Spice-like” herbal incenses. *Forensic Sci Int*. 2011, *208*, 31–35.

[13] EMCDDA, Europol. EMCDDA–Europol 2009 Annual Report. *EMCDDA*. 2009, https://www.europol.europa.eu/sites/default/files/publications/emcdda-europol_annual_report_2009.pdf

[14] M.. Leonhart. *Schedules of Controlled Substances: Temporary Placement of Five Synthetic Cannabinoids Into Schedule I.* 2010, http://www.gpo.gov/fdsys/pkg/FR-2010-11-24/pdf/2010-29600.pdf

[15] UNODC. Synthetic cannabinoids in herbal products. http://www.unodc.org/documents/scientific/Synthetic_Cannabinoids.pdf

[16] N. Uchiyama, M. Kawamura, R. Kikura-Hanajiri, Y. Goda. Identification and quantitation of two cannabimimetic phenylacetylindoles JWH-251 and JWH-250, and four cannabimimetic naphthoylindoles JWH-081, JWH-015, JWH-200, and JWH-073 as designer drugs in illegal products. *Forensic toxicology*. 2010, *29*, 25–37.

[17] G. Merola, Z. Aturki, G.D. Orazio, R. Gottardo, T. Macchia, F. Tagliaro, S. Fanali. Analysis of synthetic cannabinoids in herbal blends by means of nano-liquid chromatography. *Journal of pharmaceutical and biomedical analysis*. 2012, *71*, 45–53.

[18] S. Strano-Rossi, L. Anzillotti, E. Castrignanò, F.S. Romolo, M. Chiarotti. Ultra high performance liquid chromatography-electrospray ionization-tandem mass spectrometry screening method for direct analysis of designer drugs, “spice” and stimulants in oral fluid. *Journal of chromatography. A*. 2012, *1258*, 37–42.

[19] S. Dresen, S. Kneisel, W. Weinmann, R. Zimmermann, V. Auwarter. Development and validation of a liquid chromatography-tandem mass spectrometry method for the quantitation of synthetic cannabinoids of the aminoalkylindole type and methanandamide in serum and its application to forensic samples. *J Mass Spectrom*. 2011, *46*, 163–71.

[20] S. Kneisel, V. Auwärter. Analysis of 30 synthetic cannabinoids in serum by liquid chromatography-electrospray ionization tandem mass spectrometry after liquid-liquid extraction. *Journal of Mass Spectrometry*. 2012, *47*, 825–35.

[21] A. Wohlfarth, K.B. Scheidweiler, X. Chen, H.-F. Liu, M.A. Huestis. Qualitative Confirmation of 9 Synthetic Cannabinoids and 20 Metabolites in Human Urine Using LC-MS/MS and Library Search. *Analytical chemistry*. 2013, *85*, 3730–38.

[22] A. Wintermeyer, I. Moller, M. Thevis, M. Jubner, J. Beike, M.A. Rothschild, K. Bender. In vitro phase I metabolism of the synthetic cannabimimetic JWH-018. *Anal Bioanal Chem*. 2010, *398*, 2141–53.

[23] I. Moller, A. Wintermeyer, K. Bender, M. Jubner, A. Thomas, O. Krug, W. Schanzer, M. Thevis. Screening for the synthetic cannabinoid JWH-018 and its major metabolites in human doping controls. *Drug Test Anal*. 2011, *3*, 609–20.

[24] P. Meuleman, L. Libbrecht, R. De Vos, B. de Hemptinne, K. Gevaert, J. Vandekerckhove, T. Roskams, G. Leroux-Roels. Morphological and

biochemical characterization of a human liver in a uPA-SCID mouse chimera. *Hepatology*. 2005, *41*, 847–56.

[25] Q. Zhang, P. Ma, R.B. Cole, G. Wang. Identification of in vitro metabolites of JWH-015, an aminoalkylindole agonist for the peripheral cannabinoid receptor (CB2) by HPLC-MS/MS. *Anal Bioanal Chem*. 2006, *386*, 1345–55.

[26] T.G. Sobolevsky, I.S. Prasolov, G.M. Rodchenkov. Application of mass spectrometry to the structural identification of the metabolites of the synthetic cannabinoid JWH-018 and the determination of them in human urine. *Journal of Analytical Chemistry*. 2011, *66*, 1314–23.

[27] N. De Brabanter, S. Esposito, L. Geldof, L. Lootens, P. Meuleman, G. Leroux-Roels, K. Deventer, P. Van Eenoo. In vitro and in vivo metabolisms of 1-pentyl-3-(4-methyl-1-naphthoyl)indole (JWH-122). *Forensic Toxicology*. 2013, *31*, 212–22.

[28] T. Geiger, J. Cox, M. Mann. Proteomics on an Orbitrap benchtop mass spectrometer using all-ion fragmentation. *Molecular & cellular proteomics□: MCP*. 2010, *9*, 2252–61.

[29] S. Beuck, I. Moller, A. Thomas, A. Klose, N. Schlorer, W. Schanzer, M. Thevis. Structure characterisation of urinary metabolites of the cannabimimetic JWH-018 using chemically synthesised reference material for the support of LC-MS/MS-based drug testing. *Anal Bioanal Chem*. 2011, *401*, 493–505.

[30] Q. Zhang, P. Ma, M. Iszard, R.B. Cole, W. Wang, G. Wang, W.E.T. Al. In vitro metabolism of R(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo [1,2,3-de]1,4-benzoxazinyl]-(1-naphthalenyl) methanone mesylate, a cannabinoid receptor agonist. *Drug Metab Dispos*. 2002, *30*, 1077–86.

[31] Q. Zhang, P. Ma, W. Wang, R.B. Cole, G. Wang. Characterization of rat liver microsomal metabolites of AM-630, a potent cannabinoid receptor antagonist, by high-performance liquid chromatography/electrospray ionization tandem mass spectrometry. *Journal of mass spectrometry□: JMS*. 2004, *39*, 672–81.

[32] J.F. Denissen, B.A. Grabowski, M.K. Johnson, S.A. Boyd, J.T. Uchic, H. Stein, S. Cepa, P. Hill. The orally active renin inhibitor A-74273. In vivo and in vitro morpholine ring metabolism in rats, dogs, and humans. *Drug metabolism and disposition: the biological fate of chemicals*. *22*, 880–88.

[33] L. Lootens, P. Meuleman, O.J. Pozo, P. Van Eenoo, G. Leroux-Roels, F.T. Delbeke. uPA(+/+)-SCID Mouse with Humanized Liver as a Model for In Vivo Metabolism of Exogenous Steroids: Methandienone as a Case Study. *Clinical Chemistry*. 2009, *55*, 1783–93.

Part IV

Summary and conclusions

Chapter 9

In conclusion

Over the last years the aim of DoCoLab has been to improve the existing analytical procedures in several ways. To raise the overall efficiency of the laboratory – both with respect to time and costs – it was also tried to reduce the number of screening procedures.

This goal could be achieved although simultaneously more stringent requirements were imposed by WADA. Indeed, the number of compounds and/or corresponding metabolites is increasing while the corresponding MRPL's follow the opposite trend. Research projects - focused on metabolomics – yielded more and better target metabolites to screen for known compounds and allowed to implement newly discovered compounds in these improved screening methods.

This thesis describes some of the efforts made both in the development of a new screening method as in the implementation of a new class of compounds.

Part II: The implementation of GC-QqQ-MS

In the first part the evolutions in the GC-screening methods are described. Before the installation of the GC-MS/MS system in 2009, two GC-MS methods [1,2] were in use: one method to detect anabolic steroids and quantify the endogenous steroids and another to screen for narcotics and stimulants. This required two different sample preparation procedures and corresponding chromatographic runs of approximately 20-26 minutes each. It is self-evident that the implementation of a single GC-MS/MS method capable of detecting all these compounds, in a run of 15 minutes, improved the sample turn-around time strongly.

In **chapter 3** the first fast GC-MS/MS method is described. This method – using hydrogen as a carrier gas – is built up in two parts. In the quantitative part the steroids of the traditional steroid profile are included together with endogenous steroids, which are not affected by the intake of natural AAS (11β-OH-androsterone and 11β-OH-etiocholanolone) and markers to monitor microbiological degradation (5a-androstanedione and 5b-androstanedione) of the urine samples. Additionally, the method is also capable of quantifying salbutamol and the major metabolite of cannabis (THCA). Compared to the former methods, this quantification was not only improved due to the higher selectivity and specificity of the new technique but also by using 6-point calibration curves and deuterated internal standards instead of one-point calibration and use of a single internal standard. In the qualitative part of the method, over 140 substances can be detected at or below the required MRPL's. Although only 1 mL of urine was used, low detection limits could be reached by the combination of tandem MS detection and the injection of large volumes with the PTV-system.

While in the past, monitoring of the sample preparation was principally limited to verification of extraction recovery and derivatisation efficiency, in the new method measures were taken to monitor all major sample preparation steps in every single sample. The hydrolysis and extraction efficiency are evaluated by the addition of both free and glucuronidated deuterated internal standards, the yield of the derivatisation reaction is measured by including transitions for the mono-TMS derivatised androsterone and etiocholanolone and two markers are quantified to monitor possible microbiological sample degradation.

Technically it was possible to limit the run time to only 8 min by the combination of a short HP-Ultra 1 column and hydrogen as carrier gas

This GC-MS/MS screening method was subject of further research in order to further improve it (according to the at that time statutory WADA technical document [3]) . **Chapter 4** describes in detail both the research and the adjustments made to the method.

With daily use, it was found that the system showed stability problems when using hydrogen as a carrier gas, this was solved by switching back to the previously used helium. Although this increased the runtime to about 12 min, the extended temperature program had the advantage that the separation of some more volatile compounds became possible.

Indeed, qualitatively, the method was extended to over 150 compounds from different classes. In the quantitative part the traditional steroid profile was expanded with 6 minor metabolites, which can enlarge the distinctive power of the steroid profile in the detection of endogenous steroid abuse. The addition of these markers could allow the use of this method in the framework of the adaptive model [4] on a routine basis. Currently population based reference ranges must be exceeded before a confirmation procedure by IRMS is recommended [5]. When – by analogy with the already used hematological markers – endogenous steroid data will be included in the Athlete Biological Passport [6], individual reference ranges will decrease the margins for detection of endogenous steroid abuse [7].

The quality measurements implemented to monitor the complete sample preparation procedure were further investigated and improved. To check the hydrolysis efficiency, the ratio of androsterone-d4 glucuronide over etiocholanolone-d5 was used. Further the ratio of mono-TMS derivatised androsterone to bis-TMS derivatised androsterone proved to be the parameter of choice to monitor the derivatisation reaction. Stability experiments were performed to investigate the response and reliability of the steroid profile under harsh conditions and to identify reliable markers for microbiological degradation. Therefrom, it could be concluded that relative concentration ratios (like 5α-androstane-3,17-dion over androsterone) are the obvious parameters to estimate the effect of microbiological degradation on the concentrations of the steroid profile.

In **chapter 5**, a fast GC-MS/MS confirmation procedure for THCA is described. Therefore both the sample preparation procedure and the GC-methodology were optimized. Starting from only 1 mL of urine, the alkaline hydrolysis proved to be complete in 7 min. Compared to previously used methods, only a single extraction was needed to obtain sufficient selectivity in the MS/MS system, which was performed by 1 minute of vortexing. Microwave-assisted-derivatisation was applied to speed up the otherwise time-consuming derivatisation step. Finally the GC-runtime was reduced to 4 minutes.

In that way the combination of a simplified sample preparation procedure, large volume injection using the PTV-injector and tandem mass spectrometry allows to quantify THCA in urine in only 30 minutes.

Part III: Synthetic cannabinoids

The class S8 of the prohibited list [8] contains both natural as well as synthetic substances binding to the cannabinoid receptors. In addition to the optimization of the confirmation procedure for cannabis, this thesis also focused on other cannabinoids. It should be noted that synthetic cannabinoids are not only a doping related problem. Indeed, the introduction of a wide-range of new psychoactive substances, constitutes a growing drug problem in general. The increasing use of these products, among which the group of synthetic cannabinoids is studied in this work, should therefore be seen in a wider perspective. Although the number of positive cases in sport drugs testing are rather limited up to now [9], the popularity of these cannabimimetics is growing all over the world [10–12]. Reversing this evolution is only possible when a broad, international approach is applied. So, dissemination of correct information, a sound and uniform legislation, interception of production/dealing units and efficient detection of abuse are essential.

Chapter 6 summarizes some general considerations on these synthetic cannabinoids. An overview is given on the situation of the abuse and the current legislation. Further attention is paid to the interactions which occur in the human body, the possible health risk of (long-term) use and the current state of affairs in the field of detection is outlined.

In the doping control area as well as in many other fields, urine is the matrix of choice to detect drugs of abuse. Still, this is not straightforward since it necessitates the knowledge of the metabolic modifications to which the compound of interest is submitted prior to excretion. Synthetic cannabinoids are known to be heavily metabolized in the human body, even to such extent that the unchanged parent compound is usually not detectable in urine. This makes metabolic studies an absolute prerequisite for development of screening methods using urine. As explained in chapter 6, it is unethical to carry out human administration studies for metabolic investigation of these types of compounds. Therefore in this work a strategy - based upon the consecutive use of two models - is presented as alternative.

The application of this strategy to reveal the metabolism of JWH-122 is described in **chapter 7**. In a first step, the phase I metabolism is investigated by means of an *in vitro* model using human liver microsomes. This model has the advantage that it is easy to carry out in a standard analytical laboratory. In this work, it was found that for the cannabimimetics studied, an incubation time of 4h showed the best results, which makes it a fast procedure as well. Further, the limited amount of matrix interferences allows identifying the formed metabolites more easily than in real urine samples. Using LC-MS, 11 classes of metabolites were identified. The JWH-122 parent compound was metabolized via hydroxylations, dehydrogenations or cleavage of the *N*-pentyl side chain. Further also dihydrodiol functionalities were introduced on some metabolites and one metabolite was found carrying a carboxyl group on the *N*-pentyl side chain. Information on the location of the metabolic modifications on a given metabolite was obtained from fragmentation experiments using CID on the LC-MS/MS system.

Consecutively the presence of these metabolites was checked in an *in vivo* model, developed in cooperation with the Centre for Vaccinology, via oral administration to chimeric uPA$^{+/+}$-SCID mice. LC-MS analysis of the mouse urine allowed confirming most abovementioned metabolic modifications. Furthermore, the urine samples were analysed a second time, without enzymatic hydrolysis. From these experiments, it could be concluded that all detected metabolites were excreted as glucuronide or sulphate conjugate, with conjugation rates above 50%. By comparing the relative peak areas, it was found that the monohydroxylated JWH-122 metabolites were excreted in the highest amounts. Therefore these metabolites were selected as target compounds to screen for JWH-122 abuse.

The same strategy was also followed for JWH-200 as explained in **chapter 8**. The presence of the morpholine ring on the alkyl side chain of this naphthoylindole had a large impact on the metabolism. Indeed, next to the modifications described for JWH-122, the presence of the morpholine ring was responsible for the formation of several ring-opening metabolites. To locate these metabolic modifications both CID (LC-MS/MS) and HCD (LC-HRMS) experiments were performed. Although high resolution data acquisition makes unambiguous identification of the fragment possible, the all-ion-fragmentation technique, used in the HCD-cell, sometimes makes it difficult to distinguish compound-related fragments from others, especially in the lower concentration ranges. For the latter the more specific product ion scans on the LC-MS/MS system can offer a solution.

Although less metabolites were found via the *in vivo* model compared to *in vitro*, most metabolic modifications could be confirmed. As for JWH-122, also the JWH-200 metabolites were excreted as glucuronide or sulphate conjugates. For these experiments the strength of the HRMS detection should be highlighted. By combining the full scan high resolution data acquisition with fast polarity switching between positive and negative scan modes, all data can be collected in one single run. Afterwards it is a matter of data processing to confirm whether or not a theoretically possible phase II metabolite is present. This is particularly of interest if there is a limited volume of sample available, as for the mouse urine.

The JWH-200 metabolite, formed after consecutive morpholine cleavage and oxidation of the remaining side chain to a carboxylic group, appeared to be present in the highest amounts. In general, with this mouse model metabolites are cleared within 24h whereas this metabolite was detectable even 48h after administration. This indicates that this metabolite might be useful for prolonged detection of misuse.

It is clear that the research described in this work, which could lead to the urinary detection of both JWH-compounds, is only a small step in the whole synthetic cannabinoid story. As mentioned earlier, this problem requires a global and comprehensive approach, in which different areas of expertise will need to contribute.

Today it is impossible to estimate how the situation will evolve, so continuously improving the detection strategies of the currently known compounds and these which will – without any doubt – enter the market in the (near) future, will be necessary. Concerning the detection in urine, both research toward metabolism and method development will be essential. As can be derived from their scientific output in this area over the last years, doping control laboratories play an important part in this.

References

[1] P. Van Renterghem, P. Van Eenoo, W. Van Thuyne, H. Geyer, W. Schanzer, F.T. Delbeke. Validation of an extended method for the detection of the misuse of endogenous steroids in sports, including new hydroxylated metabolites. *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences*. 2008, *876***,** 225–35.

[2] W. Van Thuyne, P. Van Eenoo, F.T. Delbeke. Implementation of gas chromatography combined with simultaneously selected ion monitoring and full scan mass spectrometry in doping analysis. *Journal of chromatography. A*. 2008, *1210***,** 193–202.

[3] WADA. Technical Document: Minimum Required Performance Limits for Detection of Prohibited Substances: TD2010MRPL. 2010, http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-IS-Laboratories/WADA_TD2010MRPLv1.0_Minimum Required Performance Levels_Sept 01 2010_EN.pdf

[4] P. Van Renterghem, P.-E. Sottas, M. Saugy, P. Van Eenoo. Statistical discrimination of steroid profiles in doping control with support vector machines. *Analytica chimica acta*. 2013, *768***,** 41–48.

[5] WADA. WADA Technical Document - TD2004EAAS. 2004, 11. http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-IS-Laboratories/WADA_TD2004EAAS_EN.pdf

[6] WADA. Athlete biological passport - Operating guidelines. 2012, http://www.wada-ama.org/Documents/Science_Medicine/Athlete_Biological_Passport/WADA_ABP_OperatingGuidelines_version_3.0.pdf

[7] P. Van Renterghem, P. Van Eenoo, P.-E. Sottas, M. Saugy, F. Delbeke. Subject-based steroid profiling and the determination of novel biomarkers for DHT and DHEA misuse in sports. *Drug testing and analysis*. 2011, *2***,** 582–88.

[8] WADA. The 2013 Prohibited List, International Standard. 2013, http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-Prohibited-list/2013/WADA-Prohibited-List-2013-EN.pdf

[9] WADA. 2011 Laboratory Testing Figures. 2012, http://www.wada-ama.org/Documents/Resources/Testing-Figures/WADA-2011-Laboratory-Testing-Figures.pdf

[10] UNODC. Synthetic cannabinoids in herbal products. http://www.unodc.org/documents/scientific/Synthetic_Cannabinoids.pdf

[11] EMCDDA. Online sales of new psychoactive substances / “Legal highs”:Summary of results from the 2011 multilingual snapshots. *EMCDDA*. 2011, http://www.emcdda.europa.eu/attachements.cfm/att_143801_EN_SnapshotSummary.pdf

[12] Belgian monitoring centre for drugs and drug addiction. Nieuwe psychoactieve substanties in België (2005-2011). 2012, 59. http://ewsd.wiv-isp.be/Shared documents/Reports/Nieuwe psychoactieve substanties in Belgi? 2005-2011.pdf

Chapter 10:

Ter besluit

Het streven naar hogere efficiëntie was de laatste jaren één van de drijfveren voor het onderzoek rond methodeontwikkeling in DoCoLab. Enerzijds betekenen efficiëntere methodes een winst in zowel tijd als kosten, anderzijds is continue verbetering noodzakelijk om te blijven voldoen aan de steeds strenger wordende eisen. Zo wordt het aantal te bepalen componenten steeds groter, terwijl de minimaal vereiste detectielimieten hiervoor steeds worden verlaagd.

Naast het ontwikkelen van nieuwe methodes wordt veel geïnvesteerd in metabolisme onderzoek. Dit is een noodzakelijke tussenstap om tot detectie van nieuwe componenten in urine te komen. Wanneer het gaat over reeds gekende componenten verschuift de nadruk naar beter en langer detecteerbare metabolieten.

In dit werk wordt zowel de ontwikkeling van een nieuwe screeningsmethode als de implementatie van een nieuwe klasse van verboden producten besproken.

Deel II: De implementatie van GC-QqQ-MS

In het eerste deel van deze thesis wordt de evolutie op het gebied van gaschromatografie beschreven. Voor de installatie van het GC-MS/MS systeem in 2009 werd gebruikt gemaakt van twee GC-methodes [1,2], de ene voor de detectie van anabole steroïden en de kwantificering van de endogene steroïden en de andere voor het screenen op narcotica en stimulantia. Naast twee GC-methodes van 20-26 minuten waren hiervoor ook twee verschillende staalvoorbeidingsprocedures in gebruik. Het spreekt vanzelf dat de efficiëntie sterk verhoogd werd door beide methodes te vervangen door één enkele GC-MS/MS methode van slechts 15 minuten.

In **hoofdstuk 3** wordt de ontwikkeling en validatie van de eerste methode op dit nieuwe toestel beschreven. Vanuit theoretisch oogpunt bestaat deze methode uit twee delen. In het kwantitatieve deel worden de concentraties bepaald van de steroïden die deel uitmaken van het klassieke steroïdprofiel. Daarnaast worden ook twee endogene steroïden waarvan de excretie niet wordt beïnvloed door het gebruik van steroïden (11β-OH-androsterone en 11β-OH-etiocholanolone) en merkers voor microbiële degradatie (5α-androstanedione en 5β-androstanedione) kwantitatief bepaald. Naast deze zijn ook salbutamol en de belangrijkste metaboliet van cannabis

(THCA) in het kwantitatieve deel opgenomen. In vergelijking met de vroeger gebruikte methodes werd de kwantificering van deze componenten sterk verbeterd. Hierin speelde niet alleen het nieuwe GC-MS/MS systeem een rol, ook de overschakeling naar gedeutereerde inwendige standaarden en calibratiecurves over 6 niveaus zijn van belang. In het kwalitatieve deel wordt gescreend op meer dan 140 producten die gedetecteerd kunnen worden aan concentraties conform de door WADA opgelegde MRPL's. De combinatie van de selectieve tandem MS detectie met de injectie van grotere volumes via het PTV-systeem, liet toe om deze niveau's te bereiken in slechts 1 mL urine.

Ook aan kwaliteitscontrole wordt meer aandacht besteed. Bij vorige methodes was de controle op de staalvoorbereiding beperkt tot het opvolgen van het rendement van de extractie en de derivatisatie. In de nieuwe methode worden zowel geglucuronideerde als vrije gedeutereerde inwendige standaarden toegevoegd om elke stap van de staalvoorbereiding te controleren in elk individueel staal. De methode bevat verder ook transities voor de enkelvoudig gederivatiseerde (mono-TMS) androsterone en etiocholanolone om de derivatisatie reactie op te volgen en twee merkers voor eventuele microbiële degradatie van de urine. Het gebruik van een kortere HP-Ultra1 kolom met waterstof als dragergas liet toe alle componenten te scheiden in slechts 8 minuten.

Van bij de opstart van de nieuwe GC-MS/MS screeningsprocedure werd verder onderzoek gedaan met als doel deze continu te verbeteren. In **hoofdstuk 4** worden zowel het onderzoek als de uiteindelijke aanpassingen aan de methode beschreven.

Na de ingebruikname van de methode werd duidelijk dat het gebruikte dragergas verantwoordelijk was voor de ervaren stabiliteitsproblemen van de ionisatiebron. Daarom werd het waterstofgas vervangen door helium. Dit verlengt de methode tot ongeveer 12 minuten, maar maakt de scheiding van enkele meer vluchtige componenten mogelijk. Bijgevolg kon het kwalitatieve deel van de methode uitgebreid worden tot meer dan 150 componenten uit verschillende klassen. In het kwantitatieve deel werd het klassieke steroïdprofiel uitgebreid met 6 zogenaamde "minor metabolites". Eerder onderzoek in DoCoLab heeft aangetoond dat deze steroïden de kracht van het steroïdprofiel - als instrument voor de opsporing van endogeen steroïdmisbruik - kan vergroten. De kwantificering van deze merkers in de screeningsmethode is mogelijk een eerste stap naar de implementatie ervan in het Bayesiaans adaptief model [3]. Momenteel worden grenswaarden gebaseerd op populatiestatistiek gebruikt om te beslissen of een eventuele confirmatie via IRMS noodzakelijk is [4]. Wanneer echter gegevens over het endogeen steroïdprofiel worden ingevoerd in het Athlete Biological Passport [5] – zoals dat nu reeds gebeurt voor hematologische merkers – kunnen individuele grenswaarden de ruimte voor endogeen steroïdmisbruik verkleinen [6].

Wat kwaliteitscontrole betreft, werd gezocht naar optimalisatie van de gebruikte parameters. Voor de controle van de hydrolyse efficiëntie werd gebruik gemaakt van de verhouding van de inwendige standaarden androsterone-d4 glucuronide over etiocholoanolone-d5. Het enkelvoudig gederivatiseerde androsterone (mono-TMS) werd geselecteerd als beste merker om de derivatisatie op te volgen. Om een beeld te krijgen van de impact van eventuele microbiële degradatie op de concentraties van de endogene steroïden werden stabiliteitsexperimenten uitgevoerd. Hieruit kon besloten worden dat de relative verhouding van de eerder ingevoerde merkers duidelijker de echte impact van de degradatie op de concentraties van het steroïdprofiel weerspiegelen.

In **hoofdstuk 5** werd een snelle GC-MS/MS confirmatieprocedure voor THCA beschreven. Om te komen tot een snelle en efficiënte methode werden zowel de staalvoorbereiding als de GC-methode geoptimaliseerd. Er werd gebruik gemaakt van slechts 1 mL urine en een snelle alkalische hydrolyse met NaOH. In vergelijking met de oudere methodes is gebleken dat één enkele extractie volstaat om voldoende selectiviteit te krijgen op het GC-MS/MS systeem en dat met een temperatuursprogramma van slechts 4 minuten. De doorgaans lange derivatisatie kon sterk worden ingekort door microgolven te gebruiken voor de warmteoverdracht. Deze vereenvoudigde staalvoorbereiding, het gebruik van een PTV-injector en de tandem MS detectie maken het mogelijk de concentratie van THCA te bepalen in 1 mL urine in een tijdspanne van ongeveer 30 minuten.

Deel III: Synthetische cannabinoïden

Klasse S8 van de WADA lijst [7] omvat zowel natuurlijke als synthetische substanties die binden op de cannabinoïdreceptoren in het menselijk lichaam. Naast de optimalisatie van de confirmatieprocedure voor cannabis in hoofdstuk 3 (deel I), werd in deel II van dit werk ook onderzoek gedaan naar andere cannabinoïden. Het probleem van de synthetische cannabinoïden is meer dan doping alleen, het is onderdeel van een wereldwijd stijgend gebruik van een groot gamma nieuwe psychoactieve drugs. Hoewel tot op vandaag het gebruik ervan in competitie eerder beperkt is [8], stijgt de populariteit van deze cannabimimetica over de hele wereld [9–11].

Hoofdstuk 6 schetst een beeld van de huidige situatie wat betreft synthetische cannabinoïden. Het misbruik ervan werd in kaart gebracht samen met de huidige maatregelen van zowel overheden als (inter)nationale organisaties om het probleem onder controle te krijgen. Verder werd er aandacht besteed aan de interacties die plaatsvinden in het menselijk lichaam, de mogelijke gezondheidsrisico's bij (langdurig) gebruik en de ontwikkelingen op het gebied van detectie van deze substanties.

Om het gebruik van verboden producten op te sporen wordt in veel domeinen – waaronder in de dopingcontrole – gebruik gemaakt van urine als matrix. Dit is echter niet vanzelfsprekend aangezien dit de kennis vereist van de metabole processen die plaatsvinden in het menselijk lichaam. Het is reeds beschreven dat synthetische cannabinoïden zeer sterk gemetaboliseerd worden, zelfs in die mate dat de onveranderde component doorgaans niet gedecteerd wordt in urine. Bijgevolg is de studie van het metabolisme noodzakelijk om de detectie in urine mogelijk te maken. Zoals besproken in hoofdstuk 4, is het niet ethisch verantwoord deze producten met dit doel toe te dienen aan mensen. Als alternatief hiervoor werd in dit werk een strategie beschreven, gebaseerd op de combinatie van twee modellen.

De toepassing van deze strategie om het metabolisme van JWH-122 te onderzoeken werd beschreven in **hoofdstuk 7**. In een eerste stap werd gebruik gemaakt van een *in vitro* model op basis van humane levermicrosomen. Dit model is eenvoudig uit te voeren in een standaard analytisch laboratorium en neemt met een incubatietijd van slechts 4 uur weinig tijd in beslag. Verder bevat de matrix minder interfererende substanties dan urine, wat de identificatie van de aanwezige metabolieten gemakkelijker maakt. In totaal werden via LC-MS analyse 11 types metabolieten gevonden. De JWH-122 molecule werd gemetaboliseerd via hydroxylaties, dehydrogenaties of het afsplitsen van de *N*-pentyl zijketen. Naast enkele metabolieten die een dihydrodiol functie bevatten, werd ook een metaboliet gevonden met een carboxonzure functie op de *N*-pentyl keten. Informatie over de locatie van deze modificaties werd verkregen via LC-MS/MS fragmentatie experimenten (CID).

In een tweede stap werd getracht de vorming van de eerder gevonden metabolieten te bevestigen in een *in vivo* model. Hiervoor werd gebruik gemaakt van een muismodel, ontwikkeld in samenwerking met het Centrum voor Vaccinologie, waarbij chimere uPA$^{+/+}$-SCID muizen het synthetische cannabinoïd oraal toegediend kregen. Via LC-MS analyse van de gecollecteerde urine was het mogelijk de meeste metabole modificaties te bevestigen. Analyse van dezelfde stalen zonder enzymatische hydrolyse toonde aan dat alle metabolieten – weliswaar in verschillende mate - glucuronide- of sulfaatconjugatie ondergaan voor de urinaire excretie. Voor de selectie van de metaboliet die het meest in aanmerking komt om als merker te gebruiken in de screeningsmethode, werden de relatieve piekoppervlakken van alle metabolieten vergeleken.

Hieruit werd duidelijk dat de enkelvoudig gehydroxyleerde metabolieten in de grootste hoeveelheden aanwezig waren en bijgevolg te verkiezen zijn om JWH-122 gebruik in urine op te sporen.

Dezelfde strategie werd gebruikt voor JWH-200, zoals beschreven in **hoofdstuk 8**. De aanwezigheid van de morfoline ring op de alkyl zijketen van deze component had een grote impact op het metabolisme. Naast de reeds voor JWH-122 beschreven functionaliteiten, werden extra metabolieten gevormd via modificaties van de morfoline ring. Om te kunnen bepalen op welk deel van de molecule de reacties plaatsvonden, werden zowel CID (LC-MS/MS) als HCD (LC-HRMS) experimenten uitgevoerd. Gebruik maken van hoge resolutie detectie heeft als voordeel dat eenduidig de brutoformule van de gevormde fragmenten kan achterhaald worden. Echter, in de HCD collisiecel wordt alles gelijktijdig gefragmenteerd (all-ion fragmentation), wat het soms moeilijk maakt component-specificieke fragmenten te onderscheiden van andere. Dit probleem treedt vooral op bij lagere concentraties van het analiet, maar LC-MS/MS analyse kan hiervoor een oplossing bieden.

Het aantal gedetecteerde metabolieten *in vivo* lag beduidend lager dan *in vitro*, hoewel de meeste metabole modificaties konden bevestigd worden. Op dezelfe manier als voor JWH-122, werden de JWH-200 metabolieten als glucuronide of sulfaat conjugaat teruggevonden. Voor de fase II experimenten werd telkens gebruik gemaakt van HRMS detectie. Deze techniek laat toe om via de snelle wisseling tussen positieve en negatieve ionisatiemodes alle hoge resolutie data op te nemen in één enkele run. Het bevestigen van theoretisch mogelijke fase II metabolieten is nadien slechts een kwestie van data processing. Vooral wanneer men slechts beschikt over een kleine hoeveelheid monster, zoals bij de muisurine, is dit een groot voordeel.

De JWH-200 metaboliet die gevormd wordt door openvolgende afsplitsing van de morfolinering en oxidatie van de overblijvende zijketen tot een carbonzuur, was duidelijk aanwezig in de grootste hoeveelheden. Hoewel normaal bij het muismodel de klaring volledig is na 24 uur, kon deze metaboliet nog steeds gedetecteerd worden in de urine gecollecteerd tot 48 uur na toediening. Dit geeft aan dat deze metaboliet aangewezen is voor de detectie van JWH-200 in urine.

Het onderzoek naar synthetische cannabinoïden beschreven in dit werk kan leiden tot de detectie van deze componenten in urine. Daarbij dient gezegd dat dit slechts een kleine stap is in het hele cannabinoïd verhaal. Zoals hier reeds besproken, vereist dit probleem een globale aanpak, waartoe verschillende expertisedomeinen zullen moeten bijdragen.

Tot op vandaag is het moeilijk in te schatten hoe de situatie zal evolueren. Daarom zal het noodzakelijk zijn de huidige opsporingsmethoden te blijven verbeteren, zowel voor de vandaag gekende als voor de nieuwe componenten, die zonder enige twijfel op de markt zullen komen in de (nabije) toekomst. Wat betreft het opsporen in urine, zal zowel aandacht moeten gaan naar nieuw metabolisme onderzoek als naar de ontwikkeling van nieuwe methodes. Op basis van de output in de wetenschappelijke litteratuur is het duidelijk dat dopingcontrolelaboratoria hier een belangrijke rol in te spelen hebben.

Referenties

[1] P. Van Renterghem, P. Van Eenoo, W. Van Thuyne, H. Geyer, W. Schanzer, F.T. Delbeke. Validation of an extended method for the detection of the misuse of endogenous steroids in sports, including new hydroxylated metabolites. *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences*. 2008, *876*, 225–35.

[2] W. Van Thuyne, P. Van Eenoo, F.T. Delbeke. Implementation of gas chromatography combined with simultaneously selected ion monitoring and full scan mass spectrometry in doping analysis. *Journal of chromatography. A*. 2008, *1210*, 193–202.

[3] P. Van Renterghem, P.-E. Sottas, M. Saugy, P. Van Eenoo. Statistical discrimination of steroid profiles in doping control with support vector machines. *Analytica chimica acta*. 2013, *768*, 41–48.

[4] WADA. WADA Technical Document - TD2004EAAS. 2004, 11. http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-IS-Laboratories/WADA_TD2004EAAS_EN.pdf

[5] WADA. Athlete biological passport - Operating guidelines. 2012, http://www.wada-ama.org/Documents/Science_Medicine/Athlete_Biological_Passport/WADA_ABP_OperatingGuidelines_version_3.0.pdf

[6] P. Van Renterghem, P. Van Eenoo, P.-E. Sottas, M. Saugy, F. Delbeke. Subject-based steroid profiling and the determination of novel biomarkers for DHT and DHEA misuse in sports. *Drug testing and analysis*. 2011, *2*, 582–88.

[7] WADA. The 2013 Prohibited List, International Standard. 2013, http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-Prohibited-list/2013/WADA-Prohibited-List-2013-EN.pdf

[8] WADA. 2011 Laboratory Testing Figures. 2012, http://www.wada-ama.org/Documents/Resources/Testing-Figures/WADA-2011-Laboratory-Testing-Figures.pdf

[9] UNODC. Synthetic cannabinoids in herbal products. http://www.unodc.org/documents/scientific/Synthetic_Cannabinoids.pdf

[10] EMCDDA. Online sales of new psychoactive substances / “Legal highs”:Summary of results from the 2011 multilingual snapshots. *EMCDDA*. 2011, http://www.emcdda.europa.eu/attachements.cfm/att_143801_EN_SnapshotSummary.pdf

[11] Belgian monitoring centre for drugs and drug addiction. Nieuwe psychoactieve substanties in België (2005-2011). 2012, 59. http://ewsd.wiv-isp.be/Shared documents/Reports/Nieuwe psychoactieve substanties in Belgi? 2005-2011.pdf

Curriculum vitae

Personal information

Nik De Brabanter
Born on August 10th, 1986 in Ninove
Belgian nationality

Contact Address	Doping Control Laboratory Department of Clinical Biology, Microbiology and Immunology Faculty of Medicine and Health Sciences Ghent University Technologiepark 30 9052 Zwijnaarde Belgium +32 9 331 32 92
E-mail-address	NikDeBrabanter@Gmail.com

Education

2009 – 2013	**Ghent University – Doping Control Laboratory (DoCoLab)** PhD student, Promotor: Prof. Dr. ir. Peter Van Eenoo
2004 - 2009	**Ghent University** Master in Chemistry
1998 - 2004	**Sint-Aloysiuscollege Ninove** Latin-Mathematics : 1998 - 2002 Sciences-Mathematics: 2002 – 2004

Scientific contributions

1 Papers

P. Van Eenoo, W. Van Gansbeke, **N. De Brabanter**, K. Deventer, F.T. Delbeke.
A fast, comprehensive screening method for doping agents in urine by gas chromatography triple quadrupole mass spectrometry.
Journal of Chromatography A. 2011, 1218, 3306–3316.

N. De Brabanter, W. Van Gansbeke, L. Geldof, P. Van Eenoo.
An improved gas chromatography screening method for doping substances using triple quadrupole mass spectrometry, with an emphasis on quality assurance.
Biomedical Chromatography. 2012, 26, 1416–35.

N. De Brabanter, W. Van Gansbeke, F. Hooghe, P. Van Eenoo.
Fast quantification of 11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid (THCA) using microwave-accelerated derivatisation and gas chromatography–triple quadrupole mass spectrometry.
Forensic Science International. 2012, 225, 90-95.

N. De Brabanter, S. Esposito, L. Geldof, L. Lootens, P. Meuleman, G. Leroux-Roels, K. Deventer, P. Van Eenoo.
In vitro and in vivo metabolisms of 1-pentyl-3-(4-methyl-1-naphthoyl)indole (JWH-122).
Forensic Toxicology. 2013, 31, 212–222.

N. De Brabanter, S. Esposito, E. Tudela, L. Lootens, P. Meuleman, G. Leroux-Roels, K. Deventer, P. Van Eenoo.
In vivo and in vitro metabolism of the synthetic cannabinoid JWH-200.
Rapid Communications in Mass Spectrometry. 2013, 27, 1–12.

N. De Brabanter, K. Deventer, V. Stove, P. Van Eenoo.
Synthetic cannabinoids: general considerations.
Proceedings of the Belgian Royal Academies of Medicine. 2013, 2, 209-225.

O. J. Pozo, **N. De Brabanter**, A. Fabregat, J. Segura, R. Ventura, P. Van Eenoo, K. Deventer
10 years after THG discovery. Current status and bioanalytical challenges in the detection of unknown anabolic androgenic steroids in doping control analysis. Bioanalysis. 2013, 5, 2661-2677

2 Lectures

N. De Brabanter, W. Van Gansbeke, L. Geldof, P. Van Eenoo.
Improvements in GC-QqQ-MS screening, 1 year of experience.
Presented at the 30th Cologne workshop of Dope Analysis, Cologne, Germany
Proceeding: Recent Advances in Doping Analysis. 2012, 20, 73–73.

N. De Brabanter, S. Esposito, L. Geldof, L. Lootens, K. Deventer, P. Van Eenoo.
Phase I and phase II metabolism of synthetic cannabinoids, the Ghent strategy
Presented at the 31th Cologne workshop of Dope Analysis, Cologne, Germany
Proceeding: Recent Advances in Doping Analysis. 2013, *Accepted.*

3 Posters

N. De Brabanter, W. Van Gansbeke, F. Hooghe, P. Van Eenoo
Fast quantification of THCA using microwave-accelerated derivatisation and GC-MS/MS analysis.
Presented at the 30th Cologne workshop on Dope Analysis, Cologne, Germany
Proceeding: Posters of the Manfred Donike workshop□: 30th Cologne workshop on dope analysis 2012