Development of GC-C-IRMS Methods for Anti-Doping Purposes

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Development of GC-C-IRMS Methods for Anti-Doping Purposes

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5α-ol  
5α-androstan-3β-ol

5α-ol-Ac  
5α-androstan-3β-ol acetate

6αOH-ADION  
6α-hydroxy-androstenedione

6-oxo  
6-oxo-androstenedione

7βOH-DHEA  
7β-hydroxy-dehydroepiandrosterone

7-oxoDHEA  
7-oxo-dehydroepiandrosterone

11-oxoEt  
11-oxo-etiocholanolone

11-oxoEt-Ac  
11-oxo-etiocholanolone acetate

ααβ  
5α-androstane-3α,17β-diol

ααβ-Ac2  
5α-androstane-3α,17β-diacetate

βαβ  
5β-androstane-3α,17β-diol

βαβ-Ac2  
5β-androstane-3α,17β-diacetate

β-Tren  
17β-trenbolone

β-Tren-Ac  
17β-trenbolone acetate

A  
androsterone

A-Ac  
androsterone acetate

AAF  
adverse analytical findings

AAS  
androgenic anabolic steroids

ABP  
athlete’s biological passport

ACN  
acetonitrile

ADION  
4-androstenedione

AICA  
5-amino-4-imidazolecarboxyamide

AICAR  
5-amino-4-imidazolecarboxyamide ribonucleoside

ATF  
atypical finding

B  
boldenone

B-Ac  
boldenone acetate

BM  
5β-androst-1-en-17β-ol-3-one (boldenone main metabolite)

BM-Ac  
5β-androst-1-en-17β-ol-3-one acetate

CIR  
carbon isotope ratio

DEMA  
diethoxymethyl acetate

XVIII
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>DHEA</td>
<td>dehydroepiandrosterone</td>
</tr>
<tr>
<td>DHT</td>
<td>dihydrotestosterone</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N-dimethylformamide</td>
</tr>
<tr>
<td>Et</td>
<td>etiocholanolone</td>
</tr>
<tr>
<td>Et-Ac</td>
<td>etiocholanolone acetate</td>
</tr>
<tr>
<td>EpiT</td>
<td>epitestosterone</td>
</tr>
<tr>
<td>EpiT-Ac</td>
<td>epitestosterone acetate</td>
</tr>
<tr>
<td>ERC</td>
<td>endogenous reference compound</td>
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<tr>
<td>F</td>
<td>formestane</td>
</tr>
<tr>
<td>F-Ac</td>
<td>formestane acetate</td>
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<tr>
<td>GC</td>
<td>gas chromatography</td>
</tr>
<tr>
<td>GC-C-IRMS</td>
<td>gas chromatography combustion isotope ratio mass spectrometer</td>
</tr>
<tr>
<td>GC-MS</td>
<td>gas chromatography mass spectrometry</td>
</tr>
<tr>
<td>HAc</td>
<td>acetic acid</td>
</tr>
<tr>
<td>HIR</td>
<td>hydrogen isotope ratio</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>HPLC-FC</td>
<td>high performance liquid chromatography fraction collection</td>
</tr>
<tr>
<td>IF</td>
<td>international sport federation</td>
</tr>
<tr>
<td>IMP</td>
<td>inosine 5’-monophosphate</td>
</tr>
<tr>
<td>IOC</td>
<td>international olympic committee</td>
</tr>
<tr>
<td>IRMS</td>
<td>isotope ratio mass spectrometry</td>
</tr>
<tr>
<td>IS</td>
<td>internal standard</td>
</tr>
<tr>
<td>LCMS</td>
<td>liquid chromatography mass spectrometry</td>
</tr>
<tr>
<td>LLE</td>
<td>liquid liquid extraction</td>
</tr>
<tr>
<td>MRM</td>
<td>multiple reaction monitoring</td>
</tr>
<tr>
<td>MSTFA</td>
<td>N-methyl-N-(trimethylsilyl) trifluoroacetamide</td>
</tr>
<tr>
<td>MTBSTFA</td>
<td>N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide</td>
</tr>
<tr>
<td>NA</td>
<td>19-norandrosterone</td>
</tr>
<tr>
<td>NA-Ac</td>
<td>19-norandrosterone acetate</td>
</tr>
<tr>
<td>PD</td>
<td>pregnanediol</td>
</tr>
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<th>Description</th>
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<tr>
<td>PD-Ac₂</td>
<td>pregnanediacetate</td>
</tr>
<tr>
<td>PNP</td>
<td>purine nucleoside phosphorylase</td>
</tr>
<tr>
<td>SPE</td>
<td>solid phase extraction</td>
</tr>
<tr>
<td>PTV</td>
<td>programmed temperature vaporizer</td>
</tr>
<tr>
<td>QC Neg</td>
<td>negative quality control</td>
</tr>
<tr>
<td>QC Pos</td>
<td>positive quality control</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SIS</td>
<td>steroid isotopic standards</td>
</tr>
<tr>
<td>STD</td>
<td>standard</td>
</tr>
<tr>
<td>T</td>
<td>testosterone</td>
</tr>
<tr>
<td>T/E</td>
<td>testosterone/epitestosterone ratio</td>
</tr>
<tr>
<td>T-Ac</td>
<td>testosterone acetate</td>
</tr>
<tr>
<td>TC</td>
<td>target compound</td>
</tr>
<tr>
<td>u</td>
<td>average</td>
</tr>
<tr>
<td>VPDB</td>
<td>Vienna Pee Dee Belemnite</td>
</tr>
<tr>
<td>WADA</td>
<td>world anti-doping agency</td>
</tr>
</tbody>
</table>
General Introduction
Chapter 1: Introduction

1 General

1.1 History of anti-doping

In 1928 the International Association of Athletics Federations (IAAF) became the first International Sport Federation (IF) to prohibit the use of stimulating substances in sports. Many other IFs followed, but restrictions remained ineffective as no test could be performed.

In 1966 the International Cycling Union (UCI) and the International Federation of Association Football (FIFA) were among the first IFs to introduce doping tests in their world championships. [1] In the next year 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. Consequently, anti-doping tests based on gas chromatography (GC) were developed for stimulants. [2] These anti-doping tests were introduced for the first time at the Olympic Winter Games in Grenoble and at the Olympic Games in Mexico in 1968. [3] However, the initial list of prohibited substances did not contain androgenic anabolic steroids (AAS) as some committee members falsely believed that steroids did not provide an advantage in athletic performance. [4]

In the early 1970s, the use of AAS was becoming widespread as there was no way of detecting them. [5,6] Advances in the analytical field led to the development of reliable testing methods in 1974 and the IOC added AAS to its list of prohibited substances in 1976. [7,8] This resulted in a considerable increase in the number of doping violations and disqualifications, especially in strength-related sports such as weightlifting. To avoid detection during competition, a shift occurred and athletes started to administer AAS during training only. In addition, suspicions rose of state-sponsored doping programs in some countries such as for example the German Democratic Republic. [9] The most famous doping case of the 1980s however took place at the 1988 Olympic Games in Seoul. Ben Johnson tested positive for the AAS
stanozolol and this focused the world’s attention to the doping problem to an unprecedented degree. [10] As a response, the IAAF started out of competition testing in 1991. In the 1990s the testing methods became more effective, followed by a remarkable drop in the level of top results in some sports.

In 1985 beta-blockers and diuretics were included in the IOC prohibited substances list. Beta-blockers can be administered in disciplines where control of movement and calmness is required. Diuretics can be used to circumvent doping test as they increase the excretion rate of urine and reduce urinary concentrations of prohibited substances. [4,11] In 1990 the IOC added the class of peptide hormones to the prohibited list because of concerns regarding the performance enhancing capabilities and severe side-effects of growth hormone and erythropoietin (EPO). However, a reliable and effective screening test for EPO was only available in 2000. [12] Previously, indirect methodologies based upon haematocrit levels were the only indirect indicative test that could be applied. [13]

In 1998 a police raid during the Tour of France discovered a large number of prohibited medical substances. The scandal highlighted the urgent need for an independent agency, which would harmonize standards for anti-doping work and coordinate the efforts of IFs and public authorities. Up to then, debate was still taking place within several separate entities (IFs, IOC, individual governments), resulting in different definitions, policies and sanctions. This led to a lot of confusion and doping sanctions were often disputed and sometimes overruled in civil courts. In February 1999 the IOC organized the First World Conference on Doping in Sport in Lausanne and following the proposal of the Conference, the World Anti-Doping Agency (WADA) was funded on 10 November 1999.

1.2 World anti-doping agency

WADA’s main aim was the harmonization of anti-doping rules between IFs and national anti-doping organizations. Nowadays, WADA develops protocols and guidelines, finances and promotes research and education, cooperates with law
enforcement and coordinates a worldwide anti-doping program. Each year WADA publishes its prohibited list which gives a complete overview of all prohibited substances and methods. A compound can be included if it fulfills at least two out of three of the following criteria: it has potential sport enhancing characteristics, it constitutes a (potential) health risk to the athlete or it violates the spirit of sport as such. [14] This Anti-Doping Code is based on two fundamentals:

1. The protection of the athletes’ fundamental right to participate in doping-free sport and thus the promotion of health, fairness and equality for athletes worldwide.
2. Harmonized, coordinated and effective anti-doping programs at national and international level with regard to detection, deterrence and prevention of doping.

2 Endogenous steroids as doping

According to the annual report of the WADA, steroids are still very popular amongst athletes and are responsible for nearly two thirds of all adverse analytical findings (AAF). [15] AAS can be divided into two classes: endogenous and exogenous AAS. In the case of exogenous steroids, i.e. steroids not naturally produced by humans, the presence of these compounds or their metabolites in urine (or blood) specimens is strictly forbidden. Detection at any concentration results in an AAF.

Endogenous steroids, i.e. steroids naturally produced by humans, are always present in urine (or blood) specimens and represent a more complicated situation. For these compounds a different strategy is required. The most important endogenous steroid is obviously testosterone (17β-hydroxyandrost-4-en-3-one, T).
2.1 Testosterone

T is a derivative of cholesterol which is enzymatically converted in the testicles, ovaries and the adrenal cortex. Exposure of T misuse is difficult because an analytical detection of T does not prove a doping violation due to endogenous T production in the human body. Administration of naturally occurring steroids however changes the steroid concentrations and their ratios in urine. Elevated T concentrations can raise suspicion of prohibited administration but are not reliable to detect a doping violation as T is characterized by large natural fluctuations of urinary concentrations and inter-individual variability.

Detection of T misuse remained impossible until 1983. To compensate for natural variations in urinary steroid concentrations, Donike et al. proposed the ratio of T and its 17α-epimer epitestosterone (17α-hydroxyandrost-4-en-3-one, EpiT) as a marker for T abuse. [16] This T/EpiT ratio (or T/E) solved the problem of large urinary variation and proved to be a much more stable parameter than the T concentration. Donike et al. determined that most individuals had a T/EpiT value between 0.1 and 3. Consequently, in 1983 the IOC introduced a T/EpiT threshold of 6 to indicate illicit T use. [4,17]

During the years that followed, it became clear that an elevated T/EpiT ratio does not unequivocally prove a doping violation. In 1984, a Japanese volleyball player was found to have a T/EpiT ratio of 7. Follow-up doping tests later confirmed that the individual was a natural outlier and had a naturally elevated T/EpiT. [18] Later that year, a similar false positive case occurred in France. [19]

2.2 Testosterone prohormones and metabolites

Dihydrotestosterone (17β-hydroxy-5α-androst-3-one, DHT) is a direct metabolite of T and is a three times more potent androgen than T. In addition, administration of DHT cannot be detected by the T/EpiT test as formation of DHT from T is
irreversible. [20] This made DHT an attractive alternative for T abuse and new doping test needed to be developed. [21–23]

In the beginning of the 1990s, new endogenous steroids gained popularity in sports due to a boost in the nutritional supplement market. [24,25] Dehydroepiandrosterone (3β-hydroxyandrost-5-en-17-one, DHEA) for example was depicted in the media as “a fountain of youth” as it was believed to counter the effects of ageing. [26,27] Subsequently, pharmaceutical companies introduced it as dietary supplement making DHEA widely available as over-the-counter product and via internet. As DHEA is a prohormone of T, the IOC added DHEA to the list of prohibited substances in 1996.

Other prohormones such as 4-androstenedione (ADION), 5-androstenedione, 5-androstene-3β,17β-diol and 4-androstene-3β,17β-diol gained in popularity as well due to the commercialization as nutritional supplement. [28,29] Ironically, none of these steroids have real nutritional value (in contrast to food supplements containing minerals, proteins, amino acids, vitamins,....). These prohormones are weak anabolic androgens but can be metabolized to more potent AAS like DHT and T and are marketed as such. However, orally administered T prohormones are largely broken down in the liver to inactive metabolites and transformation to T is very limited. [30–32] Therefore, there is no real evidence in literature of their effectiveness as performance enhancing drug. [33–35] Nonetheless, similar to DHEA, these prohormones were all added to the IOC prohibited substances list by 1999. The chemical structures of T, DHT, DHEA and ADION are given in Figure 1.1.

When WADA was founded in 1999 under the impulse of the IOC, these compounds were obviously also included in the WADA prohibited list. There is no doubt that uncontrolled use and administration of high to very high amounts will affect an individual’s physical health. [35–37] The often unlimited accessibility of nutritional supplements containing these prohormones enables people to purchase and use
these potentially dangerous substances quite easily. This issue remains of great concern in doping analysis and for society in general.

![Chemical structures of T, DHT, DHEA and ADION](image)

**Figure 1.1: Chemical structures of T, DHT, DHEA and ADION**

During the last decade, a new type of endogenous steroids were introduced in the supplement market: hydroxylated and oxygenated analogues like formestane (4-hydroxy-androstenedione, F), 6-oxo-androstenedione (6-oxo), 11-oxo-androstenedione and 7-oxo-dehydroepiandrosterone (7oxo-DHEA). [38] F and 6-oxo have very limited direct anabolic properties. Both substances are aromatase inhibitors which block the natural conversion of androgens to estrogens and indirectly promote the build-up of T concentrations. [39–41]
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3 AAS: medical use, pharmacology and adverse effects

3.1 Medical use

Anabolic androgenic steroids have two different types of effect: anabolic, meaning that they promote anabolism (cell growth) and androgenic meaning that they affect the development and maintenance of masculine characteristics. Natural AAS are responsible for the development of the inner and outer male genital organs, deepening of the voice, stimulation of hair growth in pubic and facial regions,... [42] The anabolic properties of AAS affect protein metabolism by stimulating protein synthesis and as such they increase the lean body mass and the muscle strength.

Anabolic steroids have been used by physicians for many purposes including induction of male puberty, hormone replacement for men with low levels of T, treatment of anemia, low muscle mass due to chronic wasting conditions such as cancer and gender identity disorders. [43,44]

The androgenic – anabolic ratio is an important factor when determining the clinical application of an AAS. Steroids with a high ratio of androgenic versus anabolic effects are the preferred drugs in androgen replacement therapies. AAS with a low ratio are used for anemia and osteoporosis and to reverse protein loss following trauma, surgery or prolonged immobilization.

3.2 Pharmacology

There are three administration routes for AAS: dermatological creams, gels or skin patches, injectable preparations and oral pills. Orally administered T is rapidly absorbed but about 80% is converted in inactive metabolites. To reduce the liver’s ability to convert T in inactive metabolites, T derivatives such as methyltestosterone are orally administered instead. [45]

T has greater activity in muscle in enanthate, undecanoate or cypionate ester form. At the site of injection, these derivatives are hydrolyzed to free T. [46] Medical
injections are normally done between twice a week to once every 12 weeks, depending on the absorption rate which varies among different esters. To avoid sudden changes in the amount of drug in the bloodstream, injections are normally performed into the muscle and not in the vein. Intravenous injection has also the potential to cause an embolism in the bloodstream because T esters are dissolved in oil.

Transdermal patches, creams and gels are used to deliver a steady dose through the skin and into the bloodstream. [47] These treatments tend to be more expensive however and absorption is inefficient (roughly 10%).

AAS are fat soluble hormones and membrane permeable, influencing cells by direct action. The pharmacodynamic action begins when the steroid penetrates the membrane of the target cell and binds to an androgen receptor located in the cytoplasm. This steroid-receptor complex is translocated into the nucleus where it undergoes dimerisation and interacts with a specific DNA sequence. The attachment to this DNA strand triggers various coregulating proteins to form a transcription complex. Transcriptional activity is modulated by these coregulators which can have coactivating or corepressing action, depending on the target tissue. [48] The effects of AAS will obviously differ between various compounds because of a different affinity to the androgen receptor. In addition, steroids also induce non-genomic activity by initializing the signaling systems and changes in the ion transport.

3.3 Adverse effects

Anabolic steroid use can cause many adverse effects. The severity of these adverse effects depends greatly on gender, the dose and duration of the administration and the type of steroid. Effects can be irreversible and the unfavorable consequences are most damaging in females and adolescents. Women can suffer from male type baldness patterns, diminished breast mass, decreased menstruation, increased appetite, permanent deepening of the voice... [35] Men can suffer from reduced
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fertility, diminished sperm quality and atrophy of the testes. [49] Due to the aromatization of T into estrogens, AAS can also lead to feminization in men and cause gynaecomastia. [50] Testicular atrophy can also occur due to the suppression of natural T levels. The size of the testicles usually returns to normal within a few weeks of discontinuing AAS use as normal T production resumes.

Acne is very common among AAS users due to stimulation of the sebaceous glands and use of AAS increases the risk of cardiovascular diseases. This includes alterations in the heart structure which can cause hypertension, cardiac arrhythmias, heart attacks and heart failure. In addition, AAS can cause harmful changes in cholesterol levels and provoke an increase in body weight and an accompanying rise in blood pressure, making users more vulnerable to a cardiovascular infirmity. [51,52]

Studies have shown that high doses and prolonged administration of orally active steroids can cause liver damage as the orally administered steroids are metabolized by the liver. Hepatotoxicity is clearly correlated with 17α-alkylation of the steroid structure. This can lead to liver carcinoma and liver dysfunction. [53]

AAS are also responsible for adverse neuropsychiatric effects including increased aggression, mood changes, altered libido, depression and psychosis. [5] Long term users may develop symptoms of dependence and withdrawal on AAS discontinuation.

It has to be taken into account that most illicit steroid users combine different AAS and administer them in doses 10 to 40 times higher than those therapeutically prescribed, meaning that the described adverse effects are likely to be underestimated.
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4 Doping analysis of endogenous steroids

In the case of exogenous doping substances it is sufficient to prove that the substance is present in urine or blood to justify a doping violation. For endogenous substances like T, we need a different approach because T will always be present in every sample. To differentiate between endogenously produced steroids and synthetic analogs that have been administered, doping control laboratories use isotope ratio mass spectrometry (IRMS). [54–56] By measuring the carbon isotope ratio (CIR) it is possible to differentiate as endogenous steroids and synthetic steroids have a different CIR. IRMS is an expensive and very time consuming technique however and lacks sensitivity which means that large amounts of urine are required. This makes it impossible to perform an IRMS analysis on every urine sample. Consequently, doping control laboratories analyze all urine samples from athletes that enter their lab with a fast screening method that allows them to isolate suspicious samples. Afterwards, the suspicious samples are analyzed with a separate IRMS confirmatory method. This is the standard operating procedure required by WADA and implemented by laboratories according to the WADA technical document. [57]

4.1 Screening

For the detection of misuse of natural steroids, the measurement of urinary concentrations and ratios of several endogenous steroids has proven to be the most appropriate screening technique. In doping control laboratories, AAS screening is traditionally performed by gas chromatography mass spectrometry (GC-MS). [58–64] Because of the large inter-individual variation in urinary excretion of these endogenous steroids, single-point urinary concentrations are not suitable to confirm abuse, but they can indicate suspicious samples. The T/EpiT ratio remains the most important biomarker to establish the detection of T misuse. As epimerization from T to EpiT is negligible and EpiT production is largely independent from T, EpiT can be considered as an endogenous reference parameter in relation
to T. [65,66] In addition, administration of T suppresses the EpiT concentration, further enhancing the efficiency of the T/EpiT ratio as screening parameter. [67]

Figure 1.2: Metabolic pathways of T, DHEA and ADION
Administration of endogenous steroids such as DHT cannot be detected by the T/EpiT test as formation of DHT from T is irreversible. [20] As a response, other parameters are being monitored in the screening as well. These parameters are primarily the concentrations of endogenous steroids that can be administered such as T, EpiT, DHEA, DHT and ADION and the concentrations of their main urinary metabolites: androsterone (3α-hydroxy-5α-androst-17-one, A), etiocholanolone (3α-hydroxy-5β-androst-17-one, Et), 5β-androstane-3α,17β-diol (βαβ) and 5α-androstane-3α,17β-diol (ααβ)). [57,68] Figure 1.2 visualizes the major T metabolic pathways. The ratios of several of these steroids, e.g. A/Et and DHT/EpiT ratio, are considered as valuable indicators for anabolic steroid misuse as well. [22,23] The cluster of all these concentrations and ratios is traditionally known as the steroid profile. The urinary concentrations can be compared after correction for altered diuresis to a specific gravity of 1.020 and in order to decide which urine samples can be classified as suspicious, doping control laboratories use population reference limits. [59,60] According to the most recent WADA technical document (2014), IRMS is recommended if one of the following criteria is met:

- T/EpiT ratio > 4
- T or EpiT > 200 ng/mL (males)
- T or EpiT > 50 ng/mL (females)
- A or Et > 10 000 ng/mL and A/Et > 4 (both sex) or A/Et < 0.4 (males, in absence of inhibitors of 5α-reductase)

with all concentrations adjusted for a specific gravity of 1.020. [57]

Nowadays, most doping control laboratories employ screening methods that quantify the above mentioned endogenous steroids (T, E, DHEA, DHT, ADION, A, Et, βαβ and ααβ). These markers are known as the “traditional” steroid profile parameters.

Recent publications have shown that minor metabolites such as F, 6α-hydroxy-androstenedione (6αOH-ADION) and 7β-hydroxy-dehydroepiandrosterone (7βOH-
DHEA) are important parameters as well, capable of increasing the specificity and efficiency of the screening and indicating suspicious samples. [69–73] These oxygenated and hydroxylated metabolites are present in urine in much lower concentrations, complicating their detection and quantification in the past. Nowadays, anti-doping laboratories are equipped with more sensitive instruments which allows them to quantify these low concentration minor metabolites with high reliability. [64] The idea to monitor minor metabolites to improve detection is based on the theory that by administration of AAS, the metabolic system is very briefly overloaded, pushing the excess of administered steroid to less common metabolic pathways, resulting in elevated concentrations of the minor metabolites.

A second approach is the designation of suspicious samples based on individual reference limits instead of population reference limits. The approach was enabled by the implementation of the Athlete’s Biological Passport (ABP) that allowed anti-doping authorities to follow athletes individually over time. [57] With each measurement, the ABP progressively adapts the calculated upper and lower reference limits of the steroid profile parameters, evolving from a population based to an individual based threshold. [74] As the number of test records increases, the calculated reference limits adapt from population thresholds towards individual thresholds resulting in a more sensitive criterion. The downside of this approach is obviously that multiple samples from the same athlete are required to set up the ABP.

4.2 Boldenone, 19-norandrosterone and formestane

Boldenone (1,4-androstadiene-3-one-17β-ol, B) and 19-norandrosterone (19-nor-3α-hydroxy-5α-androstan-17-one, NA) are 2 exceptional steroids within the AAS. They are normally not naturally present in human urine, but in rare cases they can be endogenously produced in very small amounts.

Due to the close structural relation of B to endogenous steroids and the high sensitivity of the analytical techniques used to screen for the presence of B and its
metabolites in urine, the hypothesis of a natural occurrence of B in urine samples of athletes was established shortly after the first screening methods have been developed. [68] Since B is an AAS and is mentioned on the WADA list of prohibited substances this could result in false positive findings when B is detected at low concentrations. The use of a threshold concentration is a challenging task, since there have been indications that up to 23 ng/mL of these substances might be present naturally in urine. [75] According to the most recent WADA technical document, concentrations (adjusted for specific gravity) of B or its main metabolite 5β-androst-1-en-17β-ol-3-one (BM) lower than 5 ng/mL must be reported as an atypical finding (ATF) unless IRMS is performed and concentrations higher than 30 ng/mL are caused by administration of the synthetic steroid. For concentration between 5 and 30 ng/mL it is uncertain whether the origin is endogenous or exogenous and in these cases confirmation by IRMS is obligatory to determine the true nature of the substance. [76]

NA is the main metabolite of 19-nortestosterone, 19-norandrostenedione and 19-norandrostenediol and a minor metabolite of norethandrolone and ethylestrenol. [28,29,68,77–79] Detection of NA can therefore be used as proof for use of these prohibited AAS. However, similar as B, NA can also be produced endogenously in (very) low amounts. [76,80–84] To disclose an endogenous NA origin from exogenous, a cut-off value of 2 ng/mL has been established by the WADA. [85] For definitive assignment of the NA origin, IRMS needs to be performed for urinary NA concentrations between 2 and 10 ng/mL (or 15 ng/mL, depending on the situation). [86–90] Higher concentrations are considered as exogenous.

F is a slightly different situation because F is always naturally produced and can be found in every urine sample at low concentrations. [70,73,91] F is in fact a minor metabolite of ADION and given the reversible nature of the ADION - T conversion, it can also be regarded as a minor metabolite of T. [70] Because of the ability of F to suppress the estrogen production from anabolic steroids, F can be abused by athletes to withhold estrogen production as a side effect during the intake of
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anabolic steroids. Consequently, similar as with B and NA, WADA works with two thresholds: concentrations between 50 and 150 ng/mL require IRMS, lower concentrations are endogenous and higher concentrations are exogenous. [76] The chemical structures of B, BM, NA and F are given in Figure 1.3.

Figure 1.3: Chemical structures of B, BM, NA and F
5 Confirmation by IRMS

5.1 Isotopic variation

During every chemical and physical process or reaction an isotope of an element is discriminated in relation to another isotope because of the mass difference between them. This process is called fractionation and causes variation of isotopic content. Isotopic variations are generally very small and for carbon the $\delta^{13}C$ values are expressed as part-per-thousand differences from Vienna Pee Dee Belemnite (VPDB) which is the international reference standard: [92]

$$\delta^{13}C(\text{‰}) = \left[ \frac{\left( \frac{^{13}C}{^{12}C} \right)_{\text{sample}} - \left( \frac{^{13}C}{^{12}C} \right)_{\text{VPDB}}}{\left( \frac{^{13}C}{^{12}C} \right)_{\text{VPDB}}} \right] \times 1000 = \left( \frac{^{13}C}{^{12}C} \right)_{\text{sample}} - \left( \frac{^{13}C}{^{12}C} \right)_{\text{VPDB}}$$

The $^{13}C$ isotope normally occurs with an abundance of 1.11 % in nature and a difference of one unit on this per-mille scale correlates with a change of 0.001099 atom% of $^{13}C$ abundance. [93]

5.2 CIR of synthetic steroids

Differences in steroid CIR reflect isotopic fractionation patterns that are associated with photosynthesis. There are two methods by which plants can absorb CO$_2$ from the atmosphere. [94,95] C3 plants produce 3-phosphoglyceric acid, a compound containing three carbon atoms. This so called Calvin-Benson cycle results in isotopic fractionation of carbon and produces a plant with a CIR between -24 ‰ and -34 ‰. About 90 % of all plants are C3 plants, including rice, soy and wheat. C4 plants produce oxaloacetate, a compound that contains four carbon atoms. This process is called the Hatch-Slack cycle and results in plants with a CIR ranging from -6 ‰ to -19 ‰. Corn and sugarcane are examples of C4 plants. Crassulacean Acid Metabolism (CAM) plants use either the C3 or C4 pathway depending on the environment and include cacti and pineapple.
Commercially produced synthetic steroids are normally derived from soy, a C3 plant, meaning that synthetic steroid preparations will have $\delta^{13}C$ values that are close to the CIR of C3 plants. This was illustrated by reported reference intervals for synthetic steroids by de la Torre et al., Ueki et al., Cawley et al. and Brooker et al. (CIR = -23 % to -33 %) (Figure 1.4). [96–99]

![Figure 1.4: CIR of endogenous and exogenous steroids](image)

### 5.3 CIR of endogenous steroids

Humans reflect the CIR of their diet which is usually a mix of C3 and C4 plants. Human biomolecules arise from this mix of C3 and C4 precursors and endogenous steroids cover a range of $\delta^{13}C = -17 \%$ to $-27 \%$. Geographical variation on CIR of endogenous steroids is caused by different eating habits and a differing isotopic composition of food. [100,101] To compensate for the inter-individual variation in CIR of a particular steroid due to differences in diet, endogenous reference compounds (ERCs) are used. ERCs are compounds of which the CIR does not change after synthetic steroid administration because they are not involved in the androgen metabolic pathway. The primary ERC used by doping control laboratories is pregnanediol (5β-pregnane-3α,20α-diol, PD), but others such as 16-androstenol...
(5α-androst-16-en-3α-ol), 11β-hydroxyandrostosterone (3α,11β-dihydroxy-5α-androstane-17-one) and 11-oxo-etiocholanolone (11-oxoEt, 5β-androstane-3α-ol-11,17-dione) are also routinely used. The difference between the CIR of an ERC and the CIR of the target compound (TC) normalizes IRMS results in relation to the diet of the athlete. The use of these $\Delta\delta^{13}C$ values instead of absolute CIR values results in a far better detection efficiency. The $\Delta\delta^{13}C$ values provide the basis to identify doping misuse and appropriate thresholds are enforced by WADA. [76] Table 1.1 gives an overview of the current thresholds that are applicable since 2014.

<table>
<thead>
<tr>
<th>TC =</th>
<th>$T$</th>
<th>EpiT</th>
<th>A</th>
<th>Et</th>
<th>$\beta\alpha\beta$ and/or $\alpha\alpha\beta$</th>
<th>$B$, BM or F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case 1</td>
<td>&gt; 3 ‰</td>
<td></td>
<td></td>
<td>&gt; 3 ‰ (either diol)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Case 2</td>
<td></td>
<td></td>
<td></td>
<td>&gt; 3 ‰ (both diols)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Case 3</td>
<td></td>
<td>&gt; 4 ‰</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Case 4</td>
<td></td>
<td></td>
<td>&gt; 3 ‰</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Case 5</td>
<td></td>
<td></td>
<td></td>
<td>&gt; 4 ‰</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Case 6</td>
<td></td>
<td></td>
<td>2-3 ‰</td>
<td></td>
<td>&gt; 3 ‰ (either diol)</td>
<td></td>
</tr>
<tr>
<td>Case 7</td>
<td></td>
<td></td>
<td></td>
<td>3-4 ‰</td>
<td>&gt; 3 ‰ (either diol)</td>
<td></td>
</tr>
<tr>
<td>Case 8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$\Delta\delta^{13}C(\alpha\alpha\beta) &gt; 4 %$ and $\delta(\alpha\alpha\beta) \leq -27 %$</td>
<td></td>
</tr>
<tr>
<td>Case 9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&gt; 4 ‰</td>
<td></td>
</tr>
</tbody>
</table>
5.4 CIR analysis in doping control by GC-C-IRMS

Gas chromatography combustion isotope ratio mass spectrometer (GC-C-IRMS) is a highly specialized technique, said to be practiced in only a few hundred laboratories worldwide. [102] GC-C-IRMS is suitable for the analysis of carbon, nitrogen and hydrogen stable isotopes. However, as steroids do not contain nitrogen, the technique focuses primarily on carbon. The first GC-C-IRMS method for doping analyses was introduced by Becchi et al. [103–105] GC-C-IRMS instruments are designed for steroids to be separated by the GC column followed by CIR determination of the individual steroids. The GC separates the injected analytes and subsequently each individual substance enters the combustion interface consisting of an oxidation furnace and water remover. The oxidation furnace converts all carbon to CO₂. After combustion of the compounds into CO₂ and H₂O, the latter is removed by the water remover. The magnetic sector mass spectrometer is set to detect only the masses of CO₂ (m/z 44, 45, 46 representing ¹²C¹⁶O¹⁶O, ¹³C¹⁶O¹⁶O and ¹²C¹⁶O¹⁸O respectively). Comparison of their relative responses allows the software to calculate the CIR of the respective compound. [106,107]

There are considerable analytical requirements for successful CIR determination of urinary steroids. First, large sample volumes (≥ 15 mL of urine) may be required to achieve sufficient sensitivity. Secondly, an extensive sample clean up is essential as one can only determine the CIR of a pure compound and co-elutions will lead to significant errors. [108] A typical IRMS method consists of a solid phase extraction (SPE), hydrolysis, liquid liquid extraction (LLE), high performance liquid chromatography fraction collection (HPLC-FC) and acetylation. [75,91,109–113] This makes IRMS a complex and challenging analytical methodology that needs thorough consideration of information and factors influencing the analytical data as well as its interpretation. In this context, quality assurance and control is of utmost importance.
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The HPLC-FC plays a crucial role in the sample preparation. HPLC separation permits compounds that co-elute on the GC to be run individually on the GC-C-IRMS and appropriate dilution of specific HPLC fractions allows both high and low abundance urinary steroids to be run on the GC-C-IRMS within the linear range of the instrument. During the HPLC-FC it is essential that the TC or ERC is completely collected within one fraction as $^{13}$C enriched isotopomers elute slightly earlier (in most cases). [114,115] Incomplete collection of a compound would therefore have a considerable effect on the determined CIR.

In doping control laboratories, IRMS is currently mainly applied for the analysis of endogenous AAS and their metabolites. [56,116] However, IRMS possibilities are not limited to AAS and methods have been developed for other types of compounds as well, e.g. cortisone and 5-amino-4-imidazolcarboxyamide ribonucleoside (acadesine, AICAR). [117,118]

5.5 Hydrogen isotope ratio mass spectrometry

GC-C-IRMS can also be performed on steroids by using the hydrogen isotope ratio (HIR) as hydrogen constitutes the other abundant element in the steroid backbone. Piper et al. published the first extensive results on HIR of urinary steroids in 2009. [119] In 2010 Cawley et al. published results concerning the HIR of synthetic T preparations. [98]

At this stage HIR determinations of endogenous steroids are not being performed on a routine basis by doping control laboratories and CIR analysis remains the standard procedure. However, HIR determinations might be useful in cases where the carbon isotope cannot differentiate between an endogenous and exogenous origin. [55] Some rare cases have been reported where synthetic steroids exhibited CIR in the endogenous range, making differentiation almost impossible. Determination of HIR should be independent of the CIR as drinking water and not food intake is the main factor influencing the HIR. Thus, HIR determinations
potentially allow for the detection of exogenous steroids where analysis of the CIR might fail.
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6 References


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Chapter 2: Outline of the Study
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General

The research described in this work focuses on the development and implementation of new and/or improved methods and methodologies for the detection of doping abuse by using IRMS. The study was conducted at DoCoLab and can be subdivided in four major sections elucidated in parts 1 to 4 respectively.

Part 1: improved confirmation method for T

The study described in part 1 aimed at extending and improving the existing IRMS confirmation method for the detection of T. [1] New TCs and ERCs were added to the method and a new type of injection, solvent vent injection, was studied. The possibilities of this new type of injection were explored and the injection parameters were optimized in order to gain a maximum increase in sensitivity (chapter 3).

In chapter 4 an overview is given of the evolution and application of the GC-C-IRMS method in routine doping control practice during a period of three years. Gathered drug testing data is summarized and investigated and quality controls are evaluated.

Part 2: confirmation methods for NA, B and F

Research conducted in part 2 aimed at developing and validating IRMS confirmation methods for NA, B and F. These are particularly difficult steroids to analyze on IRMS because of their (very) low urinary concentrations and compliance with WADA requirements was crucial. [2,3] However, because of the implementation of solvent vent injections on the GC-C-IRMS as outlined in chapter 3, it was now possible to reach these much lower concentrations.
Chapter 2: Outline of the Study

Investigations regarding the B confirmation method are presented in chapter 5. The objective was to provide a method capable of CIR determination of both B and BM at concentrations of 5 ng/mL or lower. In a later stage, a separate method capable of CIR measurement of both NA and F was established (chapter 6).

Part 3: steroid profile thresholds for minor metabolites

Minor metabolites such as F and 6αOH-ADION beneficially contribute to the steroid profile and as more and more doping laboratories are equipped with the more sensitive GC-MS/MS instrument (compared to the single quadrupole GC-MS), the ability arises to quantify these minor metabolites that are present at low concentrations with high reliability. Consequently, an increasing amount of doping laboratories across different continents are including them in their steroid profiling screening procedure. The study in part 3 demonstrates that the increased sensitivity on both GC-MS/MS and GC-C-IRMS enables the set up of applicable thresholds by combining data from both instruments. In chapter 7 this methodology was implemented and a threshold for F was established. A similar study was performed for 6αOH-ADION and chapter 8 provides data for the introduction of a suitable threshold.

Part 4: confirmation method for AICAR

AICAR (Figure 2.1) is an endogenous substance that can be abused as doping and is mentioned on the WADA prohibited list since 2009. [4] In analogy with endogenous steroids, there is need of a urinary concentration threshold for the screening. Lower concentrations will be regarded as endogenous; samples with higher concentrations require analysis by an IRMS confirmatory method to unambiguously prove the exogenous nature of the detected AICAR. AICAR however has a completely different
Chapter 2: Outline of the Study

chemical structure than steroids and provides a substantial challenge to be analyzed on IRMS due to its polarity. When our studies regarding AICAR were commenced, WADA had not yet defined a concentration threshold and an IRMS method did not exist. In chapter 9, an AICAR concentration threshold is proposed and, as AICAR contains 4 nitrogen atoms, the potential of using the nitrogen isotope for an IRMS confirmatory method was explored.

![Figure 2.1: Chemical structure of AICAR](image-url)
Chapter 2: Outline of the Study

References


Part 1

Improved Confirmation Method for Testosterone
Chapter 3: Development of a Sensitive GC-C-IRMS Method

Adapted from
M. Polet, W. Van Gansbeke, K. Deventer, P. Van Eenoo

Development of a sensitive GC-C-IRMS method for the analysis of androgens

Chapter 3: Development of a Sensitive GC-C-IRMS Method

1 Abstract

The administration of anabolic steroids is one of the most important issues in doping control and is detectable through a change in the carbon isotopic composition of testosterone and/or its metabolites. GC-C-IRMS however, remains a very laborious and expensive technique and substantial amounts of urine are needed to meet the sensitivity requirements of the IRMS. This can be problematic because only a limited amount of urine is available for anti-doping analysis on a broad spectrum of substances. In this work we introduce a new type of injection that increases the sensitivity of GC-C-IRMS by a factor of 13 and reduces the limit of detection, simply by using solvent vent injections instead of splitless injection. This drastically reduces the amount of urine required. On top of that, by only changing the injection technique, the detection parameters of the IRMS are not affected and there is no loss in linearity.

2 Introduction

T is an endogenous steroid with strong androgenic and anabolic effects. Anabolic effects are associated with hypertrophic capacities in skeletal muscle and bone and lead to performance enhancement, while androgenic effects cause masculinization. [1,2] Obviously, T has played a significant role in doping cases and in the development of new synthetic steroids that mimic T anabolic behavior. Starting from the 1970’s the use of anabolic substances to enhance the athletic performance was banned by the IOC. WADA also included these compounds in the prohibited list. [3] Nowadays, endogenous steroids are amongst the most widespread doping substances misused in sports. In 2010 more than 50 % of the total adverse analytical and atypical findings reported by WADA accredited laboratories were attributed to endogenous steroids. [4]
Chapter 3: Development of a Sensitive GC-C-IRMS Method

EpiT is an important steroid in doping control because it is the denominator in the testosterone/epitestosterone (T/EpiT) ratio, a marker for T abuse. The intake of T increases the excretion rate of urinary T but also decreases the excretion rate of urinary EpiT. [5] As a consequence, the T/EpiT ratio is elevated and a T/EpiT > 4 is indicative for testosterone abuse. [6] Unfortunately, EpiT is sometimes used as a masking agent together with T to lower the T/EpiT ratio and circumvent the test. The T/EpiT ratio is also an indirect marker of DHEA and ADION misuse. [7,8] Obviously there are athletes with a naturally elevated T/EpiT ratio and elevated EpiT excretion rates. To exclude false positives GC-C-IRMS is used to provide evidence for a doping violation. While administered synthetic steroids are chemically identical to endogenous steroids, there are small differences in the $^{13}$C/$^{12}$C ratios. Natural variations in the abundance of $^{13}$C reflect the passage through biological and/or chemical processes in which transformations are accompanied by isotopic fractionation that results in a slight depletion or enrichment of $^{13}$C due to the difference in mass. In addition to the T/EpiT ratio, there are other WADA criteria for which GC-C-IRMS is recommended: [6]

- T or EpiT > 200 ng/mL (males)
- T or EpiT > 50 ng/mL (females)
- A or Et > 10 000 ng/mL and A/Et > 4 (both sex) or A/Et < 0.4 (males, in absence of inhibitors of 5α-reductase)

All doping control laboratories accredited by WADA have been confronted with the task to develop analytical methods and establish criteria that allow endogenous steroids to be distinguished from their synthetic copies. It has been known for some time that GC-C-IRMS is capable of meeting this challenge by comparison of the $^{13}$C/$^{12}$C ratios of the TCs with those of ERCs. [9–16] T itself and/or its main metabolites A, Et, βαβ and ααβ normally function as TCs. Typical ERC’s include PD, 11-oxoEt and 11β-hydroxyandrosterone. [17] Synthetic copies are generally derived from stigmasterol and sitosterol; plant sterols obtained from soybean (Glycine max) which have a significantly different carbon isotope composition compared to
endogenous steroids. [13,18,19] As a consequence, the administration of synthetic analogs is detectable through a change in the carbon isotopic composition of testosterone and its metabolites. To compensate for the inter-individual variation in $^{13}\text{C}/^{12}\text{C}$ ratios of a particular steroid due to differences in diet, the administration of an endogenous steroid is demonstrated when a difference of 3 ‰ or more between the $^{13}\text{C}/^{12}\text{C}$ ratio of the ERC and the TC is determined. [17]

GC-C-IRMS analysis however remains a very laborious and expensive technique because one can only determine the $^{13}\text{C}/^{12}\text{C}$ ratio of a pure compound. This means that a lot of purification steps have to be conducted and fractionation caused by one of these steps is unacceptable. On top of that, substantial amounts of urine are needed to meet the sensitivity requirements of the IRMS. This can be problematic because only a limited amount of urine is available for anti-doping analysis on a broad spectrum of substances.

Previously a method was developed for the determination of the $\delta^{13}\text{C}$ values of A, Et, $\beta\alpha\beta$ and $\alpha\alpha\beta$. [20] Although this method is capable of detecting the described steroids at reasonable concentrations, the method does not allow for the determination of EpiT misuse. Moreover, research indicated that additional target compounds can help prolong the detection time of misuse with natural steroids. Therefore the aim of this research was to expand the range of target substances and improve sensitivity to allow a more efficient analysis.

3 Experimental

3.1 Reagents and chemicals

All reagents and chemicals were the same as mentioned previously. [20] Toluene and 2,2,4-trimethylpentane were purchased from Merck (Darmstadt, Germany), n-hexane and n-pentane were obtained from Biosolve (Valkenswaard, The Netherlands). All reagents were analytical grade. T and EpiT were from Sigma
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Aldrich (St. Louis, MO, USA), testosterone acetate (T-Ac) and epitestosterone acetate (EpiT-Ac) were obtained from Steraloids (Newport, USA). All steroid standards were verified and contained less than 1 % impurities. All standard solutions were made in methanol, except 5α-androstan-3β-ol acetate (5α-ol-Ac) which was dissolved in 2,2,4-trimethylpentane and stored at 4°C.

3.2 Steroid profiling and urine sampling

An aliquot of each urine sample was prepared to check the steroid profile according to routine sample preparation procedures. [21,22] The concentrations of the target compounds allowed the calculation of the necessary sample volume using the formula presented in previous work. [20] This calculation was based upon a minimum final extract volume of 25 µL and a required extract concentration of 50 µg/mL, assuming 100 % extraction recovery. During the second part of our work the formula was adapted because PTV solvent vent injections were used which were 10 times more sensitive as splitless injections. This calculation was based upon a minimum final extract volume of 50 µL and a required extract concentration of 5 µg/mL, assuming 100 % extraction recovery. This resulted in the following new equation where the 1250 ng in the original equation is now replaced by 250 ng. \( V_{IRMS} \) is the required sample volume and \( C_x \) is the concentration of βαβ or T.

\[
V_{IRMS} = \frac{250 \, ng}{C_x}
\]

3.3 Pre-analytical steps

The pre-analytical steps were identical to those presented in our previous work. [20] Briefly, the urine was loaded on a solid phase extraction (SPE) cartridge and eluted with methanol, followed by hydrolysis. As the majority of the steroids is present in urine as glucuronides, a hydrolysis with β-glucuronidase (E. coli) is performed to obtain steroids in their free form. The next step is an extraction with diethyl ether as a second purification step. In the second part of this work we
switched from diethyl ether extractions to n-pentane extractions. Afterwards, the samples were acetylated.

3.4 High performance liquid chromatography fraction collection

To purify the sample the same semi-preparative HPLC fraction collection was executed as explained in previous work. [20] During this HPLC run, three fractions were collected: a first fraction containing androsterone acetate (A-Ac) and etiocholanolone acetate (Et-Ac), a second fraction containing 5β-androstane-3α,17β-diacetate (βαβ-Ac2) and 5α-androstane-3α,17β-diacetate (ααβ-Ac2) and a third fraction containing the ERC pregnanediacetate (PD-Ac2). For this work, an additional fraction (8.2 – 10.2 min) containing EpiT-Ac and T-Ac was collected. This fraction was also dried under nitrogen at 60°C, but afterwards it was reconstituted in 55 µL of β-Trenbolone acetate (β-Tren-Ac) (100 µg/mL) internal standard and 55 µL 50/50 MeOH/water. After transfer to a LC vial, the fraction was subjected to a second semi-preparative HPLC fraction collection procedure using a MeOH/water mobile phase (solvent A: 10/90 MeOH/H2O; solvent B: MeOH). The analysis started with 60 % B for 26 min, then 100 % B at 27 min, held for 5 min, 60 % B at 33 min and held for 5 min. β-Tren-Ac was used as retention time marker (13.66 min), fraction A (17.7 – 20.7 min) contained EpiT-Ac and fraction B (22.7 – 25.3 min) held T-Ac. Both fractions were dried under nitrogen at 60°C. All residues were quantitatively transferred (2 x 150 µL ethyl acetate) to a GC-MS vial, dried under nitrogen at 40°C and reconstituted in 5α-ol-Ac internal standard (IS). Later on, the δ13C value of the IS will be assessed to check the performance of the IRMS analysis (cf. chapter 4).

3.5 GC-MS and GC-C-IRMS

GC-MS and GC-C-IRMS analysis were conducted as reported previously. [20] In the second part of this work 13 µL solvent vent injections on a Gerstel PTV-injector (Mulheim an der Ruhr, Germany) were used instead of 1 µL splitless injections. Here, the compounds were dissolved in a 1/1 n-hexane/toluene solution and an
injection speed of 3 µL/s was used. PTV-injector settings: 70°C for 0.35 min, then the temperature was increased at 720°C/min to 330°C and hold for 0.8 min, followed by a temperature gradient of 720°C/min to 400°C. The solvent vent lasted for 0.3 min (15 mL/min at 5 psi). At 1 min, a purge vent of 300 mL/min was applied.

### 3.6 Correction for acetate adducts

In the acetylation step, additional carbon atoms from the acetate moiety are incorporated into the steroidal skeletal. Therefore the measured δ\(^{13}\)C has to be corrected using the following formula:

\[
\delta^{13}\text{C}_{\text{steroid}} = \frac{n_{sa} \delta^{13}\text{C}_{sa} - n_{a} \delta^{13}\text{C}_{\text{acorr}}}{n_{\text{steroid}}}
\]

Here, ‘n’ is the number of moles of carbon, ‘sa’ stands for steroid acetate and ‘a’ for the acetate derivative group. \(\delta^{13}\text{C}_{\text{acorr}}\) is indirectly calculated by measuring the \(\delta^{13}\text{C}\) of the reference material of each relevant analyte in the free and derivatized fractions.

### 4 Results and discussion

#### 4.1 Part 1: expansion of the method with testosterone and epitestosterone

**4.1.1 Method validation**

*Precision (repeatability and reproducibility)*

The within-batch and between-batch precision of the extraction method for the determination of raw \(\delta^{13}\)C values was tested by the analysis of steroid stripped urine spikes prepared at three concentration levels as specified in Table 3.1. One batch of six replicates at each level (low, medium or high) was then extracted by a
different operator (Operator A, B or C), thereby incorporating an additional measure of reproducibility into this determination of precision.

For each concentration level of sample, the volume of urine required for analysis was adjusted appropriately. Therefore 2.5 mL, 10 mL and 25 mL of steroid stripped urine that was spiked at a high, medium and low concentration respectively were extracted. The $\delta^{13}$C statistics are given in Table 3.1. The within-batch precision (repeatability) ranged from 0.23 ‰ – 0.30 ‰ and 0.30 ‰ – 0.52 ‰ for T-Ac and EpiT-Ac respectively. The between batch precision (reproducibility) was 0.29 ‰ and 0.52 ‰ for T-Ac and EpiT-Ac respectively.

Table 3.1: Repeatability and reproducibility of the target compounds

<table>
<thead>
<tr>
<th>Operator</th>
<th>Concentration of T and EpiT</th>
<th>EpiT-Ac ($\delta^{13}$C, ‰)</th>
<th>T-Ac ($\delta^{13}$C, ‰)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Low (50 ng/mL)</td>
<td>Average (n=6) -34.82</td>
<td>-30.97</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SD 0.30</td>
<td>0.27</td>
</tr>
<tr>
<td>B</td>
<td>Medium (125 ng/mL)</td>
<td>Average (n=6) -34.89</td>
<td>-30.83</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SD 0.38</td>
<td>0.23</td>
</tr>
<tr>
<td>C</td>
<td>High (500 ng/mL)</td>
<td>Average (n=6) -35.58</td>
<td>-31.19</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SD 0.52</td>
<td>0.30</td>
</tr>
<tr>
<td>Overall</td>
<td></td>
<td>Average (n=18) -35.09</td>
<td>-31.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SD 0.52</td>
<td>0.29</td>
</tr>
</tbody>
</table>
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Extraction recovery vs fractionation

The difference between the overall mean adjusted $\delta^{13}$C value (for the 6 aliquots at each concentration level and operator) and the underivatized standards (that were used to spike the steroid stripped urines) was 0.01 ‰ and 0.49 ‰ for T and EpiT respectively (Table 3.2). Moreover no relation was established between extraction recovery and $\delta^{13}$C. These findings indicate that the entire sample preparation procedure (including HPLC fraction collection) did not induce a significant change in the adjusted $\delta^{13}$C value of the measured analytes.

Table 3.2: Comparison between the mean adjusted $\delta^{13}$C values and the $\delta^{13}$C values of the underivatized standards

<table>
<thead>
<tr>
<th></th>
<th>EpiT ($\delta^{13}$C, ‰)</th>
<th>T ($\delta^{13}$C, ‰)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>-28.69</td>
<td>-33.21</td>
</tr>
<tr>
<td>SD</td>
<td>0.33</td>
<td>0.58</td>
</tr>
<tr>
<td>Underivatized</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>-28.70</td>
<td>-33.70</td>
</tr>
<tr>
<td>SD</td>
<td>0.14</td>
<td>0.14</td>
</tr>
</tbody>
</table>

Specificity

The specificity of the method was ensured for all steroids by GC-MS measurements. All steroids were identified by direct comparison of mass spectral data and retention times with steroid standards.

To more directly assess the specificity of the sample preparation method, a mixed standard containing many drugs and metabolites (including 120 doping substances and 58 anabolic steroids) was aliquoted, dried and derivatized with acetic anhydride using the method protocol. One aliquot was directly analyzed on the GC-MS,
another was subject to HPLC purification. The resulting fractions were run on GC-MS. Although a number of peaks were detected in the direct analysis, none were detected after HPLC purification.

As all urine samples were subjected to multiple clean up steps (SPE, LLE, HPLC) and were always screened by full scan GC-MS prior to GC-C-IRMS analysis, the peak purity and identity could be ensured for both the target compounds and the ERCs.

4.1.2 T-Ac interference

Throughout the first HPLC sample clean up, EpiT-Ac and T-Ac eluted quite early (8.2 – 10.2 min) during the analysis and a significant amount of contaminants co-eluted with them. As one of these contaminants interfered with T-Ac during the GC separation, an additional clean up step was necessary. This was achieved with a second HPLC purification step using a MeOH/H2O mobile phase (Figure 3.1).

![Figure 3.1: GC-MS analysis of T-Ac before and after the second HPLC clean up](image-url)
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With the intention of finding optimal conditions for this additional step, an effort was made during the development of the T-Ac clean up to identify the interfering compound. By analyzing a series of random urine samples it was established that the interference was endogenous and present in concentration ranges that were similar to those of EpiT and T. The interfering compound was collected separately in the non-acetylated form using HPLC-FC and derivatized with acetic anhydride later on to confirm its identity (Figure 3.2A).

To isolate the interference in non-acetylated form, 25 mL of urine was collected on a SPE cartridge, hydrolyzed and extracted according to the procedure described above. Afterwards two consecutive HPLC clean up runs were executed on the Phenomenex C18 Gemini at 35°C. The first run used a mobile phase flow of 1 mL/min and acetonitrile/water (solvent A: 10/90 CH3CN/H2O; solvent B: CH3CN). The run started with 30 % B for 19 min, then 100 % B at 20 min, held for 4 min, 30 % B at 25 min and held for 5 min. The collected fraction (11.5 -13.5 min) contained the compound of interest. The fraction was dried under nitrogen at 60°C and dissolved in 75/25 MeOH/water. The second HPLC run used a MeOH/water mobile phase (solvent D: 10/90 MeOH/H2O; solvent C: MeOH). The analysis started with 55 % C for 16 min, then 100 % C at 17 min, held for 5 min, 55 % C at 23 min and held for 5 min. Fraction 13 –15 min was collected and dried under nitrogen at 60°C.

The collected non-acetylated interference was also derivatized with MSTFA according to the procedure described by Fragkaki et al. [23] The corresponding spectrum is presented in Figure 3.2B. Comparison of the obtained spectrum with the data published by Fragkaki et al. generated 12 possible steroids (Table 3.3).
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Figure 3.2A: Spectrum of the interference in the acetylated form

Figure 3.2B: Spectrum of the interference in the silylated form
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Table 3.3: Possible steroids that could match the spectrum of the silylated interference

<table>
<thead>
<tr>
<th>12 possible steroids</th>
</tr>
</thead>
<tbody>
<tr>
<td>1                                     4-androsten-17α-ol-3-one (epitestosterone)</td>
</tr>
<tr>
<td>2                                     4-androsten-17β-ol-3-one (testosterone)</td>
</tr>
<tr>
<td>3                                     4-androsten-3α-ol-17-one</td>
</tr>
<tr>
<td>4                                     4-androsten-3β-ol-17-one</td>
</tr>
<tr>
<td>5                                     5α-androst-1-en-17β-ol-3-one (1-testosterone)</td>
</tr>
<tr>
<td>6                                     5β-androst-1-en-17β-ol-3-one (boldenone metabolite)</td>
</tr>
<tr>
<td>7                                     5α-androst-1-en-17α-ol-3-one</td>
</tr>
<tr>
<td>8                                     5β-androst-1-en-17α-ol-3-one</td>
</tr>
<tr>
<td>9                                     5α-androst-1-en-3α-ol-17-one (1-testosterone metabolite)</td>
</tr>
<tr>
<td>10                                    5β-androst-1-en-3α-ol-17-one (boldenone metabolite)</td>
</tr>
<tr>
<td>11                                    5α-androst-1-en-3β-ol-17-one</td>
</tr>
<tr>
<td>12                                    5β-androst-1-en-3β-ol-17-one</td>
</tr>
</tbody>
</table>

Seven of the twelve steroids in Table 4 were commercially available as standards but none of them exhibited a chromatographic and spectral match (steroid 1-6 and 9). Likewise, the interference couldn’t possibly be (Epi)T, 1-testosterone or a boldenone metabolite. Of the five remaining steroids, two had a conjugated π-system (steroid 7 and 8), which generates more intense UV-signals in comparison with non-conjugated compounds. Because EpiT and T have a similar conjugated π-system and because the relative intensities of (Epi)T with the interference on UV and on GC-MS are the same (Figure 3.3), it is hypothesized that the interference is steroid 7 or 8. This hypothesis is further supported by the fact that the ion at m/z 194 after trimethylsilylation has previously been assigned as typical for 1-ene-3-keto steroids. [23]
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Figure 3.3: Analysis of the same urine sample on LC-UV and GC-MS

The tentative structure assignment was also supported by LC MS/MS (TSQ Quantum Discovery max, Finnigan) (Figure 3.4). Indeed due to the relative high proton affinity of conjugated carbonyl groups, steroids containing this type of function are expected to show an abundant [M+H]+. The observed number of losses of water was also equal to the number of oxygen atoms in the structure. [24,25] This however does not unequivocally confirm our hypothesis and further studies are required. The interference was also analyzed with a LC high resolution MS (Exactive, Thermo Scientific) for additional verification. The acquired mass was 289.2162 Da (mass accuracy 0.14 ppm).
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4.2 Part 2: improvement of the method

4.2.1 n-pentane extraction

To further refine the method the extraction solvent was switched from diethyl ether to n-pentane. Indeed, it is well known that n-pentane extractions are much more selective than diethyl ether extractions and this means a reduction in background and further elimination of possible interferences. This is clearly illustrated in Figure 3.5 which shows the UV-signal measured during the HPLC fraction collection of a urine sample.

Figure 3.4: LC MS/MS spectrum of the interference; A) collision energy = 20 eV, B) collision energy = 30 eV
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Figure 3.5: HPLC analysis of a urine sample; A: diethyl ether extraction, B: n-pentane extraction; above: 195 nm, below: 245 nm

Even though the urine samples are cleaned up extensively (SPE, extraction and HPLC fraction collection) there could still be other compounds present. Especially for urine samples with a high degree of contamination, there will never be a guarantee that a perfectly clean sample will be obtained. Obviously, in rare cases this could make the measurement of the δ\(^{13}\)C value very difficult or even impossible. By using n-pentane extractions the chance for such interferences is further reduced.

4.2.2 Solvent vent injection on a PTV

Validation of solvent vent injections

To assess if the solvent vent injections on the PTV induce any fractionation, 6 acetate standards (2.56 µg/mL) dissolved in 1/1 n-hexane/toluene were analyzed (13 µL injections). The obtained δ\(^{13}\)C values were compared with the δ\(^{13}\)C values that were collected during the analysis of 6 acetate standards (33.3 µg/mL) dissolved in iso-octane with splitless injection (1 µL injections). The acetate standards that were used during solvent vent injections were diluted 13 times in comparison with the acetate standards that were used during the splitless injection.
As such, equal peak intensities could be obtained (cf. Linearity of the IRMS). As a consequence, the solvent vent injections increase the sensitivity of the IRMS by a factor 13. The results are presented in Table 3.4 and indicate that the PTV solvent vent injection did not induce any fractionation as the $\delta^{13}C$ values for both injections modes were the same.

**Linearity**

The linear range of the instrument was assessed by the analysis of 22 aliquots of a steroid acetate mixed standard over a concentration range of approximately 0.8 – 8 $\mu$g/mL (in vial), equivalent to approximately 8 – 80 ng of steroid on column. A summary of the results is shown in Table 3.5. The linearity (expressed as the slope of the regression line (‰/V)) ranged from -0.0604 ‰/V to -0.2377 ‰/V indicating that 0.5 V – 5 V is a suitable interval for appropriate $\delta^{13}C$ determination.
### Table 3.4: Comparison between splitless injections and solvent vent injections

<table>
<thead>
<tr>
<th></th>
<th>Acetate standard (33.3 µg/mL) splitless injection</th>
<th>Acetate standard (2.56 µg/mL) solvent vent injection</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>δ¹³C(‰)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5a-ol-Ac</td>
<td>-32.63</td>
<td>-32.72</td>
</tr>
<tr>
<td>5-en</td>
<td>-30.33</td>
<td>-30.33</td>
</tr>
<tr>
<td>Et-Ac</td>
<td>-21.82</td>
<td>-21.85</td>
</tr>
<tr>
<td>A-Ac</td>
<td>-37.03</td>
<td>-36.73</td>
</tr>
<tr>
<td>βαβ-Ac₂</td>
<td>-31.79</td>
<td>-31.37</td>
</tr>
<tr>
<td>ααβ-Ac₂</td>
<td>-38.26</td>
<td>-38.26</td>
</tr>
<tr>
<td>EpiT-Ac</td>
<td>-34.36</td>
<td>-34.30</td>
</tr>
<tr>
<td>T-Ac</td>
<td>-33.71</td>
<td>-33.70</td>
</tr>
<tr>
<td>PD-Ac₂</td>
<td>-22.89</td>
<td>-22.90</td>
</tr>
<tr>
<td><strong>Intensity (mV)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>average</td>
<td>1518</td>
<td>1301</td>
</tr>
<tr>
<td>SD</td>
<td>67</td>
<td>52</td>
</tr>
<tr>
<td><strong>Intensity splitless /Intensity solvent vent</strong></td>
<td>1.17</td>
<td>1,17</td>
</tr>
</tbody>
</table>
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Table 3.5: IRMS linearity

<table>
<thead>
<tr>
<th></th>
<th>Et-Ac</th>
<th>A-Ac</th>
<th>βαβ-Ac₂</th>
<th>EpiT-Ac</th>
<th>PD-Ac₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average (δ¹³C,‰)</td>
<td>-20.12</td>
<td>-35.89</td>
<td>-30.84</td>
<td>-33.32</td>
<td>-21.62</td>
</tr>
<tr>
<td>SD (δ¹³C,‰)</td>
<td>0.36</td>
<td>0.42</td>
<td>0.38</td>
<td>0.49</td>
<td>0.33</td>
</tr>
<tr>
<td>Slope (‰/V)</td>
<td>0.0740</td>
<td>-0.2377</td>
<td>0.1186</td>
<td>0.1680</td>
<td>-0.0604</td>
</tr>
<tr>
<td>min (V)</td>
<td>0.58</td>
<td>0.50</td>
<td>0.31</td>
<td>0.50</td>
<td>0.76</td>
</tr>
<tr>
<td>max (V)</td>
<td>5.02</td>
<td>5.19</td>
<td>4.59</td>
<td>5.33</td>
<td>6.77</td>
</tr>
</tbody>
</table>

Precision (repeatability and reproducibility) and fractionation

To assess the sample preparation process for possible fractionation, 2 batches of 6 spiked steroid stripped urines (Table 3.6) were analyzed. The first batch (10 mL aliquots) was analyzed with splitless injections, the second batch (2 mL aliquots) was analyzed according to the new procedure with solvent vent injections. The raw δ¹³C value obtained for the steroid stripped urine spikes was adjusted using the correction factor and is compared in Table 3.7. The differences ranged from 0.02 ‰ (A-Ac) till 0.49 ‰ (T-Ac). These results indicate that the new method does not induce any additional fractionation compared to the method with splitless injection. The precision has remained similar as well (0.02 - 0.49 ‰ compared to 0.26 - 0.57 ‰). The levels of precision obtained for the extraction of samples compare very favourably with the results obtained for the repeated analysis of reference standards (linearity determination) indicating the sample preparation process does not significantly contribute additional variation.
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Table 3.6: Concentration of the target compounds

<table>
<thead>
<tr>
<th></th>
<th>Et</th>
<th>A</th>
<th>βαβ</th>
<th>EpiT</th>
<th>T</th>
<th>PD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration (ng/mL)</td>
<td>2500</td>
<td>2500</td>
<td>250</td>
<td>125</td>
<td>125</td>
<td>125</td>
</tr>
</tbody>
</table>

Table 3.7: Precision and fractionation of the target compounds

<table>
<thead>
<tr>
<th></th>
<th>Et-Ac</th>
<th>A-Ac</th>
<th>βαβ-Ac₂</th>
<th>EpiT-Ac</th>
<th>T-Ac</th>
<th>PD-Ac₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent injection Average (δ¹³C, ‰)</td>
<td>-27.71</td>
<td>-33.20</td>
<td>-29.53</td>
<td>-33.65</td>
<td>-28.87</td>
<td>-30.54</td>
</tr>
<tr>
<td>SD (δ¹³C, ‰)</td>
<td>0.43</td>
<td>0.53</td>
<td>0.26</td>
<td>0.32</td>
<td>0.57</td>
<td>0.46</td>
</tr>
<tr>
<td>Splitless injection Average (δ¹³C, ‰)</td>
<td>-27.86</td>
<td>-33.22</td>
<td>-29.72</td>
<td>-33.87</td>
<td>-28.38</td>
<td>-30.10</td>
</tr>
<tr>
<td>SD (δ¹³C, ‰)</td>
<td>0.49</td>
<td>0.02</td>
<td>0.13</td>
<td>0.48</td>
<td>0.37</td>
<td>0.33</td>
</tr>
</tbody>
</table>

Limit of detection

With the aid of a PTV a sensitive GC-C-IRMS analysis was successfully developed which reduces the required amount of urine and the limit of detection. The δ¹³C value can be determined at 7 - 10 ng/mL for T and EpiT and 5 - 6 ng/mL for βαβ and ααβ, starting from 25 mL of urine. Figure 3.6 shows the GC-C-IRMS analysis of a real urine sample (starting from an aliquot of 18 mL) with a ααβ concentration of 5.94
Chapter 3: Development of a Sensitive GC-C-IRMS Method

ng/mL. The signal to noise ratio is excellent and the signal intensity is 685 mV, which is within the linear range of our IRMS (500 – 5000 mV).

![Graph showing GC-C-IRMS analysis of 18 mL of urine with an ααβ concentration of 5.94 ng/mL.](image)

Figure 3.6: GC-C-IRMS analysis of 18 mL of urine with an ααβ concentration of 5.94 ng/mL

5 Conclusions

Because the amount of urine received from an athlete is limited, doping control laboratories have to make their analysis as sensitive as possible so all the required doping tests can be executed. If less urine is consumed, then there is more available for additional tests. In this work we introduce a new type of injection which
increases the sensitivity of GC-C-IRMS by a factor of 13, simply by changing the injection technique. Indeed, with the aid of a PTV the limit of detection (7 - 10 ng/mL for T and EpiT; 5 - 6 ng/mL for βαβ and ααβ) of several important endogenous steroids will allow a decrease in the needed urine volume to confirm atypical findings. In the past, improving the sensitivity of the IRMS meant optimizing the detection parameters and reducing the linearity. With a PTV, the detection parameters remain fixed and there is no loss in linearity. Additionally, the developed method not only allows for an improved detection of misuse of testosterone or its prohormones, but also for the use of epitestosterone as masking agent.
Chapter 3: Development of a Sensitive GC-C-IRMS Method

6 References


Chapter 3: Development of a Sensitive GC-C-IRMS Method


Chapter 3: Development of a Sensitive GC-C-IRMS Method


Part 1: Improved Confirmation Method for Testosterone

Chapter 4: Three Years of IRMS in Routine Doping Control

Adapted from

M. Polet, P. Van Eenoo

GC-C-IRMS in routine doping control practice: three years of drug testing data, quality control and evolution of the method

Analytical and Bioanalytical Chemistry, in press, DOI: 10.007/s00216-014-8374-7
1 Abstract

In order to detect the misuse of endogenous anabolic steroids, doping control laboratories require methods that allow differentiation between endogenous steroids and their synthetic copies. GC-C-IRMS is capable of measuring the carbon isotope ratio of urinary steroids and this allows differentiation between both. GC-C-IRMS and its application to doping control has evolved a lot during the last decade and so have the WADA technical documents that describe how GC-C-IRMS should be applied. In this paper, three years of drug testing data of our GC-C-IRMS method in routine doping control practice is described, with an emphasis on the new 2014 WADA technical document and its implementation. Useful data for other doping control laboratories is presented focusing on general method setup, quality control and data collected from routine samples.

2 Introduction

The administration of synthetic steroids is one of the most important issues facing sports. Synthetic copies of endogenous steroids are pharmacologically and chemically identical to their endogenous analogues, but there are small differences in the CIR. Natural variations in the abundance of $^{13}$C reflect the passage through biological and/or chemical processes in which transformations are accompanied by isotopic fractionation that results in a slight depletion or enrichment of $^{13}$C due to the difference in mass. CIRs are expressed as $\delta^{13}$C values against the international standard VPDB. [1] To measure the CIR of a compound, doping control laboratories use GC-C-IRMS. [2–14] Many laboratories derivatize their compounds before analysis. The CIR of the acetylated compounds needs to be corrected for the extra carbon atoms that have been incorporated into the steroidal skeletal. Therefore the measured $\delta^{13}$C value has to be corrected using the following formula:
Chapter 4: Three Years of IRMS in Routine Doping Control

\[ \delta^{13}C_{\text{steroid}} = \frac{(n_{sa}\delta^{13}C_{sa}) - (n_{a}\delta^{13}C_{acorr})}{n_{\text{steroid}}} \]

Here, ‘n’ is the number of moles of carbon, ‘sa’ stands for steroid acetate and ‘a’ for the acetate derivative group. \( \delta^{13}C_{\text{acorr}} \) is indirectly calculated by measuring the \( \delta^{13}C \) value of the reference material of each relevant analyte in the free and derivatized fractions.

GC-C-IRMS remains very laborious and expensive because one can only determine the CIR of a pure compound. [15] To minimize the amount of samples that needs to be analyzed on GC-C-IRMS and maximize the detection of doping violations, all urine samples that enter a doping control laboratory are first screened by a fast Gas Chromatography triple quadrupole Mass Spectrometer (GC-MS/MS) method that quantifies all important endogenous steroids. [16] Afterwards, samples with suspicious endogenous steroid concentrations and/or ratios are forwarded to IRMS according to WADA regulations. [17] The testosterone/epitestosterone (T/EpiT) ratio is the most important parameter for detecting T or T prohormone administration and its threshold is currently set at 4 by WADA as most individuals have values that vary between 0.1 and 3. [18,19]

After administration of a synthetic anabolic steroid the CIR of that steroid and its metabolites will exhibit depleted CIR in relation to an ERC. ERCs are steroids that are not involved in the pathway of anabolic steroids and therefore their CIR will not be affected by application of an exogenous anabolic steroid. PD is used as primary ERC, but others such as 5α-androst-16-en-3α-ol, 11β-hydroxyandrosterone and 11-oxoEt are also routinely used. ERCs normalize GC-C-IRMS results in relation to the diet of the individual athlete and WADA has set a minimum threshold of 3 ‰ difference between ERC and TC in order to prove an adverse analytical finding by synthetic steroid abuse. [17] TCs are steroids that can be administered as doping substance or their metabolites (for example A, Et, ααβ, βαβ,...).
Chapter 4: Three Years of IRMS in Routine Doping Control

The main goal of this chapter is to provide useful data for other doping control laboratories and illustrate how we deal with GC-C-IRMS measurements, with an emphasis on the new 2014 WADA technical document and its implementation. [17] In an extensive method as GC-C-IRMS, localizing a malfunction can obviously be very time consuming. The different quality controls incorporated in the GC-C-IRMS method are described and an elucidation is given on how these can help identifying the source of deviating $\delta^{13}$C values.

3 Experimental

3.1 Reagents and chemicals

All used reagents and chemicals are mentioned in previous papers. [20,21]

3.2 GC-C-IRMS confirmation procedure and its evolution

All samples were processed according to our routine IRMS confirmation procedure. The first samples were analyzed in February 2011. Since then, the method has been adapted on several occasions in order to improve quality control, simplify the sample preparation, expand the number of TCs and ERCs and to comply with WADA requirements (Table 4.1). [17] The original method started with aliquotation of a system blank, quality control negative (QC Neg), QC positive (QC Pos) and the routine samples, followed by SPE, hydrolysis, liquid liquid extraction (LLE) with diethyl ether, acetylation and a High Performance Liquid Chromatography fraction collection (HPLC-FC). Four TCs (A-Ac, Et-Ac, $\alpha\alpha\beta$-Ac$_2$ and $\beta\alpha\beta$-Ac$_2$) and one ERC (5$\beta$-pregnane-3$\alpha$,20$\alpha$-diacetate, PD-Ac$_2$) were collected, analyzed on GC-MS to check for peak impurities and finally the CIRs were determined on GC-C-IRMS by one single injection per fraction. The details of the procedure are described in a previous publication. [20]
Chapter 4: Three Years of IRMS in Routine Doping Control

Table 4.1: Summary of the method evolution

<table>
<thead>
<tr>
<th>Date</th>
<th>Method Evolution</th>
</tr>
</thead>
<tbody>
<tr>
<td>February 2011</td>
<td>Launch routine method (A, Et, βαβ, ααβ and PD)</td>
</tr>
<tr>
<td>September 2011</td>
<td>T, EpiT, n-pentane LLE, new QC Neg</td>
</tr>
<tr>
<td>December 2011</td>
<td>New QC Pos</td>
</tr>
<tr>
<td>February 2012</td>
<td>IRMS sensitivity x 10 (10 µL solvent vent injections)</td>
</tr>
<tr>
<td>September 2012</td>
<td>$\delta^{13}$C values Acetate STD</td>
</tr>
<tr>
<td>May 2013</td>
<td>11-oxoEt + new calibration procedure</td>
</tr>
<tr>
<td>January 2014</td>
<td>QC STD MIX</td>
</tr>
</tbody>
</table>

In September 2011, the method was slightly extended: an additional fraction containing T-Ac and EpiT-Ac was collected during the HPLC-FC. This fraction needed a second HPLC clean up due to interference of T-Ac. During the first HPLC-FC this T-Ac/EpiT-Ac fraction was always collected, but in order to keep the sample preparation as simple as possible, it only underwent the second HPLC-FC, GC-MS and GC-C-IRMS analysis if suspicious CIRs were determined for the primary compounds (A-Ac, Et-Ac, βαβ-Ac$_2$, ααβ-Ac$_2$ and PD-Ac$_2$). In addition, the diethyl ether LLE was replaced by a n-pentane LLE. More details are available in chapter 3.[21] At this stage, a new QC Neg was collected and stored at -80°C. The new QC Neg comprised a volume of more than 3 L which allows us to use the same QC Neg for several years. If long term shifts in CIR would occur, they can be detected and visualized with this QC Neg. In December 2011, a new QC Pos was made and stored at -80°C for the same purpose. The new QC Pos was obtained by spiking QC Neg with sufficient amounts of A, Et, βαβ, ααβ, T and EpiT.

In February 2012, we switched from 1 µL splitless injections to 10 µL solvent vent injections by using a PTV (Programmed Temperature Vaporizer) on our GC-MS and GC-C-IRMS equipment. These 10 µL solvent vent injections increased our IRMS sensitivity by a factor of 10 and allowed us to simplify our sample preparation procedure by reducing the required urine volume for IRMS analysis by a factor of 5.
For a TC with a urinary concentration of 50 ng/mL we now only needed 5 mL of urine instead of 25 mL in the past. More information is available in chapter 3. [21] From now on, we consume 7 mL of QC Neg and 3 mL of QC positive per batch, prolonging the lifetime of our QCs.

From September 2012 on, we have started to actively record all $\delta^{13}C$ values of all compounds in our Acetate Standard (Acetate STD). The Acetate STD is a mixture of acetylated standards of all our TCs, PD-Ac$_2$ and the IS (5α-ol-Ac) and is analyzed on GC-C-IRMS before, during and after every batch. It has always been included in our IRMS confirmation procedure, but was merely used to check and set the retention times of the compounds, estimate the GC chromatographic performance (peak tailing and separation) and evaluate the $\delta^{13}C$ value of our IS as a deviating $\delta^{13}C$ value could indicate problems during the combustion process.

Since May 2013, a second ERC (11-oxoEt) has been included in the method. The sample preparation procedure remained the same, but an additional fraction containing 11-oxoEt-Ac was collected during the HPLC-FC (6.6 - 8.1 min). This additional fraction was always collected, but in order to keep the sample preparation as simple as possible, it was only analyzed on GC-MS and GC-C-IRMS if suspicious CIR were determined for the primary compounds or in another exceptional case. The IRMS calibration procedure was also adjusted. Instead of calibrating the CO$_2$ reference gas with an n-alkane mixture ($C_{17}$ – $C_{25}$), CU-USADA 33-1 was used. During the calibration procedure an additional check was performed using CU-USADA 34-1 and 5α-androstane (IU 5α-A), provided by the Biogeochemical Laboratories of the Indiana University (Bloomington, IN, USA). These adaptations were made to comply with the requirements of the new 2014 WADA technical document that would come into effect in September 2014. [17] From January until September 2014 the guidelines document was in force, but both documents are essentially the same. [22]
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Since January 2014, an extra QC (QC STD MIX) was processed and analyzed with every batch. QC STD MIX was prepared by spiking 3 mL of steroid stripped urine with 150 µL of a 12.5 µg/mL standard solution containing A, Et, βαβ, ααβ, T, EpiT, PD and 11-oxoEt. QC STD MIX was added to the method in order to comply with WADA regulations. [17] However, a recent communication from WADA (dated 8th September 2014) has indicated that it is not necessary to subject reference materials with known CIR to the full sample preparation procedure, only that they must be injected prior to sample analysis.

3.3 Routine doping samples

From February 2011 until May 2014 we analysed 609 routine urine samples from athletes on GC-C-IRMS. 279 samples (45.8 %) were investigated on IRMS because of an elevated T/EpiT ratio, the other 330 samples (54.2 %) required IRMS investigation due to an elevated concentration of A, Et, βαβ, ααβ, T or DHEA (or combinations) or because this was requested by the sport federation.

3.4 Negative reference population samples

The collection and analysis of negative reference population samples occurred at different points in time during the period 2010 – 2013. In 2010, a first reference population consisting of 27 male (aged 26.0 ± 5.5 year) and 25 female (aged 26.3 ± 8.0 year) volunteers who declared not to have used any prohibited substance was analyzed. The remainder of the negative reference population samples originated from healthy university staff that needed to undergo the yearly health check at the university hospital. These were collected and analyzed in 2011 - 2013.
4 Results and discussion

4.1 Calibration

Ideally, the CO₂ reference pulse is calibrated against an isotopic standard with a chemical structure that is very similar or equal to that of the analytical compounds that need to be analyzed on IRMS. It is important that reference materials have similar chemical properties because the combustion efficiency may vary, changing the isotopic composition of the gases. [23] To harmonize reported values and achieve uniform results between the different anti-doping laboratories worldwide, the IRMS system needs to be calibrated against a steroid reference material according to the new 2014 WADA regulations. [17]

In 2008, two steroid isotopic standards (SIS), CU-USADA 33-1 and CU-USADA 34-1, have been assigned certified CIRs that are traceable to the international reference material. [24] CU-USADA 33-1 contains 5α-ol-Ac, A-Ac, 11-oxoEt-Ac and 5α-cholestan (5α-Chol), CU-USADA 34-1 contains Et, A and PD (Table 4.2).

Since May 2013, our CO₂ reference pulse has been calibrated against CU-USADA 33-1 eight times. This SIS was chosen because it contains acetylated steroids, similar or identical to the ones we analyze in our GC-C-IRMS method. We calibrate with CU-USADA 33-1 in threefold and use the average assigned value as the new \( \delta^{13}C \) value for our CO₂ reference pulse. Immediately before and after the calibration, CU-USADA 33-1 is analyzed as well to check how much the measured \( \delta^{13}C \) values of the steroids deviate from the certified \( \delta^{13}C \) values by using respectively the old and the new \( \delta^{13}C \) value of the CO₂ reference pulse (Table 4.3). Afterwards, as additional check, CU-USADA 34-1 and IU 5α-A (5α-androstan) are analyzed with the new CO₂ reference pulse \( \delta^{13}C \) value for the same purpose.
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Table 4.2: Certified CIRs for CU-USADA 33-1, CU-USADA 34-1 and IU 5α-A

<table>
<thead>
<tr>
<th>Certified Value</th>
<th>$\delta^{13}C$ (‰)</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>CU/USADA 33-1</td>
<td>5α-ol-Ac</td>
<td>-30.61</td>
</tr>
<tr>
<td></td>
<td>5α-Chol</td>
<td>-24.77</td>
</tr>
<tr>
<td></td>
<td>A-Ac</td>
<td>-33.04</td>
</tr>
<tr>
<td></td>
<td>11oxo-Et-Ac</td>
<td>-16.7</td>
</tr>
<tr>
<td>CU/USADA 34-1</td>
<td>E</td>
<td>-28.91</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>-27.06</td>
</tr>
<tr>
<td></td>
<td>PD</td>
<td>-31.49</td>
</tr>
<tr>
<td>IU</td>
<td>5α-A</td>
<td>-31.64</td>
</tr>
</tbody>
</table>

For CU-USADA 33-1, the bias with the certified values is acceptable, except for 11-oxoEt-Ac. The standard deviation (SD) and bias are unacceptable large for this compound. Similar observations were made for 11-oxoEt-Ac in CU-USADA 33-1 in a paper of Piper et al. [25] The effect was partially attributed to an incomplete transfer from the GC column onto the hot zone of the combustion furnace. Other effects caused by the GC such as losses linked to the injection were also indicated as possible contributors to the atypical behaviour. An amount-depended shift was also observed, but this does not apply for our measurements as we always inject the same amount during the calibration. 11-oxoEt-Ac is also present in our QC Neg, QC Pos and QC STD MIX but here we do not see a similar phenomenon and normal deviations are obtained (Table 4.4). The four steroids present in CU-USADA 33-1, including 11-oxoEt-Ac are used to set the CO$_2$ reference pulse $\delta^{13}C$ value, but despite this atypical behaviour of 11-oxoEt-Ac, a correct $\delta^{13}C$ value for the reference pulse is obtained (acceptable bias for the other compounds). We assume
that during the calibration process, the other three steroids compensate for the atypical behaviour of 11-oxoEt-Ac.

CU-USADA 34-1 consists of three non-acetylated standards. Free steroids cause more tailing during the GC analysis and this results in larger SDs. Overall, the bias is slightly worse in comparison with CU-USADA 33-1. The bias and SD for IU 5α-A were in line with CU-USADA 33-1.

Calibrating with the new reference material CU-USADA 33-1 did not induce a shift in the CO₂ reference gas value. The obtained δ¹³C values were in agreement with previously obtained values (using the alkane reference material). Neither was there a shift in the CIR of the IS, QC Neg and QC Pos (Figure 4.1, Figure 4.2A and B).
## Chapter 4: Three Years of IRMS in Routine Doping Control

### Table 4.3: Measured CIRs for CU-USADA 33-1, CU-USADA 34-1 and IU 5α-A

<table>
<thead>
<tr>
<th></th>
<th>$\delta^{13}$C CU-USADA 33-1 (%)</th>
<th>$\delta^{13}$C CU-USADA 34-1 (%)</th>
<th>$\delta^{13}$C IU 5α-A (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>before calibration ($n = 8$)</td>
<td>after calibration ($n = 8$)</td>
<td>total ($n = 16$)</td>
</tr>
<tr>
<td></td>
<td>Sα-ol-Ac</td>
<td>Sα-Chol</td>
<td>A-Ac</td>
</tr>
<tr>
<td>SD</td>
<td>0.17</td>
<td>0.28</td>
<td>0.39</td>
</tr>
<tr>
<td>bias with certified value</td>
<td>0.15</td>
<td>-0.03</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>-30.69</td>
<td>-25.05</td>
<td>-33.31</td>
</tr>
<tr>
<td>SD</td>
<td>0.15</td>
<td>0.27</td>
<td>0.64</td>
</tr>
<tr>
<td>bias with certified value</td>
<td>-0.08</td>
<td>-0.28</td>
<td>-0.27</td>
</tr>
<tr>
<td>total ($n = 16$)</td>
<td>-30.57</td>
<td>-24.92</td>
<td>-33.09</td>
</tr>
<tr>
<td>SD</td>
<td>0.20</td>
<td>0.29</td>
<td>0.56</td>
</tr>
<tr>
<td>bias with certified value</td>
<td>0.04</td>
<td>-0.15</td>
<td>-0.05</td>
</tr>
</tbody>
</table>
4.2 System stability

As recommended in a paper of Piper et al., the CIR of the IS has been monitored on a continuous basis as this compound is highly sensitive towards long term shifts and changes in the combustion process. [26] The data is presented in Figure 4.1 with consecutive numbers instead of the date of measurement as data collection was carried out on a regular basis (n = 579, 3 – 4 measurements per week). The obtained average value was -32.30 ‰ with a SD of 0.39 ‰ and according to the Shapiro Wilk test (W = 0.996, p = 0.221) spread according to a normal distribution. The first 30 measurements were characterised by an average of -32.38 ‰ and a SD of 0.47 ‰, the last 30 measurements had an average of -32.23 ‰ and SD of 0.51 ‰. According to the t-test at p = 0.05 there is no significant difference between the first and last 30 measurements (p = 0.236), illustrating that no clear significant shift occurred. The same methodology was applied on the 30 measurements before and after an alteration in the IRMS method. According to the t-test at p = 0.05 there were no significant differences: p = 0.818 for switching to n-pentane for the LLE, p = 0.131 for replacing the combustion reactor, p = 0.264 for altering the injection mechanism and p = 0.109 for the application of a new calibration procedure. Contrary to the expectations, the changes made to the method (different injection type and different calibration procedure) did not induce a clear shift.

In the publication of Piper et al. two factors contributed to two shifts: replacement of the CO2 tank for the reference pulse and a change in the oxidation protocol for reoxidizing the combustion reactor. [26] Since the launch of our method it has not yet been necessary to replace the CO2 tank. Our oxidization protocol has remained the same as well: we oxidize for 12 s before each measurement. If the IRMS has not been used for a longer period of time (> 3 days), we start the batch with 5 blanks to ensure that the combustion reactor is saturated with enough oxygen before the actual measurements begin. We do not perform any other oxidation protocols (i.e. oxidize for a longer time period once a week). So far we only had to replace the
combustion reactor once (November 2011), but this did not induce a measurable shift either.

Figure 4.1: CIR of the IS from February 2011 until May 2014. Event A: new LLE; event B: replacement combustion reactor; event C: solvent vent injections; event D: new calibration procedure. The corresponding dashed line represents the Gaussian least-square fitted linear slope ($y = 0.0002x - 32.36$)

4.3 Quality control

4.3.1 System blank

So far, there has not been a case where the system blank indicated any problems or contaminations.

4.3.2 QC Neg and QC Pos

QC Neg and QC Pos were prepared and analyzed with every batch of samples. The obtained CIR for the TCs and ERCs were plotted in a quality control chart and
compared with the data acquired in the previous batches. If the CIR of one of the TCs or ERCS was outside the 2-sigma limits, all data from that batch were rejected and the batch was reanalyzed. [27] The cause of the out-of-control data would be sought and solved and the batch would be reanalyzed. Five times we have had out-of-control data: on one occasion we were dealing with a broken combustion reactor and in a second incident we used a HPLC column that had exceeded its lifetime. The peaks had become so wide that they started to elute outside the collected fractions during HPLC-FC. This was especially the case for PD-Ac₂ (late eluting compound) and was more pronounced in QC Neg than QC Pos (same concentration of PD but a 7 mL aliquot compared to 3 mL for QC Pos). PD-Ac₂ had a value of 0.5 ‰ and 0.3 ‰ outside the 2-sigma limit for QC Neg and QC Pos respectively. On three other occasions the GC column had exceeded its lifetime.

The collected data for QC Neg (starting from September 2011) and QC Pos (starting from December 2011) are presented in Table 4.4 and visualized in Figure 4.2A and B. For QC Neg the largest SD was found to be 0.81 ‰ (ααβ), for QC Pos the largest SD was 0.87 ‰ (EpiT). Overall, the obtained precisions were in line with what can be expected for GC-C-IRMS measurements. [26,28] Differences in precision between compounds are obviously caused by chemical and physical dissimilarities, which may for example lead to different chromatographic behaviour (i.e. tailing compounds are prone to more variation). However, differences in precision are also sample dependent. QC Pos is the same urine as QC Neg (e.i. same matrix) but spiked with the TCs. In QC Neg, ααβ is present in quite low concentrations, leading to a low peak intensity (but still within the acceptable linearity range) which will be susceptible to more variation. In QC Pos ααβ is present in higher concentrations and has a lower SD (0.68 ‰). Likewise, a sample (and its TCs and ERCs) defined by a higher background will be prone to more variation. This is something one has to keep in mind when defining “the overall precision of a method” (cf. section 4.4 “Uncertainty”).
Table 4.4: Statistical data gathered for QC Neg, QC Pos, QC STD MIX and the Acetate STD

<table>
<thead>
<tr>
<th>δ^{13}C (%)</th>
<th>Et</th>
<th>A</th>
<th>βαβ</th>
<th>ααβ</th>
<th>EpiT</th>
<th>T</th>
<th>11-oxoEt</th>
<th>PD</th>
</tr>
</thead>
<tbody>
<tr>
<td>QC Neg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n =</td>
<td>61</td>
<td>61</td>
<td>61</td>
<td>54</td>
<td>11</td>
<td>11</td>
<td>7</td>
<td>61</td>
</tr>
<tr>
<td>u</td>
<td>-23.1</td>
<td>-23.3</td>
<td>-22.8</td>
<td>-23.8</td>
<td>-25.3</td>
<td>-24.5</td>
<td>-22.9</td>
<td>-22.7</td>
</tr>
<tr>
<td>SD</td>
<td>0.38</td>
<td>0.61</td>
<td>0.56</td>
<td>0.81</td>
<td>0.78</td>
<td>0.69</td>
<td>0.31</td>
<td>0.56</td>
</tr>
<tr>
<td>u+2xSD</td>
<td>-22.4</td>
<td>-22.1</td>
<td>-21.7</td>
<td>-22.2</td>
<td>-23.7</td>
<td>-23.2</td>
<td>-22.3</td>
<td>-21.5</td>
</tr>
<tr>
<td>u-2xSD</td>
<td>-23.9</td>
<td>-24.6</td>
<td>-23.9</td>
<td>-25.4</td>
<td>-26.9</td>
<td>-25.9</td>
<td>-23.5</td>
<td>-23.8</td>
</tr>
<tr>
<td>QC Pos</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n =</td>
<td>53</td>
<td>53</td>
<td>53</td>
<td>49</td>
<td>10</td>
<td>10</td>
<td>7</td>
<td>53</td>
</tr>
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<td>0.53</td>
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<td>0.83</td>
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<td>-28.4</td>
<td>-25.7</td>
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<td>-27.1</td>
<td>-26.2</td>
<td>-22.1</td>
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<tr>
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<td>-27.8</td>
<td>-30.9</td>
<td>-30.6</td>
<td>-29.5</td>
<td>-23.9</td>
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<tr>
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<td>0.47</td>
<td>0.19</td>
<td>0.64</td>
<td>0.42</td>
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<td>0.63</td>
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<tr>
<td>u+2xSD</td>
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<tr>
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<td>-32.8</td>
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<tr>
<td>SD</td>
<td>0.61</td>
<td>0.42</td>
<td>0.57</td>
<td>0.61</td>
<td>0.49</td>
<td>0.61</td>
<td>0.35</td>
<td>0.35</td>
</tr>
<tr>
<td>u+2xSD</td>
<td>-19.6</td>
<td>-34.9</td>
<td>-29.7</td>
<td>-35.3</td>
<td>-32.4</td>
<td>-31.6</td>
<td>-21.2</td>
<td>-21.2</td>
</tr>
<tr>
<td>u-2xSD</td>
<td>-22.0</td>
<td>-36.6</td>
<td>-32.0</td>
<td>-37.8</td>
<td>-34.3</td>
<td>-34.0</td>
<td>-22.7</td>
<td>-22.7</td>
</tr>
</tbody>
</table>
Figure 4.2A: Quality control chart with CIR of Et, A, βαβ, ααβ and PD from QC Neg
Figure 4.2B: Quality control chart with CIR of Et, A, $\beta\alpha\beta$, $\alpha\alpha\beta$ and PD from QC Pos
4.3.3 QC STD MIX

QC STD MIX has basically the same function as QC Neg and Pos: It allows the analyst to identify deviating CIR for one or multiple compounds and to visualize long term shifts. QC STD MIX however has the advantage that the standards used to spike the steroid free urine are disposable and can be analyzed directly on GC-C-IRMS without sample preparation. The standards can be injected with or without acetylation which allows the analyst to for example identify acetylation problems if these would occur. QC STD MIX data is given in Table 4.4.

4.3.4 Acetate STD

The Acetate STD has always been included in our IRMS confirmation procedure, but was merely used to check and set the retention times of the compounds, estimate the GC chromatographic performance (peak tailing and separation) and evaluate the \( \delta^{13}C \) value of our IS as a deviating \( \delta^{13}C \) value could indicate problems during the combustion process (cf. section 4.2 “System stability”). When our IRMS method was launched, we assumed that if the \( \delta^{13}C \) value of the IS was within specifications, the GC-C-IRMS was working properly for \( \delta^{13}C \) value determination of the other compounds as well (if there weren’t any chromatographic issues, impurities,…). However, in some occasions, the \( \delta^{13}C \) value of one or more acetylated standards started to deviate from their normal value and the deviation progressed in time whereas the \( \delta^{13}C \) values of the IS and the other acetylated standards remained correct, indicating that incomplete combustion was not the problem. All compounds were still baseline separated, there was no peak tailing or other chromatographic issue and there were no visible impurities present. In these cases replacement of the GC column however solved the issue and restored the \( \delta^{13}C \) values back to their normal values. We suspect that as the GC column gets older, (undetectable) column bleeding that elutes at specific retention times starts to influence the combustion process of compounds eluting at that retention time with a deviating \( \delta^{13}C \) value as a result. In our experience, the GC column lifetime rarely
exceeds 600 – 700 injections for GC-C-IRM purposes. However, to minimize costs we transfer this “old” column to our GC-MS instrument as it still fulfils the chromatographic requirements for this analysis.

In this light, measuring the $\delta^{13}C$ values of all compounds in our Acetate STD facilitates troubleshooting to a great extent. Correct $\delta^{13}C$ values for all compounds in our Acetate STD guarantees that the GC-C-IRM is working properly for CIR determination of all TCs and ERCs (and not only the IS). In the past, when deviating CIR were obtained in the QC Neg and QC Pos, we were uncertain if the problem was situated in the sample preparation or originated from a malfunctioning GC-C-IRM instrument. In our experience, in 90 % of the cases, deviating CIR are invoked by an “old” GC column. For the remaining 10 % there were various causes. One time they were caused by an HPLC column that had exceeded its lifetime. On a second occasion, deviating $\delta^{13}C$ values were the result of a broken combustion reactor. In a third incident, deviating CIR resulted from a malfunctioning IRMS detector and an electronic control board needed to be replaced.

Some laboratories include additional quality controls in order to for example evaluate the efficiency of the hydrolysis or the derivatization. Because issues during the hydrolysis or derivatization will be traceable with our current quality controls, we have chosen not to include them in our already extensive method and we consider the presented strategy as sufficient.

4.4 Uncertainty

Since 2014, WADA demands a maximum combined measurement uncertainty $(u_c)$ of 1 ‰. [17] To the best of our knowledge, only one publication has dealt with detailed uncertainty calculations for IRMS purposes. [29] These calculations however focused at certifying a reference material, rather than the combined measurement uncertainty of an IRMS method. The basic rule for the calculation of the combined uncertainty is the “square root of the sum of the squares rule”.

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Uncertainty components \( u(x_1)...u(x_n) \), are combined as shown in the following equation [30]:

\[
  u_c = \sqrt{u(x_1)^2 + u(x_2)^2 + \cdots + u(x_n)^2}
\]

For GC-C-IRMS, there are no clear rules on how these separate uncertainty components need to be defined exactly. The consequence is that doping control laboratories use different methodologies to calculate their \( u_c \), leading to a situation where some doping control laboratories have no problem maintaining their \( u_c \) beneath 1 \(^\circ\), whereas other laboratories have great difficulties. In those cases, the apparent difference in combined uncertainty is not necessarily related to the quality of the GC-C-IRMS method, but caused by a difference in calculation methodology. An example of such a methodology might be the following (Table 4.5):

\[
  u_c = \sqrt{u(\text{calibration bias})^2 + u(\text{reference value})^2 + u(\text{method precision})^2}
\]

The calibration bias is the difference between the certified value for the SIS and the measured SIS value as calculated in Table 4.3 (-0.15 \(^\circ\); the largest bias, disregarding 11-oxoEt-Ac). The second uncertainty component is the SD on the true certified SIS value as given in Table 4.2 (0.14 \(^\circ\); again the largest value). The largest contribution to \( u_c \) is the method precision that can for example be defined as the SDs for QC Neg (Table 4.4). As mentioned in section 4.3.2 “QC Neg and QC Pos”, these SDs are not only component dependent, but also sample (and matrix) dependent. Laboratories that do not use real urine but spiked steroid stripped urine (or even water) for the calculation of this uncertainty component will most likely have lower SDs and a lower combined measurement uncertainty. Applying this methodology would in our case result in a maximum \( u_c \) of 0.84 \(^\circ\).
Table 4.5: Combined uncertainty calculation according to the square root of the sum of the squares rule

<table>
<thead>
<tr>
<th></th>
<th>Et</th>
<th>A</th>
<th>βαβ</th>
<th>ααβ</th>
<th>EpiT</th>
<th>T</th>
<th>11-oxoEt</th>
<th>PD</th>
</tr>
</thead>
<tbody>
<tr>
<td>u(calibration bias)</td>
<td>-0.15</td>
<td>-0.15</td>
<td>-0.15</td>
<td>-0.15</td>
<td>-0.15</td>
<td>-0.15</td>
<td>-0.15</td>
<td>-0.15</td>
</tr>
<tr>
<td>u(reference value)</td>
<td>0.14</td>
<td>0.14</td>
<td>0.14</td>
<td>0.14</td>
<td>0.14</td>
<td>0.14</td>
<td>0.14</td>
<td>0.14</td>
</tr>
<tr>
<td>u(method precision)</td>
<td>0.38</td>
<td>0.61</td>
<td>0.56</td>
<td>0.81</td>
<td>0.78</td>
<td>0.69</td>
<td>0.31</td>
<td>0.56</td>
</tr>
<tr>
<td>uc</td>
<td>0.43</td>
<td>0.64</td>
<td>0.60</td>
<td>0.84</td>
<td>0.81</td>
<td>0.72</td>
<td>0.37</td>
<td>0.60</td>
</tr>
</tbody>
</table>

In general, the $u_c$ will contain contributions from:

1) precision of measurements

2) bias of experimental processes

3) the uncertainty of CIR in reference materials used to fix and normalize the $\delta$-scale

4) the algorithms applied to correct and normalize the data. [23]

The issue is that this requires the separate uncertainty components to be independent. IRMS works with values that are normalized in relation to VDPB, meaning that the uncertainty in normalized results for samples ($\delta_{true \ (sample)}$) will have contributions from the precision of the measurements of the reference standards and sample, and the uncertainty in the reference value. This makes determination of the uncertainty of $\delta_{true \ (sample)}$ complicated because of correlation. Fortunately the correlation can be circumvented by calculating the uncertainty directly as described below. [23]
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The equation for calculating $\delta_{\text{true (sample)}}$ can be written as:

$$
\delta_{\text{true (sample)}} = \delta_{\text{true (RS1)}} + \left[ (\delta_{\text{raw (sample)}} - \delta_{\text{raw (RS1)}}) \times \left( \frac{\delta_{\text{true (RS1)}} - \delta_{\text{true (RS2)}}}{\delta_{\text{raw (RS1)}} - \delta_{\text{raw (RS2)}}} \right) \right]
$$

RS1 and RS2 are the reference standards that were used for normalization using their measured and true values. In our GC-C-IRMS method we have four RSs in CU-USADA 33-1, RS1 represents the steroid with the highest CIR and RS2 the steroid with the lowest CIR (or vice versa) so that the entire calibration range is covered. $\delta_{\text{true}}$ is the true CIR in relation to VDPB, $\delta_{\text{raw}}$ is the non-normalized value.

For this equation it is not possible to use the simple rules for combining uncertainties and the spreadsheet-based calculation as described by Kragten is the most straightforward approach. [31] The Kragten spreadsheet that applies for our IRMS method is presented in Table 4.6 and a clear explanation on how to set up these spreadsheets is given in the work of Carter and Barwick. [23]

In Kragten sheet A, RS1 and RS2 are respectively 11-oxoEt-Ac and A-Ac from CU-USADA 33-1. Their $\delta_{\text{true}}$ and its uncertainty can be found in Table 4.2, $\delta_{\text{raw}}$ and its uncertainty were obtained from the same measurements as in Table 4.3, but before normalization with the reference value. For “sample”, $\alpha\alpha\beta$-Ac$_2$ of the QC Neg measurements was chosen (Table 4.4) as this compound was characterized by the largest SD. The $\delta_{\text{raw}}$ was extracted from the data and the uncertainty calculated. The Kragten sheet calculates $\delta_{\text{true (sample)}}$ and its $u_c$. Keep in mind that $\delta_{\text{true (sample)}}$ is the value for $\alpha\alpha\beta$-Ac$_2$ whereas the value given in Table 4.4 is the corrected value for $\alpha\alpha\beta$. The original non-corrected value for $\alpha\alpha\beta$-Ac$_2$ was -28.88 ‰, meaning that there is a difference of 0.65 ‰ between the value calculated by the Kragten sheet and the value generated by the IRMS software. This is likely caused by the IRMS software that uses all four steroids in CU-USADA 33-1 and in addition we are uncertain on how the conversion algorithms functions exactly.
In Kragten sheet B, ααβ-Ac₂ has been replaced by 11-oxoEt-Ac from QC Neg (lowest SD). The original non-corrected value for 11-oxoEt-Ac was \(-25.75\) ‰, meaning that there is a difference of \(0.79\) ‰ between the value calculated by the Kragten sheet and the value generated by the IRMS software.

In Kragten sheet C and D, 11-oxoEt-Ac has been replaced by 5α-Chol as RS1. As mentioned in section 4.1 “Calibration” 11-oxoEt-Ac showed atypical behaviour and replacement by 5α-Chol should give a better representation of \(u_c\). 5α-Chol was chosen as it has the second highest CIR of CU-USADA 33-1. In sheet C, “sample” is again ααβ-Ac₂ and in sheet D 11-oxoEt-Ac. The difference between \(\delta_{\text{true (sample)}}\) calculated by the Kragten sheet and the value generated by the IRMS software now drops to \(0.29\) and \(0.17\) ‰ for ααβ-Ac₂ and 11-oxoEt-Ac respectively.

In all four sheets \(u_c\) remains beneath 1 ‰ (0.96, 0.72, 0.80 and 0.44 ‰ for A, B, C and D respectively). Sheet C however gives the most representative value as it uses ααβ-Ac₂ (largest SD) and excludes 11-oxoEt-Ac (atypical behaviour during calibration).
Table 4.6: Combined uncertainty calculation with a Kragten spreadsheet. A and B: RS1 = 11-oxoEt-Ac, RS2 = A-Ac; C and D: RS1 = 5α-Chol, RS2 = A-Ac. A and C: sample = ααβ-Ac2; B and D: sample = 11-oxoEt-Ac. The calculated uc is given in bold.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>value (δ13C, ‰)</th>
<th>uncertainty (δ13C, ‰)</th>
</tr>
</thead>
<tbody>
<tr>
<td>δtrue (RS1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>δtrue (RS1)</td>
<td>-16.70</td>
<td>-16.64</td>
</tr>
<tr>
<td>δtrue (RS2)</td>
<td>-33.04</td>
<td>-33.02</td>
</tr>
<tr>
<td>δraw (RS1)</td>
<td>13.62</td>
<td>13.62</td>
</tr>
<tr>
<td>δraw (RS2)</td>
<td>-0.76</td>
<td>-0.76</td>
</tr>
<tr>
<td>δraw (sample)</td>
<td>3.47</td>
<td>3.47</td>
</tr>
<tr>
<td>δtrue (sample)</td>
<td>-28.23</td>
<td>0.96</td>
</tr>
</tbody>
</table>

| Difference      | 0.02            | 0.01                  |

| B               |                 |                       |
| δtrue (RS1)     | -16.70          | -16.70                |
| δtrue (RS2)     | -33.04          | -33.04                |
| δraw (RS1)      | 13.62           | 13.62                 |
| δraw (RS2)      | -0.76           | -0.76                 |
| δraw (sample)   | 6.35            | 6.35                  |
| δtrue (sample)  | -24.96          | 0.72                  |

| Difference      | 0.03            | 0.01                  | -0.58 | -0.33 | 0.27 |
4.5 Routine doping samples

Table 4.7 gives an overview of the number of samples that were analyzed from February 2011 until May 2014. 97 out of 609 samples (15.9 %) were female. 8 out of 609 samples (1.3 %) were positive, all of these were males. From 2011 until 2013, 40 % of all IRMS investigations were due to an elevated T/EpiT ratio (>4). In 2014 almost 80 % of all IRMS requests were triggered by a T/EpiT ratio above 4. This increase was caused by the new 2014 WADA regulations that demanded that urine samples with a T/EpiT above 4 in the screening would be analyzed on IRMS (after approval of the corresponding sport federation). The old WADA rules required that
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An elevated T/EpiT (>4) was first confirmed with a second independent analysis before the corresponding sport federation received a request whether IRMS needed to be performed on the suspicious sample or not. [32] Taking the measurement uncertainty of the T/EpiT confirmation analysis in account, this meant that only T/EpiT ratios above 4.43 were labelled as suspicious.

Table 4.7: Overview of routine samples confirmed by GC-C-IRMS

<table>
<thead>
<tr>
<th></th>
<th>Number of samples</th>
<th>T/EpiT &gt; 4</th>
<th>Number of female samples</th>
<th>negative</th>
<th>Positive</th>
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</thead>
<tbody>
<tr>
<td>2011</td>
<td>209</td>
<td>63 (30.1 %)</td>
<td>25 (12.0 %)</td>
<td>206</td>
<td>3 (1.4 %)</td>
</tr>
<tr>
<td>2012</td>
<td>171</td>
<td>74 (43.3 %)</td>
<td>20 (11.7 %)</td>
<td>170</td>
<td>1 (0.6 %)</td>
</tr>
<tr>
<td>2013</td>
<td>157</td>
<td>86 (54.8 %)</td>
<td>26 (16.6 %)</td>
<td>154</td>
<td>3 (1.9 %)</td>
</tr>
<tr>
<td>2014 (until May)</td>
<td>72</td>
<td>56 (77.8 %)</td>
<td>26 (36.1 %)</td>
<td>71</td>
<td>1 (1.4 %)</td>
</tr>
<tr>
<td>total</td>
<td>609</td>
<td>279 (45.8 %)</td>
<td>97 (15.9 %)</td>
<td>601</td>
<td>8 (1.3 %)</td>
</tr>
</tbody>
</table>

The highest T/EpiT that resulted in a negative IRMS result was 18.5. For this sample, all Δδ^{13}C values were between -0.2 and 0.4 % and all δ^{13}C values were above -23.4 %, making it unlikely that this was a false negative as the most enriched synthetic preparation that has ever been reported had a CIR of -22.9 %. [4,5,33,34] All T/EpiT > 19 were due to anabolic steroid abuse (Table 4.8). One sample with a T/EpiT beneath 4 (T/EpiT = 2.4) led to an adverse analytical finding. All other adverse analytical findings had T/EpiT ratios between 10 and 83. From 2011 until 2013, 30 % (195 out of 609) of all IRMS requests were triggered by a T/EpiT between 4 and 6; in 2014 this number increased to 50 % (37 out of 72), but in four years time none of
these samples exhibited $\Delta \delta^{13}C$ values above the WADA IRMS threshold. These data support the proposition of Mareck et al. to raise the $T/EpiT$ threshold to 6 and only recommend an IRMS analysis for samples showing $T/EpiT > 6$. [19] This would reduce the time and money spent on negative IRMS confirmations considerably and increase the overall efficiency of the IRMS confirmation procedure.

Recently, WADA has implemented the steroidal module of the athlete biological passport (ABP). [35] The steroidal module monitors selected urinary steroid concentrations of an athlete over time in order to detect steroid doping. With each measurement, the ABP progressively adapts the calculated upper and lower reference limits, evolving from a population based to an individual based threshold. [36] As the number of test records increases, the calculated reference limits adapt from population thresholds (i.e. $T/EpiT > 4$) towards individual thresholds resulting in a more sensitive criterion. Over time, the use of the ABP will lead to a change in the types of samples submitted to IRMS analysis. More $T/EpiT < 4$ samples will undergo an IRMS analysis, while other ones with $T/EpiT > 4$ will no longer require testing. This fundamental change will be a big factor for IRMS analysis moving forward.

Table 4.8 gives an overview of all positive samples. These adverse analytical findings were conclusive and according to WADA guidelines. There were other samples that were suspicious and had one or multiple values close to the 3‰ threshold. Nonetheless, these cases did not fulfil the criteria and were reported as negative.
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Table 4.8: Steroid profile and CIR of the positive samples

<table>
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<th></th>
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<th></th>
<th></th>
<th></th>
<th align="left">11-oxoEt</th>
<th>PD</th>
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<tbody>
<tr>
<td></td>
<td align="left">(ng/mL)</td>
<td>Et</td>
<td>A</td>
<td>βαβ</td>
<td>ααβ</td>
<td>T/EpiT</td>
<td>Et</td>
<td>A</td>
<td>βαβ</td>
<td>ααβ</td>
<td>T</td>
<td>11-oxoEt</td>
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<td>526</td>
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<td>-29.3</td>
<td>-24.2</td>
<td>-30.4</td>
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<td>-25.0</td>
<td>-26.8</td>
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<td>-31.5</td>
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<td>-30.4</td>
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<td></td>
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</tr>
</tbody>
</table>

As pointed out in our previous publication, βαβ and ααβ are more sensitive parameters than A and Et. [20] This can be attributed to lower endogenous dilution, longer detection times and a higher biological sensitivity due to their position in the metabolic pathway. In all positive samples, at least one of the androstanediols had a higher Δδ^{13}C value than Et and A; and in all samples at least one of the androstanediols had a Δδ^{13}C value above 3 %. In sample number 4 for example, neither A or Et has a Δδ^{13}C value above 3 whereas ααβ does. This illustrates and confirms that measuring only A and Et and not the androstanediols during the IRMS confirmation deteriorates the efficiency of the analysis.

Table 4.9 gives an overview of the collected data for the negative urine samples. The data for T and EpiT have been omitted as these TCs were only measured in cases that resulted in suspicious IRMS results in the first place. They were eventually labelled as negative but there is a high chance for false negatives within
these samples. For the remaining TCs and ERCs it is obviously impossible to exclude the occurrence of any false negatives.

In a reference population with 56 subjects (36 males and 20 females) Flenker et al. pointed out that the difference between PD and A had an approximately symmetrical scatter around zero. In contrast, Et was on average $+/-\ 1\ %$ more depleted in relation to PD and A. [7] The same phenomenon is observed in our data. The effect was attributed to isotopic fractionation between the $5\alpha$- and $5\beta$-pathway, where the $5\beta$-compound becomes more depleted. Kinetic isotope effects originate from either rate limitation or branching (or a combination). Reduction of the A-ring is rate limiting and generates two branches: $5\alpha$- and $5\beta$-metabolites. [37] However, our data shows only marginal difference between the average CIR of $\alpha\alpha\beta$ and $\beta\alpha\beta\ (0.1\ %)$. The average CIR for Et, $\alpha\alpha\beta$ and $\beta\alpha\beta$ are very similar to each other. The average CIR for A and PD are very similar as well but $+/-\ 1\ %$ more enriched in relation to the androstanediols and Et. Piper et al. analyzed a population twice (one time with an old and one time with an optimized new HPLC-FC). [25] Interestingly, with the old HPLC-FC, a difference of $+/-\ 1\ %$ between $\alpha\alpha\beta$ and $\beta\alpha\beta$ was observed. With their optimized HPLC-FC, the average CIR for Et, $\alpha\alpha\beta$ and $\beta\alpha\beta$ were very similar to each other as is the case in our population and A and PD were also $+/-\ 1\ %$ more enriched, illustrating that there can be analytical causes for observed dissimilarities between laboratories.
Chapter 4: Three Years of IRMS in Routine Doping Control

Table 4.9: Overview of CIR obtained for the negative routine samples

<table>
<thead>
<tr>
<th></th>
<th>δ13C (%)</th>
<th>Δδ13C (%)</th>
</tr>
</thead>
<tbody>
<tr>
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<td>1.1</td>
</tr>
<tr>
<td>Max</td>
<td>-19.8</td>
<td>-19.0</td>
</tr>
<tr>
<td>Min</td>
<td>-27.1</td>
<td>-26.9</td>
</tr>
</tbody>
</table>

In a study of Cawley et al. with a reference population of 167 subjects, the average CIR of Et was 0.3‰ depleted in relation to A. [6] The average Δδ13C value for Et and A with 11-oxoEt was 2.3‰ and 2‰, whereas our population resulted in 0.2‰ and -0.3‰ respectively. The data of Cawley suggest that 11-oxoEt is enriched with +/- 2‰ in comparison with the TCs making it less convenient to use as ERC in combination with a WADA 3‰ threshold. In our population however 11-oxoEt is suitable as ERC as 11-oxoEt is on average 0.8‰ depleted in relation to PD.

At this stage it is unclear on how the parallels and differences between laboratories and populations need to be clarified in a consistent manner. The origin could be
biological and physiological; and related to the population. There could also be an analytical cause, resulting in (small) deviations between laboratories. In this context, analyzing an identical small population (10 – 20 samples) by different laboratories might bring clarification. In any case, it is clear that every doping laboratory needs to verify the reference population limits for its relevant TCs and ERCs with their own reference population. Table 4.10 gives an overview of the collected data for the negative reference population and these values (average and SD) are very close to the values gather with the negative routine samples.

**Table 4.10: Overview of CIR obtained for the negative reference population samples**

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<thead>
<tr>
<th></th>
<th>Et</th>
<th>A</th>
<th>βαβ</th>
<th>ααβ</th>
<th>11-oxoEt-PD</th>
<th>A-Et</th>
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<td>87</td>
<td>64</td>
<td>121</td>
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<td>-24.5</td>
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<td>-23.4</td>
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<td>0.1</td>
</tr>
<tr>
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<td>0.9</td>
<td>1.3</td>
<td>1.2</td>
<td>1.0</td>
<td>1.2</td>
<td>0.7</td>
<td>0.9</td>
</tr>
<tr>
<td>Max</td>
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<td>-21.3</td>
<td>-21.3</td>
<td>-21.6</td>
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<td>-20.8</td>
<td>2.9</td>
<td>1.9</td>
</tr>
<tr>
<td>Min</td>
<td>-27.1</td>
<td>-25.8</td>
<td>-27.7</td>
<td>-27.2</td>
<td>-25.7</td>
<td>-26.9</td>
<td>-1.8</td>
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<table>
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<th>PD-βαβ</th>
<th>PD-ααβ</th>
<th>11-oxoEt</th>
<th>11-oxoEt</th>
<th>11-oxoEt</th>
<th>11-oxoEt</th>
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</thead>
<tbody>
<tr>
<td>Count</td>
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<td>87</td>
<td>64</td>
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</tr>
<tr>
<td>Average</td>
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<td>0.0</td>
<td>1.0</td>
<td>0.9</td>
<td>0.4</td>
<td>-0.1</td>
<td>0.6</td>
<td>0.6</td>
</tr>
<tr>
<td>SD</td>
<td>0.8</td>
<td>1.0</td>
<td>0.7</td>
<td>0.8</td>
<td>0.9</td>
<td>0.9</td>
<td>1.0</td>
<td>1.1</td>
</tr>
</tbody>
</table>
5 Conclusions

Our GC-C-IRMS confirmation method was performed on 609 routine doping samples. The data was investigated, presented and interpreted, covering different areas relevant to doping control. Special emphasis was given to the calibration, quality control and measurement uncertainty, in light of the new 2014 WADA technical document.

The method revealed 8 cases of endogenous anabolic steroid abuse. The gathered data proves that using a T/EpiT ratio threshold of 6 instead of 4 would increase the efficiency of the IRMS confirmation procedure. The introduction of the steroidal module of the ABP however will most likely invoke a fundamental change in the types of samples that will require IRMS analysis in the future. In addition, we demonstrated the reliability and validity of the GC-C-IRMS confirmation protocol.
6 References


Chapter 4: Three Years of IRMS in Routine Doping Control


Chapter 4: Three Years of IRMS in Routine Doping Control


Part 2

Confirmation Methods for Norandrosterone, Boldenone and Formestane
Chapter 5: Confirmation Method for NA, F and 6αOH-ADION
1 Introduction

NA is the main metabolite of 19-nortestosterone, 19-norandrostenedione and 19-norandrostenediol and a minor metabolite of norethandrolone and ethylestrenol. [1–6] The use of these anabolic androgenic steroids is banned from sports and these compounds can be found on the WADA prohibited list. [7] Excretion of NA is also possible after administration of contraceptives containing norethisterone and lynestrenol. [8,9]

In addition, NA can also be produced endogenously in low amounts. [10–15] To disclose an endogenous from exogenous origin, a cut-off value of 2 ng/mL has been established by the WADA. [16] For definitive assignment of the NA origin, GC-C-IRMS needs to be performed for urinary NA concentrations between 2 and 10 ng/mL (or 15 ng/mL, depending on the situation, cf. Figure 5.1). [17–21] AAF and ATF are defined by WADA as adverse analytical finding and atypical finding, respectively.

F possesses only weak androgenic properties, but it is an irreversible aromatase inhibitor used in the treatment of breast cancer in postmenopausal women. [22–24] Because of this ability to suppress the estrogen production from anabolic steroids, F can be abused by athletes to withhold estrogen production as a side effect during the intake of anabolic steroids. Consequently, F is mentioned on the WADA prohibited list. [7]

ADION is an endogenous steroid that can be converted into testosterone in the human body, meaning that athletes can administer it to increase their testosterone levels and as a result enhance their physical performance. It has been shown that C4-hydroxylation is a major metabolic pathway following ADION administration, thereby resulting in urinary excretion of F. [25] Given the reversible nature of the ADION - T conversion, increased F concentrations are also found after the administration of T, making F a minor metabolite.
As studies have shown that F is produced endogenously in small amounts [25–27], a threshold for urinary excreted F of 100 ng/mL was introduced. [28] Recently, the threshold has been increased to 150 ng/mL. [29] Concentrations higher than 150 ng/mL are considered as AAF and lower concentrations could be due to endogenous production and need further investigation to prove the exact origin through determination of the carbon isotope ratio. However, because the current screening
methods are a lot more sensitive, F is detected in very small to small amounts in as good as every urine sample. In 2014 an additional threshold of 50 ng/mL was introduced. Concentrations beneath 50 ng/mL are considered as endogenous and no further investigation on GC-C-IRMS is required. [30,31]

The main aim of this work was to develop and validate a GC-C-IRMS method for NA and F. 6α-hydroxy-androstenedione (6αOH-ADION) has also been added to the method for future purposes.

2 Experimental

2.1 Reagents and chemicals

NaH₂PO₄·H₂O, Na₂HPO₄·2H₂O, K₂CO₃ and toluene were purchased from Merck (Darmstadt, Germany). Ethyl acetate, LCMS grade methanol and NaHCO₃ were obtained from Fisher Scientific (Leicestershire, UK) and methyl tert-butyl ether, n-hexane, LCMS grade water and acetonitrile from Biosolve (Valkenswaard, The Netherlands). β-Glucuronidase (E. coli) was purchased from Roche Diagnostics (Mannheim, Germany); helium, carbon dioxide and oxygen from Air Liquide (Bornem, Begium); acetic anhydride, pyridine, PD, F, A, PD-Ac₂, 11-oxoEt, 11-oxoEt-Ac and β-Tren from Sigma Aldrich (St. Louis, MO, USA); β-Tren-Ac, NA, 5α-ol-Ac and 6αOH-ADION from Steraloids (Newport, RI, USA). All steroid standards contained less than 1 % impurities. All standard solutions were prepared in methanol.

2.2 Steroid profiling with GC-MS/MS and pre-analytical steps

An aliquot of each urine sample was prepared to check the steroid profile according to routine sample preparation procedures. [32,33] The concentration of the TC allowed us to calculate the necessary sample volume using the following formula. \( V_{\text{IRMS}} \) is the required sample volume and \( C_x \) is the concentration of NA, F or 6αOH-ADION. For concentrations lower than 10 ng/mL, 25 mL of urine was used.
Chapter 5: Confirmation Method for NA, F and 6αOH-ADION

\[ V_{IRMS} = \frac{250 \, ng}{C_x} \]

A detailed description of the sample preparation procedure was published earlier. [34] In brief: urine was loaded on a conditioned C18 solid phase extraction cartridge, washed with 5 mL of H₂O and 5 mL of 10/90 CH₃OH/ H₂O; the compounds of interest were eluted with 5 mL CH₃OH; the dried residue was reconstituted in 1 mL of a 0.1 M pH 7 phosphate buffer and 50 µL of β-glucuronidase enzyme was added for the hydrolysis; afterwards 1 mL NaHCO₃/K₂CO₃ buffer (pH 9.5) was added. To increase the recovery 5 mL of methyl tert-butyl ether was added instead of n-pentane for the extraction; the organic phase was transferred to a new tube and evaporated. The residue was reconstituted in 50 µL of β-Tren-Ac (100 µg/mL) and 50 µL of 50/50 CH₃OH/H₂O and transferred to an LC vial. The retention time of β-Tren-Ac was used to verify that for every sample the correct fractions were collected.

2.3 High performance liquid chromatography fraction collection

To purify the sample, three semi-preparative HPLC fraction collections were performed on a Thermo Scientific Surveyor (Bremen, Germany) with an injection volume of 100 µL. The first separation was achieved using a Zorbax Extend-C18 (250 mm x 4.6 mm x 5 µm) column (Palo Alto, CA, USA) maintained at 35°C. The mobile phase flow rate was 1 mL/min using an CH₃CN/H₂O gradient (solvent A: H₂O; solvent B: CH₃CN) that started with 10 % of solvent B, then 100 % B at 24 min, held until 28 min, 10 % B at 29 min and held until 35 min. Photodiode array detection was performed at 195, 245 and 345 nm. β-Tren-Ac was used as retention time marker (19.4 min) to check the collection windows. Four fractions were automatically collected with a Gilson FC 204 (Gilson, Middleton, WI, USA); fraction A (FA: 10.9 – 12.2 min) contained 6αOH-ADION, fraction B (FB: 14.1 – 15.3 min) contained 11-oxoEt, fraction C (FC: 16.7 – 18.6 min) contained F and NA and fraction D (FD: 18.7 – 20.7 min) contained PD and A. The collection windows were set sufficiently wide to
collect the entire peak of the compounds of interest. Each batch of analyses started with a retention time check for β-Tren-Ac. Afterwards, 50 µL of β-Tren (100 µg/mL) was added to FA and FC. All fractions were dried under nitrogen at 60°C.

Acetylation took place by adding 50 µL of acetic anhydride and 50 µL of pyridine (60 min at 80°C). The acetylation reagents were evaporated under nitrogen at 60°C and the residue of FA and FC was reconstituted in 100 µL of 75/25 CH₃OH/H₂O.

A second HPLC fraction collection using a Phenomenex Gemini C6-Phenyl (150 mm x 4.6 mm x 5 µm) column (Torrance, California, USA) was conducted on the acetylated 6αOH-ADION (fraction FA). The mobile phase flow was 1 mL/min using an CH₃OH/H₂O gradient (solvent A: H₂O; solvent B: CH₃OH) that started with 10 % of solvent B, 50 % B at 1 min, then 75 % B at 19 min, 100 % B at 20 min and held until 24 min, 10 % B at 25 min and held until 30 min. Photodiode array detection was performed at 195, 245 and 345 nm. β-Tren-Ac was used as retention time marker (19.9 min) to check the collection windows. One fraction containing 6αOH-ADION-Ac (13.0 – 14.0 min) was automatically collected with a Gilson FC 204 (Gilson, Middleton, WI, USA). Each batch of analyses started with a retention time check of β-Tren-Ac.

A third HPLC fraction collection using a Zorbax Extend-C18 (250 mm x 4.6 mm x 5 µm) column (Palo Alto, CA, USA) was conducted on the acetylated NA and F (fraction FC). The mobile phase flow was 1 mL/min using an CH₃OH/H₂O gradient (solvent A: H₂O; solvent B: CH₃OH) that started with 10 % of solvent B, 50 % B at 1 min, then 100 % B at 20 min, held until 24 min, 10 % B at 25 min and held until 30 min. Photodiode array detection was performed at 195, 245 and 345 nm. β-Tren-Ac was used as retention time marker (19.9 min) to check the collection windows. Two fractions containing F-Ac (11.5 – 12.6 min) and NA-Ac (18.4 – 19.5 min) were automatically collected with a Gilson FC 204 (Gilson, Middleton, WI, USA). Each batch of analyses started with a retention time check for β-Tren-Ac.
Chapter 5: Confirmation Method for NA, F and 6αOH-ADION

All fractions were dried under nitrogen at 60°C. All residues were quantitatively transferred (2 x 150 µL ethyl acetate) to a GC-MS vial, dried under nitrogen at 40°C and reconstituted in an appropriate volume of internal standard solution 5α-ol-Ac (1.66 µg/mL, in 1/1 n-hexane/toluene) for the GC-MS and GC-C-IRMS analysis.

2.4 GC-MS

An Agilent 6890 gas chromatograph coupled to a 5975B VI MSD from Agilent Technologies (Palo Alto, CA, USA) was used. Helium was used as carrier gas. 13 µL solvent vent injections on a Gerstel PTV injector (Mülheim an der Ruhr, Germany) were used. The compounds were dissolved in a n-hexane-toluene (1:1) solution and an injection speed of 3 µL/s was used. PTV injector settings: 70°C for 0.35 min, then the temperature was increased at 720°C/min to 330°C and hold for 0.8 min, followed by a temperature gradient of 720°C/min to 400°C. The solvent vent lasted for 0.3 min (15 mL/min at 5 psi). At 1 min, a purge vent of 300 mL/min was applied. The GC column (30 m x 0.25 mm x 0.25 µm) was an Agilent J&W DB-17MS. The column temperature was held at 70°C for 1 minute, increased to 250°C with a rate of 30°C/min and then increased to 280°C with a rate of 2°C/min. The final temperature gradient consisted of a rate of 5°C/min to 310°C. The MSD acquired data in full scan mode from m/z 57 to 645 using Chemstation software (Agilent, Waldbronn, Germany).

Each fraction was analyzed by full scan GC-MS to determine the relative retention of the acetylated steroids versus IS 5α-ol-Ac according to WADA criteria. [35] The acetylated steroids were identified, the peak purity checked and the concentration was assessed using one-point calibration in order to estimate the ideal sample volume for GC-C-IRMS analysis.
Chapter 5: Confirmation Method for NA, F and 6αOH-ADION

2.5 GC-C-IRMS

An Agilent 7890A gas chromatograph (Palo Alto, CA, USA) was coupled to a Thermo GC-Isolink, a Thermo ConflolV interface and a Thermo Scientific MAT253 IRMS (Bremen, Germany) to determine the δ\textsuperscript{13}C value of the compounds of interest. The GC conditions were exactly the same as those described for full-scan GC-MS analysis. Data was acquired using ISODAT NT 3.0 software (Thermo Scientific, Bremen, Germany). The IRMS was calibrated as reported previously. [36]

2.6 Correction for acetate adducts

In the acetylation step, additional carbon atoms from the acetate moiety are incorporated into the steroidal skeletal. Therefore the measured δ\textsuperscript{13}C value has to be corrected using the following formula:

\[
\delta^{13}C_{\text{steroid}} = \frac{n_{sa}\delta^{13}C_{sa} - n_{a}\delta^{13}C_{\text{acorr}}}{n_{\text{steroid}}}
\]

Here, ‘n’ is the number of moles of carbon, ‘sa’ stands for steroid acetate and ‘a’ for the acetate derivative group. \(\delta^{13}C_{\text{acorr}}\) is indirectly calculated by measuring the \(\delta^{13}C\) value of the reference material of each relevant analyte in the free and derivatised fractions.

3 Results and discussion

3.1 Method validation: precision (repeatability and reproducibility)

The within-batch and between-batch precision of the extraction method for the determination of raw \(\delta^{13}C\) was tested by the analysis of steroid stripped urine spikes (with NaCl added) prepared at three concentration levels as specified in Table 5.1 and analysed by three different operators (A, B and C). The recoveries for NA, 6αOH-ADION and F in low batch 1 were too low because no NaCl was added.
Chapter 5: Confirmation Method for NA, F and 6αOH-ADION

The recovery of NA in low batch 2 of steroid stripped urines (with added NaCl) was still too low for δ^{13}C determination. However, in real urines samples (free of NA), spiked with NA at 2 ng/mL, the recovery was sufficient for δ^{13}C determination. The presence of a urinary matrix most likely enhances the NA recovery during SPE and/or LLE. These values were used for the NA low batch 2.

The raw δ^{13}C values for the 4 batches at each concentration level and operator are tabulated in Table 5.2. Each batch consisted of 6 urines.

Table 5.1: Concentration levels of the different validation batches

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<tr>
<th>operator</th>
<th>batch</th>
<th>NA</th>
<th>6αOH-ADION</th>
<th>F</th>
<th>A</th>
<th>11-oxoEt</th>
<th>PD</th>
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<td>2</td>
<td>2</td>
<td>1000</td>
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<td>100</td>
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<td>5</td>
<td>5</td>
<td>1000</td>
<td>500</td>
<td>100</td>
</tr>
<tr>
<td>B</td>
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<td>25</td>
<td>25</td>
<td>25</td>
<td>2500</td>
<td>1250</td>
<td>250</td>
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<tr>
<td>C</td>
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<td>250</td>
<td>250</td>
<td>250</td>
<td>10000</td>
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<td>1000</td>
</tr>
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</table>
Table 5.2: Repeatability and reproducibility of 6αOH-ADION-Ac, F-Ac, NA-Ac, A-Ac, 11-oxoEt-Ac and PD-Ac₂

<table>
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<tr>
<th></th>
<th>δ¹³C (%)</th>
<th>6αOH-ADION-Ac</th>
<th>F-Ac</th>
<th>NA-Ac</th>
<th>11-oxoEt-Ac</th>
<th>A-Ac</th>
<th>PD-Ac₂</th>
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</thead>
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<td><strong>Low 1</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>operator A</td>
<td>average</td>
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<td>/</td>
<td>/</td>
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<td>SD</td>
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<td>0.45</td>
<td>0.34</td>
<td>0.26</td>
<td>0.31</td>
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<tr>
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<td>0.33</td>
<td>0.36</td>
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<td>0.14</td>
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<tr>
<td><strong>High</strong></td>
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<td>0.17</td>
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<tr>
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<td>0.48</td>
<td>0.44</td>
<td>0.47</td>
<td>0.43</td>
<td>0.31</td>
<td>0.37</td>
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</table>
Chapter 5: Confirmation Method for NA, F and 6αOH-ADION

The within-batch precision (repeatability) ranged from 0.39 ‰ – 0.43 ‰, 0.17 ‰ – 0.51 ‰, 0.33 ‰ – 0.45 ‰, 0.24 ‰ – 0.48 ‰, 0.10 ‰ – 0.37 ‰ and 0.14 ‰ – 0.41 ‰ for 6αOH-ADION-Ac, F-Ac, NA-Ac, 11-oxoEt-Ac, A-Ac and PD-Ac2, respectively.

The between batch precision (reproducibility) was 0.48 ‰, 0.44 ‰, 0.47 ‰, 0.43 ‰, 0.31 ‰ and 0.37 ‰ for 6αOH-ADION-Ac, F-Ac, NA-Ac, 11-oxoEt-Ac, A-Ac and PD-Ac2, respectively.

To confirm the precision, 15 “dirty” urine samples (with high concentrations of endogenous steroids and other urinary compounds) free of NA (25 mL) were spiked with the same NA standard that was used for the steroid stripped urines (9 samples at 2 ng/mL, 6 samples at 10 ng/mL) and analysed according to the described procedure.

The NA-Ac raw δ13C values of the urine samples are tabulated in Table 5.3. The average raw δ13C value of the urine samples is compared with the average raw δ13C of the steroid free validation batches. The precision and δ13C value of the urine samples is comparable to that of the validation batches with steroid stripped urine. This confirms that the method can clean up the urinary matrix sufficiently, without significant loss of precision at the low concentrations.

3.2 Method validation: limit of detection

The δ13C value of 6αOH-ADION can be determined at a urinary concentration of 5 ng/mL starting from 25 mL of urine. The δ13C value of NA and F can be determined at a urinary concentration of 2 ng/mL starting from 25 mL of urine. At these concentrations the peak height is within the linear range of our IRMS (300 – 5000 mV) and the background is sufficiently low for accurate measurements.
Chapter 5: Confirmation Method for NA, F and 6αOH-ADION

Table 5.3: Precision of NA-Ac in real spiked urine samples

<table>
<thead>
<tr>
<th>NA-Ac δ¹³C (‰)</th>
<th>real urine spiked with 2 ng/mL NA</th>
<th>real urine spiked with 10 ng/mL NA</th>
<th>average</th>
<th>SD</th>
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<tr>
<td>-32.29</td>
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<td>-32.05</td>
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<td>-31.84</td>
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</tr>
<tr>
<td>-32.23</td>
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<td>-32.32</td>
<td></td>
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</tr>
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<td>-31.87</td>
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<td>-33.43</td>
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<tr>
<td>-32.75</td>
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<td>-32.69</td>
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<td></td>
</tr>
<tr>
<td>average</td>
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<td>0.49</td>
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<tr>
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<tr>
<td>total real urines</td>
<td>average</td>
<td>-32.37</td>
<td>0.54</td>
<td></td>
</tr>
<tr>
<td>total steroid stripped urines</td>
<td>average</td>
<td>-32.48</td>
<td>0.47</td>
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</tbody>
</table>

4 Conclusions

Due to the evolution and progress that has been made in the development of more sensitive screening methods over the last years, lower and lower concentrations of steroids such as NA and F can be detected. As a result, an increased number of suspicious urine samples is found and it is important to have a GC-C-IRMS method that can determine the endogenous or exogenous origin, even at these low concentrations. The developed method is able to determine the δ¹³C values of NA and F in urine samples and amounts as low as 2 ng/mL (using 25 mL of urine) could be determined with good repeatability and reproducibility.
5 References


Chapter 5: Confirmation Method for NA, F and 6αOH-ADION


Chapter 5: Confirmation Method for NA, F and 6αOH-ADION


Chapter 5: Confirmation Method for NA, F and 6αOH-ADION


Part 2: Confirmation Methods for NA, B and F

Chapter 6: Confirmation Method for Boldenone
1 Introduction

B is a steroid with androgenic activity that differs from T by only one double bond at the 1-position. B and B esters are for sale as anabolic preparations and are being misused in cattle breeding and equine doping. [1] B improves growth and feed conversion and can be abused to improve the muscle development. Because of these properties B is also prohibited in human sports. In humans, the metabolism of B has been elucidated earlier and despite the fact that B is not clinically approved for human application, there have been a relatively high number of adverse analytical and atypical findings for B and/or its main metabolite (5β-androst-1-en-17β-ol-3-one, BM). [2,3] Over the years several other steroids, including 1-androstenedione, 1-androstenediol, quinbolone and boldione, which are metabolized to B or its metabolites have been introduced into the market as regular pharmaceutical preparations or as ‘nutritional’ supplements. [4–6] In addition, it has been shown that the aromatase inhibitor androst-1,4,6-triene-3,17-dione is also metabolized to B. [7]

Screening for misuse of B or its precursors is in general performed by GC-MS or LC-MS. [8–10] However, due to its close structural relation to endogenous steroids and the high sensitivity of the analytical techniques used to screen for the presence of B and its metabolites in urine, the hypothesis of a natural occurrence of B in urine samples of athletes was established shortly after the first screening methods had been developed. [11] The possibility of endogenous B production in humans or other natural causes, including faecal bacterial activity, has been discussed for many years. [12] Formation of 1-dehydro steroids is a minor pathway in many species and is species dependent. Moreover, the biosynthetic route of formation of these compounds has not been fully determined. [13] Since B is on the list of prohibited substances of WADA this could result in false positive findings when B is detected at low concentrations. [14] The differentiation between an endogenous or synthetic origin of natural occurring steroids is a challenging issue for doping control laboratories. The use of a threshold concentration is a challenging task, since there
have been indications that up to 23 ng/mL of B or BM might be present naturally in urine. [12] By determining the \(^{13}\text{C}/^{12}\text{C}\) ratio of B and/or BM with the aid of GC-C-IRMS it is, similar as for testosterone, possible to differentiate between an exogenous or endogenous origin at low concentration levels. [12] To compensate for the inter-individual variation in \(^{13}\text{C}/^{12}\text{C}\) ratios of a particular steroid due to differences in diet, the \(\delta_{^{13}\text{C}}\) values of the TCs B and BM are compared to the \(\delta_{^{13}\text{C}}\) values of ERCs such as PD from which the \(\delta_{^{13}\text{C}}\) value does not change by administering B, B esters or a B precursor.

Unfortunately, GC-C-IRMS remains a laborious and expensive technique because the compounds need to be pure to determine the \(^{13}\text{C}/^{12}\text{C}\) ratio. IRMS methodologies imply an extensive sample clean-up process which is not susceptible to isotopic fractionation effects. Moreover, substantial amounts of urine are needed to meet the sensitivity requirements of the IRMS, which is far less sensitive than the LC-MS or GC-MS procedures used for screening purposes. This can be problematic because only a limited amount of urine is available for anti-doping analysis on a broad spectrum of substances and it is especially the case for compounds like B and BM because they can be present in very low concentrations. Based upon our previous work, it is now possible to reach those very low concentrations with the aid of solvent vent injections. [15] This is done with a reasonable amount of urine and without having to change anything about the IRMS detection parameters.

### 2 Material and methods

#### 2.1 Reagents and chemicals

\(\text{NaH}_{2}\text{PO}_{4} \cdot \text{H}_{2}\text{O}, \text{Na}_{2}\text{HPO}_{4} \cdot 2\text{H}_{2}\text{O}, \text{K}_{2}\text{CO}_{3}, \text{toluene and 2,2,4-trimethylpentane were}
\) purchased from Merck (Darmstadt, Germany). Ethyl acetate, cyclohexane, LC-MS grade methanol and \(\text{NaHCO}_{3}\) were obtained from Fisher Scientific (Leicestershire,
Chapter 6: Confirmation Method for Boldenone

UK) and n-pentane, n-hexane, LC-MS grade acetonitrile and LC-MS grade water from Biosolve (Valkenswaard, The Netherlands). β-Glucuronidase (E. coli) was purchased from Roche Diagnostics (Mannheim, Germany); helium, carbon dioxide and oxygen from Air Liquide (Bornem, Belgium); acetic anhydride, pyridine, 5β-pregnan-3α,20α-diol (PD), 5β-pregnan-3α,20α-diol diacetate (PD-Ac₂), 17β-trenbolone (β-Tren), 5-androstene-3β,17α-diol (5-en) and boldenone from Sigma Aldrich (St. Louis, MO, USA); 17β-Trenbolone acetate (β-Tren-Ac), 5α-androst-an-3β-ol (5α-ol), 5α-androst-an-3β-ol acetate (5α-ol-Ac) and boldenone acetate (B-Ac) from Steraloids (Newport, RI, USA). BM (5β-androst-1-en-17β-ol-3-one) was obtained from the Laboratory for Doping Analysis German Sports University Cologne. All steroid standards contained less than 1% impurities. All standard solutions were made in methanol, except 5α-ol-Ac which was dissolved in 2,2,4-trimethylpentane and stored at 4°C.

2.2 Steroid profiling and urine sampling

An aliquot of each urine sample was prepared to check the steroid profile according to routine sample preparation procedures. [9,10] The concentrations of the target compounds allowed us to calculate the necessary sample volume (with a maximum of 25 mL) using the following formula. $V_{IRMS}$ is the required sample volume and $C_x$ is the concentration (ng/mL) of B or BM.

$$V_{IRMS} = \frac{250 \text{ ng}}{C_x}$$

2.3 Pre-analytical steps

A system blank and a negative and positive quality control were analyzed concurrently with each batch of urine samples. All the samples were centrifuged. Before the urine was loaded on SPE cartridge (Bond Elut C18, 500 mg, 3 mL, Agilent technologies, Palo Alto, CA, USA), two conditioning steps (5 mL CH₃OH, 5 mL H₂O) were carried out. Two washing steps were conducted (5 mL H₂O, 5 mL 10:90
CH$_3$OH-H$_2$O) and the compounds of interest were eluted (5 mL CH$_3$OH). The methanolic extract was evaporated to dryness under nitrogen at 60°C and reconstituted in 0.1 M phosphate buffer (pH 7; 1 mL). 50 µL of β-glucuronidase was added and hydrolysis took place in an oven at 56°C for 60 min. The sample was cooled to room temperature before NaHCO$_3$/K$_2$CO$_3$ buffer (pH 9.5; 1 mL) and 5 mL n-pentane were added. LLE was performed for 20 min, followed by centrifugation. Afterwards the tubes were stored at -80°C for 15 min, the organic phase was transferred to a new tube and the organic phase was evaporated under nitrogen at 40°C. The residue was reconstituted in 50 µL of β-Tren-Ac (100 µg/mL) and 50 µL of 50:50 CH$_3$OH-H$_2$O and transferred to an LC vial. The retention time of β-Tren-Ac was used to verify that for every sample the correct fractions were collected.

2.4 High performance liquid chromatography fraction collection

To purify the sample, a semi-preparative HPLC fraction collection was performed on a Thermo Scientific Surveyor (Bremen, Germany) with an injection volume of 100 µL. Separation was achieved using a Phenomenex Gemini C18 (150 mm x 4.6 mm i.d.; 5 µm) column (Torrance, CA, USA) maintained at 35°C.

The mobile phase flow rate was 1 mL/min using a methanol-water gradient (solvent A = 10:90 CH$_3$OH-H$_2$O; solvent B = CH$_3$OH) that started with 100 % of solvent A, then 45 % B at 1 min, 100 % B at 19 min, held until 24 min, 100 % A at 25 min and held until 30 min. Photodiode array detection was performed at 195, 245 and 345 nm.

Each batch analysis started with a retention time check for β-trenbolone acetate. It was used as retention time marker (13.6 min) to check the collection windows. Three fractions were automatically collected with a Gilson FC 204 (Gilson, Middleton, WI, USA); FB (9.6 – 10.7 min) contained B, FBM (12.1 – 13.1 min) contained BM and FPD (16.2 – 17.1 min) contained PD. The collection windows were set sufficiently wide to collect the entire peak of the compounds of interest. 50 µL of β-Tren (100 µg/mL) was added to all fractions. In order to monitor any occurring fractionation caused by the acetylation step, it was necessary to add a
control standard before the acetylation and to determine the $\delta^{13}$C value afterwards. To fractions FB and FBM 25 µL of 5α-ol (100 µg/mL) and to fraction FPD 25 µL of 5-en (100 µg/mL) were added. All fractions were dried under nitrogen at 60°C.

Acetylation took place by adding 50 µL of acetic anhydride and 50 µL of pyridine (60 min at 80°C). The acetylation reagents were evaporated under nitrogen at 60°C and the residue was reconstituted in 100 µL of 75:25 CH$_3$OH-H$_2$O.

A second HPLC fraction collection was conducted on the acetylated compounds. The mobile phase flow rate was 1 mL/min using an acetonitrile-water gradient (solvent C = 10:90 CH$_3$CN-H$_2$O; solvent D = CH$_3$CN) that started with 100% of solvent C, then 55% D at 1 min, 100% D at 18 min, held until 24 min, 100% C at 25 min and held until 30 min. Photodiode array detection was performed at 195, 245 and 345 nm.

β-Trenbolone acetate was used as retention time marker (7.5 min) to check the collection windows. Five fractions were automatically collected with a Gilson FC 204 (Gilson, Middleton, WI, USA); F1 (7.5 – 8.5 min) contained B-Ac, F2 (9.8 – 10.7 min) contained BM-Ac, F3 (17.1 – 19.0 min) contained PD-Ac$_2$, F4 (14.0 – 15.2 min) contained 5-en-Ac$_2$ and F5 (20.7 – 22.7 min) contained 5α-ol-Ac. The collection windows were set sufficiently wide to collect the entire peak of the compounds of interest. Each batch analysis started with a retention time check of β-Tren-Ac. All fractions were dried under nitrogen at 60°C. All residues were quantitatively transferred (2 x 150 µL ethyl acetate) to a GC-MS vial, dried under nitrogen at 40°C and reconstituted in an appropriate volume of internal standard solution 5α-ol-Ac (1.66 µg/mL, in n-hexane-toluene (1:1)) for the GC-MS and GC-C-IRMS analysis.

2.5 GC-MS

To ensure the absence of any co-eluting and disturbing matrix compounds, all fractions were scanned by GC-MS. The GC-MS chromatographic conditions were equivalent to those of the GC-C-IRMS. An Agilent 6890 GC coupled to a 5973B VI
MSD from Agilent Technologies (Palo Alto, CA, USA) was used. Helium was used as carrier gas. Solvent vent injections (13 μL) on a Gerstel PTV-injector (Mulheim an der Ruhr, Germany) were used. Here, the compounds were dissolved in an n-hexane/toluene (1:1) solution and an injection speed of 3 μL/s was used. PTV-injector settings: 70°C for 0.35 min, then the temperature was increased at 720°C/min to 330°C and hold for 0.8 min, followed by a temperature gradient of 720°C/min to 400°C. The solvent vent lasted for 0.3 min (15 mL/min at 5 psi). At 1 min, a purge vent of 300 mL/min was applied. The GC column (30 m x 0.25 mm i.d.; 0.25 μm) was an Agilent J&W DB-17ms. The column temperature was held at 70°C for 1 min, increased to 250°C with a rate of 30°C/min and then increased to 280°C with a rate of 2°C/min. The final temperature gradient consisted of a rate of 5°C/min to 300°C. The MSD acquired data in full scan mode from m/z 57 to 645 using Chemstation software (Agilent, Waldbronn, Germany).

Each fraction was analyzed by full scan GC-MS to determine the relative retention of the acetylated steroids versus 5α-ol-Ac IS according to WADA criteria. [16] The acetylated steroids were identified using electron ionization spectral comparison with standards, the peak purity checked and the concentration was assessed using one-point calibration in order to estimate the ideal sample volume for GC-C-IRMS analysis.

2.6 GC-C-IRMS

An Agilent 7890A GC was coupled to a Thermo GC-Isolink, a Thermo ConfloIV interface and a Thermo Scientific MAT253 IRMS (Bremen, Germany) to determine the $\delta^{13}$C value of the compounds of interest. The GC conditions were exactly the same as those described for full-scan GC-MS analysis. Data was acquired using ISODAT NT 3.0 software (Thermo Scientific, Bremen, Germany). The IRMS was calibrated as reported previously. [17]
2.7 Quality control for GC-C-IRMS

The reproducibility and stability of the GC-C-IRMS measurements were monitored by the determination of the $\delta^{13}$C value of 5α-ol-Ac in every sample. This value was compared with the mean $\delta^{13}$C value of 5α-ol-Ac (-32.37 ± 0.37 ‰), determined by analyzing more than 700 5α-ol-Ac standards over a time period of one year. A water blank sample was extracted and analyzed with every batch of urine samples. This did not contain any peaks other than the internal standards, demonstrating that there was no contamination. A positive and negative quality control urine were aliquoted and analyzed with every batch of urine samples as well.

2.8 Correction for acetate adducts

In the acetylation step, additional carbon atoms from the acetate moiety are incorporated into the steroidal skeletal. Therefore the measured $\delta^{13}$C has to be corrected using the following formula:

$$\delta^{13}C_{\text{steroid}} = \frac{(n_{sa} \delta^{13}C_{sa}) - (n_{a} \delta^{13}C_{\text{acorr}})}{n_{\text{steroid}}}$$

Here, ‘n’ is the number of moles of carbon, ‘sa’ stands for steroid acetate and ‘a’ for the acetate derivative group. $\delta^{13}C_{\text{acorr}}$ is indirectly calculated by measuring the $\delta^{13}$C of the reference material of each relevant analyte in the free and derivatized fractions.
3 Results and discussion

3.1 Method validation

Specificity

No interferences were detected in the system blank samples that were analyzed with all batches. A blank urine sample spiked with all available reference standards of anabolic steroids, included in the general screening methods, did not lead to interferences neither.

As all urine samples were subjected to multiple clean-up steps (SPE, LLE, HPLC) and were always screened by full scan GC-MS prior to GC-C-IRMS analysis, the peak purity and identity could be ensured for both the target compounds and the ERC.

For all compounds, identification by direct comparison of mass spectral data and retention times with steroid standards according to WADA’s criteria on chromatography and mass spectrometry warranted correct detection. [18]

Moreover, as shown in Figures 6.1, 6.3 and 6.4, the noise in the regions where B-Ac and BM-Ac elute is extremely low, facilitating correct and easy integration of the peaks belonging to the target compounds, even in ‘dirty’ urine samples (meaning with high concentrations of endogenous steroids and other urinary compounds and a high density), which warrants a correct and easy processing of data.

Precision (repeatability and reproducibility)

The within-batch and between-batch precision of the extraction method for the determination of raw $\delta^{13}$C values was tested by the analysis of steroid stripped urine spikes ($n = 6$) prepared at three concentration levels as specified in Table 6.1. For each concentration level of sample, the volume of urine required for analysis was adjusted appropriately. Therefore 2.5 mL, 10 mL and 25 mL of steroid stripped urine that was spiked at a high, medium and low concentration respectively were extracted. The within-batch precision (repeatability) ranged from 0.38‰ – 0.51‰,
Chapter 6: Confirmation Method for Boldenone

0.32 ‰ – 0.45 ‰, and 0.25 ‰ – 0.42 ‰ for B-Ac, BM-Ac and PD-Ac₂ respectively. The between batch precision (reproducibility) was 0.45 ‰, 0.38 ‰ and 0.50 ‰ for B-Ac, BM-Ac and PD-Ac₂ respectively. Additionally there seems to be no variation depending on the concentration in repeatability. To proof that the method also works for ‘dirty’ urine samples with low concentrations of B and its metabolite, 6 very ‘dirty’ urine samples (25 mL) were spiked with 10 ng/mL of B and BM and analyzed according to the procedure explained above (Figure 6.1). The same experiment was repeated by spiking ‘dirty’ urine at the 3 ng/mL level (Table 6.1). Despite the fact that samples with higher backgrounds are prone to more variation, the δ¹³C values and precision were comparable to that of the other batches. This confirms that the method can clean up the urinary matrix sufficiently and it shows that a very good linearity in the low signal range was obtained.

Table 6.1: Repeatability and reproducibility of the target compounds

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<th>B-Ac</th>
<th>BM-Ac</th>
<th>PD-Ac₂</th>
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<td><strong>Low</strong> (10 ng/mL)</td>
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</tr>
<tr>
<td>Average</td>
<td>-29.98</td>
<td>-30.56</td>
<td>-35.67</td>
</tr>
<tr>
<td>SD</td>
<td>0.38</td>
<td>0.40</td>
<td>0.38</td>
</tr>
<tr>
<td><strong>Medium</strong> (50 ng/mL)</td>
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</tr>
<tr>
<td>Average</td>
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<td>-30.79</td>
<td>-35.08</td>
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<tr>
<td>SD</td>
<td>0.51</td>
<td>0.45</td>
<td>0.25</td>
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<tr>
<td><strong>High</strong> (100 ng/mL)</td>
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<td></td>
</tr>
<tr>
<td>Average</td>
<td>-29.47</td>
<td>-30.60</td>
<td>-34.60</td>
</tr>
<tr>
<td>SD</td>
<td>0.39</td>
<td>0.32</td>
<td>0.42</td>
</tr>
<tr>
<td><strong>Overall</strong></td>
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<tr>
<td>Average</td>
<td><strong>-29.65</strong></td>
<td><strong>-30.64</strong></td>
<td><strong>-35.23</strong></td>
</tr>
<tr>
<td>SD</td>
<td><strong>0.45</strong></td>
<td><strong>0.38</strong></td>
<td><strong>0.50</strong></td>
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<table>
<thead>
<tr>
<th></th>
<th>B-Ac</th>
<th>BM-Ac</th>
<th>PD-Ac₂</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dirty urines</strong> (10 ng/mL)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Average</td>
<td>-30.06</td>
<td>-30.25</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>0.37</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td><strong>Dirty urines</strong> (3 ng/mL)</td>
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<td></td>
</tr>
<tr>
<td>Average</td>
<td>-30.19</td>
<td>-30.87</td>
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</tr>
<tr>
<td>SD</td>
<td>0.59</td>
<td>0.52</td>
<td></td>
</tr>
</tbody>
</table>
Figure 6.1: GC-C-IRMS analysis of a spiked urine sample

Comparison of the $\delta^{13}$C values of the standards with the corrected $\delta^{13}$C values obtained from the spiked steroid stripped urines showed that no isotopic fractionation is taking place during the sample preparation procedure (Table 6.2). The difference between the overall mean adjusted $\delta^{13}$C (for the 6 aliquots at each concentration level and operator) and the underivatized standards (that were used to spike the steroid free urines) was -0.20 ‰ and -0.26 ‰ for B and BM respectively. These findings indicate that the sample preparation procedure (including HPLC fraction collecting) did not induce a significant change in the adjusted $\delta^{13}$C of the measured analytes.
Chapter 6: Confirmation Method for Boldenone

Table 6.2: Repeated analysis of the spiked steroid stripped urines and the standards (n=6)

<table>
<thead>
<tr>
<th></th>
<th>(\delta^{13})C (‰)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>B</td>
</tr>
<tr>
<td>Spiked urines</td>
<td>-27.2</td>
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<tr>
<td>Average</td>
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<tr>
<td>SD</td>
<td>0.5</td>
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<tr>
<td>standards</td>
<td>-27.0</td>
</tr>
<tr>
<td>Average</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>0.23</td>
</tr>
</tbody>
</table>

**Linearity**

The linear range of the instrument was assessed by the analysis of 26 aliquots of B-Ac and BM-Ac over a concentration range of approximately 0.5 – 20 µg/mL (in vial), equivalent to approximately 5 – 200 ng of steroid on column. A summary of the results is tabulated below and Figure 6.2 shows the regression analysis plot for B-Ac. Within this range the slope of the regression line is acceptable (0.11 and 0.28 ‰/V), indicating that at least 0.3 V – 3 V is a suitable interval for appropriate \(\delta^{13}\)C determination for the target compounds. BM-Ac was not available as a standard and was made by an in house acetylation of the BM standard. The average and standard deviation (SD) of BM-Ac were practically the same as those measured during the precision experiments. This shows that there is no isotopic fractionation and that there is no additional variation taking place during the sample preparation.

Table 6.3: Linearity of the target compounds

<table>
<thead>
<tr>
<th></th>
<th>B-Ac</th>
<th>BM-Ac</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average (%)</td>
<td>-31.25</td>
<td>-30.64</td>
</tr>
<tr>
<td>SD (%)</td>
<td>0.24</td>
<td>0.39</td>
</tr>
<tr>
<td>Slope (‰/V)</td>
<td>0.11</td>
<td>0.28</td>
</tr>
<tr>
<td>Min (V)</td>
<td>0.294</td>
<td>0.212</td>
</tr>
<tr>
<td>Max (V)</td>
<td>5.905</td>
<td>3.070</td>
</tr>
</tbody>
</table>
**Chapter 6: Confirmation Method for Boldenone**

**B-Ac**

$$y = 0.1106x - 31.48$$

![Graph](graph.png)

**Figure 6.2: linearity plot of B-Ac**

**Limit of detection**

With the aid of a PTV a sensitive GC-C-IRMS analysis was successfully developed which reduces the required amount of urine and the limit of detection. This made it possible to develop a method that is sensitive enough to determine the $\delta^{13}$C value of B and its metabolite, even for those samples with very small amounts of analyte. The limit of detection for B and its metabolite is below 2 – 3 ng/mL by using 25 mL of urine. To reach even lower concentrations, higher amounts of urine can be used. Figure 6.3 shows a representative GC-C-IRMS analysis of a urine sample (25 mL) that is spiked with boldenone glucuronide at a concentration of exactly 3 ng/mL. The signal to noise ratio is excellent and the signal intensity is 405 mV, which is within the established linear range of our IRMS (300 – 3000 mV).
Chapter 6: Confirmation Method for Boldenone

**Figure 6.3:** GC-C-IRMS analysis of 25 mL of urine spiked with boldenone-glucuronide (3 ng/mL)

### 3.2 Analysis of suspicious urine samples

To confirm the validity of our method, a number of urine samples containing B and/or its metabolite were analysed. The results are summarized in Table 6.4. $\delta^{13}$C values of B and its metabolite were considered exogenous if the $\Delta \delta^{13}$C values exceeded the 3 % threshold established by the WADA. [19] It has been reported earlier that a concentration threshold is not a helpful tool for a definitive discrimination between an endogenous and an exogenous origin. [12] This is further confirmed by our findings.

Sample 5 was a urine sample that was collected after the administration of androst-1,4,6-trien-3,17-dione. Both the parent steroid, as well as its metabolites androst-
Chapter 6: Confirmation Method for Boldenone

1,4,6-triene-17β-ol-3-one, B and BM were detected. [7] In the study that elucidated androst-1,4,6-triene-3,17-dione metabolism, the described IRMS-methodology could not successfully clean up the sample and separate B (or its metabolite) from interferences. Using the described methodology here, the $\delta^{13}$C value of BM could be successfully and reliably determined and indicated an exogenous origin. Sample 1 and 2 have high $\delta^{13}$C values for PD and sample 3 has rather low $\delta^{13}$C values for B and BM. This indicates that it is possible that the administration of preparations with low $\delta^{13}$C values to an individual with a high natural $\delta^{13}$C value for the ERC could result in false negatives. Figure 6.4 shows the GC-C-IRMS analysis of sample 6. Sample 7 is an example of an athlete with endogenous values for B and BM.

Table 6.4: Investigated doping samples containing B and/or BM

<table>
<thead>
<tr>
<th>Sample</th>
<th>B (ng/mL)</th>
<th>BM (ng/mL)</th>
<th>$\delta^{13}$C (%o) B</th>
<th>$\delta^{13}$C (%o) BM</th>
<th>$\delta^{13}$C (%o) PD</th>
<th>$\Delta\delta^{13}$C (%o) PD-B</th>
<th>$\Delta\delta^{13}$C (%o) PD-BM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>10</td>
<td>20</td>
<td>-29.05</td>
<td>-29.38</td>
<td>-25.87</td>
<td>3.18</td>
<td>3.51</td>
</tr>
<tr>
<td>Sample 2</td>
<td>20</td>
<td>30.58</td>
<td>-30.58</td>
<td>-26.36</td>
<td>4.22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample 3</td>
<td>351</td>
<td>53</td>
<td>-25.63</td>
<td>-27.52</td>
<td>-22.37</td>
<td>3.26</td>
<td>5.15</td>
</tr>
<tr>
<td>Sample 4</td>
<td>1430</td>
<td>63</td>
<td>-34.67</td>
<td>-33.08</td>
<td>-21.97</td>
<td>12.7</td>
<td>11.11</td>
</tr>
<tr>
<td>Sample 5</td>
<td>18</td>
<td>25</td>
<td>-33.12</td>
<td>-33.41</td>
<td>-18.75</td>
<td>14.37</td>
<td>14.66</td>
</tr>
<tr>
<td>Sample 6</td>
<td>5</td>
<td>5</td>
<td>-29.54</td>
<td>-28.95</td>
<td>-25.32</td>
<td>4.22</td>
<td>3.63</td>
</tr>
<tr>
<td>Sample 7</td>
<td>8</td>
<td>8</td>
<td>-24.37</td>
<td>-22.91</td>
<td>-23.13</td>
<td>1.24</td>
<td>-0.22</td>
</tr>
</tbody>
</table>
4 Conclusions

As the sensitivity of screening methods in doping control has increased drastically over the years, lower and lower concentrations of steroids such as boldenone are being detected. This increases the number of suspicious urine samples and the chance of detecting steroid abuse, but also implies that a higher amount of endogenous B and BM samples will be found. Obviously, it is crucial to have a GC-C-IRMS method that can differentiate between an endogenous and an exogenous origin, especially at those low levels. Our method is able to determine the $\delta^{13}$C values of B and BM in urine samples, even at low concentrations. The validity of our method was carefully examined and concentrations of B and BM as low as 2 ng/mL (using 25 mL of urine) could be determined with good repeatability and
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reproducibility. Lower concentrations can be reached by using more urine. No evidence of any fractionation could be found.

The amount of received urine from an athlete for doping tests is limited, which means doping control laboratories have to make their analysis as sensitive as possible in order to perform the required doping tests. If less urine is consumed, then there is more available for additional tests. Moreover, because solvent vent injections are used, all the IRMS parameters remain the same for all our compounds (T, epiT, A, Et, βαβ, ααβ, B, BM and also F and NA) by which spending time and effort in resetting and optimizing the IRMS every time different compounds are analyzed is avoided.
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5 References


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Part 3

Steroid Profile

Thresholds for Minor Metabolites
Chapter 7: Steroid Profile Threshold for Formestane

Adapted from

M. Polet, P. Van Renterghem, W. Van Gansbeke, P. Van Eenoo

Profiling of urinary formestane and confirmation by isotope ratio mass spectrometry

Chapter 7: Steroid Profile Threshold for Formestane

1 Abstract

F is an irreversible aromatase inhibitor with the ability to suppress the estrogen production from anabolic steroids. Consequently, F is mentioned on the WADA prohibited list and because studies have shown that F is produced endogenously in small amounts, a threshold for urinary excreted F of 150 ng/mL was introduced. Lower concentrations could be due to endogenous production and need further investigation to prove the exact origin through determination of the carbon isotope ratio.

However, because the current screening methods are a lot more sensitive, F is detected in practically every urine sample. A strict implementation of this WADA rule would imply that almost every urine sample needs additional investigation to verify an exogenous or endogenous origin. The main aim of this study was to propose and introduce a lower concentration limit of 25 ng/mL beneath which the detected F is considered as being endogenous and no further investigation is needed. The data presented in this paper suggests that this threshold provides a good balance between a sufficiently large detection window without having to perform IRMS analyses on negative urine samples.

2 Introduction

F possesses only weak androgenic properties, but it is an irreversible aromatase inhibitor used in the treatment of breast cancer in postmenopausal women. [1–3] Because of this ability to suppress the estrogen production from anabolic steroids, F can be abused by athletes to withhold estrogen production as a side effect during the intake of anabolic steroids. Consequently, F is mentioned on the WADA prohibited list. [4]
ADION is an endogenous steroid that can be converted into testosterone in the human body, meaning that athletes can administer it to increase their testosterone levels and as a result enhance their physical performance. It has been shown that \( \text{C}_4 \)-hydroxylation is a major metabolic pathway following ADION administration, thereby resulting in urinary excretion of F. [5] Given the reversible nature of the ADION - T conversion, increased F concentrations are also found after the administration of T. This makes F a suitable marker for ADION administration during GC-MS screening.

In this context, it is important to be able to distinguish between a F administration and F as a metabolite originating from the intake of T or a T prohormone, because the consequences for an athlete are different for aromatase inhibitor misuse than for the administration of anabolic steroids as both belong to a different class. [4]

As studies have shown that F is produced endogenously in small amounts [5–7], a threshold for urinary excreted F of 100 ng/mL was introduced. [8] Recently, the threshold has been increased to 150 ng/mL. [9] Concentrations higher than 150 ng/mL are considered as adverse analytical findings and lower concentrations could be due to endogenous production and need further investigation to prove the exact origin through determination of CIR. However, because the current screening methods are a lot more sensitive, F is detected in very small to small amounts in practically every urine sample. A strict implementation of this WADA rule would imply that almost every urine sample needs additional investigation to verify an exogenous or endogenous origin. The main aim of this study was to propose a lower limit beneath which the detected F is considered as being endogenous and no further investigation is needed.

By means of GC-C-IRMS it is possible to ascertain the CIR of steroids in urine samples [10–14] and based on previous developments it is possible to do so for very low urinary concentrations. [15–17] By comparison of the \(^{13}\text{C}/^{12}\text{C}\) ratios of ERCs like PD with that of the TC it is possible to differentiate between an exogenous or
endogenous origin. To compensate for the inter-individual variation in $^{13}\text{C}/^{12}\text{C}$ ratios of a particular steroid due to differences in diet, the administration of an endogenous steroid is demonstrated when a difference of 3 ‰ or more between the $^{13}\text{C}/^{12}\text{C}$ ratio of the ERC and the target compound is determined. [18] As pointed out earlier, it is possible that this threshold does not apply for some pairs of ERC and TC. [14,19–21] A second aim of this paper was to prove that this threshold is correct for F and PD in our lab and for our population as well, in agreement with the previous study. [6] The third aim was to develop a simple and sensitive method to measure these $^{13}\text{C}$ values, but without loss of precision at low concentrations and to be able to distinguish between F administration and F as a metabolite from the misuse of anabolic steroids through the combination of steroid profiling (with our proposed lower concentration limit) and GC-C-IRMS.

3 Experimental

3.1 Reagents and chemicals

Na$_2$HPO$_4$·H$_2$O, Na$_3$HPO$_4$·2H$_2$O, K$_2$CO$_3$, toluene and 2,2,4-trimethylpentane were purchased from Merck (Darmstadt, Germany). Ethyl acetate, LC-MS grade methanol and NaHCO$_3$ were obtained from Fisher Scientific (Leicestershire, UK) and n-pentane, n-hexane and LC-MS grade water from Biosolve (Valkenswaard, The Netherlands). β-Glucuronidase (E. coli) was purchased from Roche Diagnostics (Mannheim, Germany); helium, carbon dioxide and oxygen from Air Liquide (Bornem, Belgium); acetic anhydride, pyridine, PD, PD-Ac$_2$, β-Tren, 5-androstene-3β,17α-diol (5-en) and F from Sigma Aldrich (St. Louis, MO, USA); β-Tren-Ac, EpiT-Ac and 5α-ol-Ac from Steraloids (Newport, RI, USA). All steroid standards contained less than 1 % impurities. All standard solutions were made in methanol.
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3.2 Reference populations and excretion studies

50 routine doping control samples (36 males and 14 females, F concentration range 2.48 – 44 ng/mL) that were analyzed at the Doping Control Laboratory (DoCoLab) and not found suspicious for the intake of any doping substance, were further investigated regarding the CIR of F and PD. 47 urine samples were chosen randomly, 3 additional samples (2 males and 1 female) were selected because of their relatively high F concentration (25, 27 and 44 ng/mL). The collected data was used to confirm the applicability of the 3 % threshold from WADA.

A second reference population (n = 3031) was used to propose a lower concentration limit beneath which the detected F is considered as being endogenous and no further investigation on IRMS is needed. Further details and information regarding the sample collecting and data analysis can be found in a previous study. [7]

To one healthy male volunteer (subject 1, 23 years) 40 mg of oral testosterone undecanoate (TU, Testocaps, Organon, The Netherlands; assessed by Adam Cawley [22] at δ^{13}C = -27.7 ± 0.6 ‰; excretion study 2009) was administered. One healthy male volunteer (subject 2A, 26 years; excretion study 1997) ingested 50 mg ADION (δ^{13}C = -30.7 ± 0.17 ‰, n = 3). For the subject 2A excretion study, urine samples were collected at 0, 2, 4, 6, 9 and 14 h. For the TU excretion study, at day 1 a urine sample was collected at 0, 2, 4, 5, 8, 10 and 12 h. Additional urines samples were collected after 24 h and the next 5 days, the participants delivered urine samples every morning, noon and evening. A third excretion study was performed to check the possibility of a proposed hypothesis (further explanation later on in text). In this administration study (subject 2B, 41 years; excretion study 2012) the volunteer was the same person as subject 2A and the same 50 mg of ADION were also ingested. Urine samples were collected at 0, 3, 5, 7, 8.5, 9, 10, 12, 24, and 30 h. All samples were stored at -20°C. Blood tests were taken to confirm that none of the volunteers suffered from any liver problems or had an androgen insufficiency. The Ethical
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Committee of the Ghent University Hospital (B67020064707) approved the study and all volunteers gave informed consent. The steroid profile of all samples was measured by GC-MS/MS using the procedure explained in a previous study. [23,24] CIR determination of T was conducted using the previously described method. [16]

3.3 Steroid profiling with GC-MS/MS and pre-analytical steps

An aliquot of each urine sample was prepared to check the steroid profile according to routine sample preparation procedures. [23,24] The concentrations of the target compounds allowed us to calculate the necessary sample volume using the following formula. \( V_{IRMS} \) is the required sample volume and \( C_x \) is the concentration of F. For concentrations lower than 10 ng/mL, 25 mL of urine was used.

\[
V_{IRMS} = \frac{250 \, ng}{C_x}
\]

A system blank, QC negative and QC positive were aliquoted with each batch of urine samples. A detailed description of the sample preparation procedure was published earlier. [16] In short: urine was loaded on a conditioned C18 solid phase extraction cartridge, washed with 5 mL of H₂O and the compounds of interest were eluted with 5 mL CH₃OH; the dried residue was reconstituted in 1 mL of a 0.1M pH 7 phosphate buffer and 50 µL of β-glucuronidase enzyme was added for the hydrolysis; afterwards 1 mL NaHCO₃/K₂CO₃ buffer (pH 9.5) and 5 mL n-pentane were added for the extraction; the organic phase was transferred to a new tube and evaporated; 50 µL of pyridine and 50 µL of acetic anhydride were added for the acetylation; The residue was dried, reconstituted in 100 µL of 75/25 CH₃OH/H₂O and transferred to a LC vial.
3.4 High performance liquid chromatography fraction collection

To purify the sample, a semi-preparative HPLC fraction collection was performed on a Thermo Scientific Surveyor (Bremen, Germany) with an injection volume of 100 μL. Separation was achieved using a Phenomenex Gemini C18 (150 mm x 4.6 mm x 5 μm) column (Torrance, California, USA) maintained at 35°C. The mobile phase flow rate was 1 mL/min using an CH₃OH/ H₂O gradient (solvent A: 10/90 CH₃OH/ H₂O; solvent B: CH₃OH) that started with 100% of solvent A, then 45% B at 1 min, 65% B at 13 min and 100% B at 20 min, held until 24 min, 100% A at 25 min and held until 30 min. Photodiode array detection was performed at 195, 245 and 345 nm. β-Trenbolone acetate was used as retention time marker (17.7 min) to check the collection windows. Two fractions were automatically collected with a Gilson FC 204 (Gilson, Middleton, WI, USA); FA (11.6 – 12.6 min) contained formestane acetate (F-Ac) and FB (23.1 – 25.1 min) contained PD-Ac₂. The collection windows were set sufficiently wide to collect the entire peak of the compounds of interest. Each batch analysis started with a retention time check of β-Tren-Ac. All fractions were dried under nitrogen at 60°C. All residues were quantitatively transferred (2 x 150 μL ethyl acetate) to a GC-MS vial, dried under nitrogen at 40°C and reconstituted in an appropriate volume of internal standard solution (5α-ol-Ac 1.66 μg/mL, epiT-Ac 2.56 μg/mL, in n-hexane-toluene (1:1)) for the GC-MS and GC-C-IRMS analysis.

3.5 GC-MS

An Agilent 6890 GC coupled to a 5975B VI MSD from Agilent Technologies (Palo Alto, CA, USA) was used. Helium was used as carrier gas. 13 μL solvent vent injections on a Gerstel PTV injector (Mülheim an der Ruhr, Germany) were used. Here, the compounds were dissolved in a n-hexane-toluene (1:1) solution and an injection speed of 3 μL/s was used. PTV injector settings: 70°C until 0.35 min, then the temperature was increased at 720°C/min until 330°C and hold for 0.8 min, followed by a temperature gradient of 720°C/min until 400°C. The solvent vent
lasted until 0.3 min (15 mL/min at 5 psi). At 1 min, a purge vent of 300 mL/min was applied. The GC column (30 m x 0.25 mm x 0.25 μm) was an Agilent J&W DB-17MS. The column temperature was held at 70°C for 1 minute, increased until 250°C with a rate of 30°C/min and then increased until 280°C with a rate of 2°C/min. The final temperature gradient consisted of a rate of 5°C/min until 310°C. The MSD acquired data in full scan mode from m/z 57 to 645 using Chemstation software (Agilent, Waldbronn, Germany).

Each fraction was analyzed by full scan GC-MS to determine the relative retention of the acetylated steroids versus IS 5α-ol-Ac according to WADA criteria. [18] The acetylated steroids were identified, the peak purity checked and the concentration was assessed using one-point calibration in order to estimate the ideal sample volume for GC-C-IRMS analysis.

3.6 GC-C-IRMS

An Agilent 7890A GC (Palo Alto, CA, USA) was coupled to a Thermo GC-Isolink, a Thermo ConfloIV interface and a Thermo Scientific MAT253 IRMS (Bremen, Germany) to determine the δ^{13}C value of the compounds of interest. The GC conditions were exactly the same as those described for full-scan GC-MS analysis. Data was acquired using ISODAT NT 3.0 software (Thermo Scientific, Bremen, Germany). The IRMS was calibrated as reported previously. [25]

3.7 Correction for acetate adducts

In the acetylation step, additional carbon atoms from the acetate moiety are incorporated into the steroidal skeletal. Therefore the measured δ^{13}C has to be corrected using the following formula:

$$
\delta^{13}C_{steroid} = \frac{(n_{Ac} \delta^{13}C_{Ac}) - (n_{Ac} \delta^{13}C_{acorr})}{n_{steroid}}
$$
Here, ‘n’ is the number of moles of carbon, ‘sa’ stands for steroid acetate and ‘a’ for the acetate derivative group. $\delta^{13}C_{acorr}$ is indirectly calculated by measuring the $\delta^{13}C$ of the reference material of each relevant analyte in the free and derivatized fractions.

4 Results and discussion

4.1 Method validation

*Precision (repeatability and reproducibility)*

The within-batch and between-batch precision of the extraction method for the determination of raw $\delta^{13}C$ was tested by the analysis of steroid stripped urine spikes prepared at three concentration levels as specified in Table 7.1. One batch of six replicates at each level was then extracted by a different operator, thereby incorporating an additional measure of reproducibility into this determination of precision. For each concentration level of sample, the volume of urine required for analysis was adjusted appropriately. Therefore 2.5 mL, 10 mL and 25 mL of steroid stripped urine that was spiked at a high, medium and low concentration respectively were extracted. The $\delta^{13}C$ statistics are given in Table 1. The within-batch precision (repeatability) ranged from 0.13 ‰ – 0.31 ‰ and 0.26 ‰ – 0.46 ‰ for F-Ac and PD-Ac₂, respectively. The between batch precision (reproducibility) was 0.24 ‰ and 0.45 ‰ for F-Ac and PD-Ac₂ respectively.
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Table 7.1: Repeatability and reproducibility of F-Ac and PD-Ac₂

<table>
<thead>
<tr>
<th>Operator</th>
<th>Concentration of F</th>
<th>Concentration of PD</th>
<th>δ¹³C value (%)</th>
<th>F-Ac</th>
<th>PD-Ac₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Level 1 (10 ng/mL)</td>
<td>Level 1 (50 ng/mL)</td>
<td>Average</td>
<td>-32.42</td>
<td>-35.42</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SD</td>
<td>0.27</td>
<td>0.37</td>
</tr>
<tr>
<td>B</td>
<td>Level 2 (100 ng/mL)</td>
<td>Level 2 (500 ng/mL)</td>
<td>Average</td>
<td>-32.36</td>
<td>-35.14</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SD</td>
<td>0.13</td>
<td>0.46</td>
</tr>
<tr>
<td>C</td>
<td>Level 3 (500 ng/mL)</td>
<td>Level 3 (2500 ng/mL)</td>
<td>Average</td>
<td>-32.32</td>
<td>-35.75</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SD</td>
<td>0.31</td>
<td>0.26</td>
</tr>
<tr>
<td>Overall</td>
<td></td>
<td></td>
<td>Average</td>
<td>-32.37</td>
<td>-35.44</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SD</td>
<td>0.24</td>
<td>0.45</td>
</tr>
</tbody>
</table>

Limit of detection

The δ¹³C value of F can be determined at a urinary concentration of 2 - 2.5 ng/mL starting from 25 mL of urine. At this concentration the peak height is still within the linear range of our IRMS (300 – 5000 mV) and the background is sufficiently low for accurate measurements. A chromatogram that represents a bad situation is given in Figure 7.1. F was present at low concentrations (5 ng/mL) in a urine sample with high concentrations of endogenous steroids (that could interfere if the sample clean up would be insufficient) and high concentrations of other urinary compounds that would produce a high background during the IRMS analysis. Figure 7.1 shows that even under these conditions we get an F-Ac peak free of interferences, illustrating the effectiveness of the sample clean up.
Figure 7.1: IRMS analysis of a urine sample (5 ng/mL of F) with high concentrations of endogenous steroids and other urinary compounds

4.2 Threshold proposal

The main aim was to propose a new concentration threshold. Data collected in our previous study [7] was used to propose this lower concentration limit beneath which the detected F is considered as being endogenous and no further investigation on IRMS will be needed. In this study 3031 urine samples were analyzed, 1531 samples showed F concentrations that were above the detection limit of the method at that time (≥ 5 ng/mL). Based on these data a lower concentration limit of 25 ng/mL corresponding to the 99 % reference limit of the population was proposed. [7] Figure 7.2 shows the concentrations of those 1531 samples. By using 25 ng/mL as limit, 15 out of 3031 urine samples (0.49 %) are
considered as being suspicious and would have been forwarded for IRMS analysis. In practice this would mean that on average 1 out of 200 urine samples with endogenous F values will be analyzed on IRMS. Lowering the limit would imply analyzing more negative samples at a high financial burden. Using a higher limit will obviously lower the detection sensitivity for certain positive samples.

![Figure 7.2](image)

**Figure 7.2:** Plot of the F concentrations in 1531 urine samples. The bold line represents the 25 ng/mL threshold

### 4.3 Applicability of the 3 ‰ WADA rule

50 urine samples were analyzed to demonstrate the applicability of the 3 ‰ WADA rule. Figure 7.3 plots the obtained $\Delta\delta^{13}C$ values for PD – F values versus the F concentration. As expected, there was no relation between concentration and $\Delta\delta^{13}C$. The distribution of $\Delta\delta^{13}C$ was tested using the Anderson-Darling normality test at $p = 0.05$. The $\Delta\delta^{13}C$ values showed a Gaussian distribution ($p = 0.55$) with an average of -0.60 ‰ and a standard deviation of 0.77 ‰. Because the values are Gaussian distributed, the reference limit could be calculated by adding the three-fold standard deviation to the average. This results in a reference limit of 1.71 ‰ which is within the 3 ‰ WADA rule.
Chapter 7: Steroid Profile Threshold for Formestane

Figure 7.3: Scatter plot of $\Delta\delta^{13}$C (PD – F) versus the F concentration of 50 urine samples

4.4 Excretion study: time profiles of T/EpiT ratios and F and T concentrations

As described earlier [5,26], ingestion of ADION or TU leads to an increase of the urinary excretion rates of both T and F (Figure 7.4). Subject 1 had pre-administration concentrations of ~ 100 ng/mL for T (T/EpiT ~ 1.5 - 2) and 3 - 4 ng/mL for F. Subject 2A had pre-administration concentrations of ~ 20 ng/mL for T (T/EpiT ~ 1) and 6 - 7 ng/mL for F. For both subjects the T/EpiT ratio remains above 4 between 2 and 10 hours after administration with a maximum T/EpiT of 11.50 (subject 1) and 12.85 (subject 2A). The F concentration rises above the 25 ng/mL threshold between 2 and 4 hours post administration with a maximum of 63 ng/mL (subject 1) and 80 ng/mL (subject 2A).

It has been shown in the past that ADION administration has a large influence on the urinary F excretion: up to 30 times the basal F concentration for a single dose
administration (100 mg) and up to 70 times for multiple dose administrations. [5] In our excretion study with subject 2A, the administered dose was less and so was the influence on the F concentration. Nonetheless, this influence is still present and results in urinary F concentrations above our established 25 ng/mL limit.

![Figure 7.4: Urinary concentration versus time plot after administration of 40 mg TU (A) and 50 mg ADION (B) at t = 0 h. The black diamonds represent the F concentration and the asterisks the T concentration. The bold line represents the 25 ng/mL threshold.](image)

Unfortunately, because the F concentration did not increase as much as anticipated, the detection time for both subjects was quite short as well. Increasing the administration dose would increase the detection time, but based on these results, it seems unlikely that the detection time would be longer than the T/EpiT detection time (Figure 7.5). Until now, despite the disadvantages, the T/EpiT ratio remains the most sensitive and adequate biomarker to detect misuse and trigger the IRMS confirmation. [27–29] However, in some cases, steroid profile parameters such as the T/EpiT ratio are not effective enough to detect misuse. The T/EpiT ratio of
subjects with low (<1) natural values for example, does not increase after an ADION or T administration. [5,30,31] This can be caused by a genetic deletion morphism that affects androgen disposition by lowering T glucuronidation. [32] In such cases, monitoring minor metabolites such as F can lead to an IRMS confirmation for urines which would otherwise have been false negatives.

Figure 7.5: Plot of the T/EpiT ratio after administration of 40 mg TU (A) and 50 mg ADION (B) at t = 0 h

4.5 Excretion study: time profiles of CIR values

The $\Delta \delta^{13}$C value time profiles of subject 1 and 2A are shown in Figure 7.6. For subject 1, the basal level for PD - T was around 1 % and for PD - F around -1.5 %. For subject 2A, the basal levels for PD - T and PD - F were around 0 %. Administration of TU (subject 1) resulted in PD - T values above 3 % from 2 – 10 hours post administration with a maximum of 8.1 % after 4 hours. Administration of ADION (subject 2A) resulted in a maximum PD - T of 8.6 % after 2 hours. In both subjects the $\Delta$ value for PD - F remained above 3 % from 2-4 hours post administration.
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Figure 7.6: Δδ^{13}C value versus time plot after administration of 40 mg TU (A) and 50 mg ADION (B) at t = 0 h. The black diamonds represent PD - F and the asterisks the PD - T. The bold line represents the 3 ‰ WADA limit.

As stated by Piper et al. [6] and Cawley et al. [5], the δ^{13}C value of urinary F rarely reaches the δ^{13}C value of the administered steroid (F in the study of Piper et al., ADION in the study of Cawley). Endogenous dilution of the CIR cannot be the
Chapter 7: Steroid Profile Threshold for Formestane

explanation as this is very small for F. It seems likely that F metabolizes to other compounds with isotopic fractionation as a result. This causes enriched values for F and depleted values for its metabolites. Interestingly, the same phenomena, but to a much greater extent was observed. The reason for this was not completely clear. The urine samples are stored at -20°C but are 15 years old and have been subjected to many frosting-defrosting cycles. It is not impossible that a big proportion of the urinary F has been converted over the years. This would also explain why our urinary F concentrations are lower than expected. To verify this possibility, the same excretion study (subject 2B) was performed with the same person, 15 years later and with the same ADION.

An additional reason why the PD - F values are less elevated in comparison with other papers and their populations, is because in our population and subjects the basal δ13C values of F seem to be higher than the δ13C values of PD and because the δ13C values of the administered steroids are considerably lower.

4.6 Excretion study subject 2B

As expected, the urinary F concentrations were higher (maximum = 180 ng/mL at 5h) and remained above 25 ng/mL between 2 and 8h post administration (Figure 7.7A). The T/EpiT ratio had the same detection window and also remained above its threshold between 2 and 8h.

The basal level for PD - F was around -0.5 ‰. The administration of ADION (subject 2B) resulted in a Δ value for PD - F above 3 ‰ between 2 and 8h and a maximum of 5.9 ‰ after 5 hours (Figure 7.7B). The PD - T values for subject 2B remained very similar to the obtained data from subject 2A. Overall, in comparison with subject 2A, PD - F showed higher values (resulting in longer detection times) but still did not reach the δ13C value of the administered ADION in contrast to T that does reach this δ13C value. These results seem to imply that F is more susceptible to further metabolism and that it is associated with a stronger kinetic isotope effect. However, having in mind interindividual differences, the limited value of a single
excretion study has to be underlined and further investigation is needed to support this hypothesis, but falls outside the scope of this study.

Figure 7.7: Time plot after administration of 50 mg ADION at $t = 0$ h.

A: The black diamonds represent the urinary $F$ concentrations and the asterisks the $T/EpiT$ ratios. The bold and dashed line show the 25 ng/mL and $T/EpiT = 4$ threshold, respectively.

B: The black diamonds represent the urinary $F$ concentrations and the asterisks PD – $F$ value. The bold and dashed line show the 25 ng/mL threshold and the 3 % limit, respectively.
5 Conclusions

A simple urine pretreatment procedure, leading to extracts free of interferences and permitting precise and accurate IRMS analysis was developed, validated and implemented in doping control. 50 urine samples were analyzed with an average PD – F value of -0.60 ‰ and a standard deviation of 0.77 ‰. This results in a reference limit of 1.71 ‰ which is within the 3 ‰ WADA rule.

A combined approach of GC-MS(/MS) and GC-C-IRMS provides specific detection of endogenous steroid misuse for improved doping analysis. [33] The main aim of this study was to propose a threshold of 25 ng/mL beneath which the detected F is considered as being endogenous and no further investigation on GC-C-IRMS will be required. Our data suggests that this threshold provides a good balance between a sufficiently large detection window and not having to perform IRMS analyses on negative urine samples.
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6 References


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Chapter 8: Steroid Profile Threshold for 6αOH-ADION

Adapted from

M. Polet, P. Van Renterghem, W. Van Gansbeke, P. Van Eenoo

Studies on the minor metabolite 6α-hydroxy-androstenedione for doping control purposes and its contribution to the steroid profile

Drug Testing and Analysis, 6, 10 (2014), p 978-84.
1 Abstract

Recent publications have shown that the concentrations of minor metabolites such as F and 6αOH-ADION are import parameters, capable of increasing the specificity and efficiency of steroid abuse screening. The importance of such minor metabolites has been recognised for some time, but setting up concentration thresholds is not that straightforward with a single quadrupole gas chromatograph mass spectrometer because of the low concentrations and this is especially the case for 6αOH-ADION.

The main aim of this study was to propose a concentration threshold above which the detected 6αOH-ADION is considered as being suspicious IRMS is recommended. 2128 routine doping control samples from athletes analyzed at our lab and not found suspicious for the intake of any doping substance were used to determine the baseline concentrations of 6αOH-ADION. For this purpose, the more sensitive GC-MS/MS was used, capable of quantifying these low concentrations with high reliability. A urinary concentration threshold of 5 ng/mL was set. Concentrations above this threshold are considered as suspicious and are forwarded to IRMS for confirmation in routine practice. In addition, an IRMS method was developed, capable of determining the $^{13}$C value of 6αOH-ADION. If a urine sample has an elevated 6αOH-ADION concentration and normal $^{13}$C values for the traditional IRMS target compounds, we are still able to check the $^{13}$C value of 6αOH-ADION.

Six excretion studies were executed to stress the applicability of the threshold by visualising the concentration and $\delta^{13}$C value time profiles of 6αOH-ADION.
2 Introduction

The use of anabolic androgenic steroids has been prohibited by the International Olympic Committee (IOC) in 1976, leading to the development of various approaches to detect its misuse. [1] While for exogenous steroids their presence in a doping control sample suffices for a positive finding, this is not the case for misuse of endogenous steroids. In doping control, the concentrations and concentration ratios of several endogenous steroids in urine are measured, but because of the large inter-individual variation in urinary excretion of these steroids, they are not suitable to confirm abuse. However, they can indicate suspicious samples. In 1983, to compensate for natural variations in urinary steroid excretions, Donike et al. proposed the ratio of the T and EpiT concentration as a marker for synthetic testosterone. [2] Currently, a T/EpiT ratio greater than 4 is considered as suspicious and is defined as an atypical analytical finding. [3] Unfortunately, this threshold cannot fully exclude false negative and false positive screening results due to those large inter-individual urinary excretion profiles. Some people have naturally high T/EpiT ratios above 4, others have for example a genetic deletion morphism that severely limits T glucuronidation, causing very low T/EpiT ratios that will never exceed the threshold of 4 after T misuse. [4]

In addition to the T/EpiT ratio, other steroid concentrations are monitored in the screening as well. These parameters are primarily the concentrations of steroids that can be administered such as T, E, DHEA, DHT and ADION and the concentrations of their main urinary metabolites A, Et, βαβ and ααβ. [5,6] The concentration ratios of several of these steroids, e.g. A/Et and DHT/EpiT ratio, are considered as valuable indicators for anabolic steroid misuse as well. [7,8] The cluster of all these concentrations and ratios is traditionally known as the steroid profile and is measured with the aid of GC-MS.

Currently, steroid profiles showing ratios or concentrations of one or multiple steroids outside of population reference values are classified as atypical analytical
Chapter 8: Steroid Profile Threshold for 6αOH-ADION

findings and are forwarded to a confirmatory method that can unequivocally distinguish between an endogenous and exogenous origin. [9] Because of the isotopic difference between synthetic and endogenous compounds, GC-C-IRMS is capable of steroid abuse detection. [10–17] The technique exploits the difference in CIR between C3 and C4 plants. A mix of these determines the CIR of human biomolecules. Commercially produced synthetic steroids are derived from soy, a C3 plant that has a significantly lower CIR than human biomolecules which arise metabolically from mixed C3 and C4 precursors.ERC’s are compounds of which the CIR does not change after synthetic steroid administration, because they are for example situated higher in the metabolic chain. By comparison of the $^{13}$C/$^{12}$C ratios of ERCs like PD and 11-oxoEt with that of a TC like T, it is possible to differentiate between an exogenous or endogenous origin. To compensate for the inter-individual variation in CIR of a particular steroid due to differences in diet, the administration of an endogenous steroid is demonstrated when a difference of 3 ‰ or more between the $^{13}$C/$^{12}$C ratio of the ERC and the TC is determined. [9]

Concerning the steroid profile, publications have shown that the concentrations of minor metabolites such as F, 6αOH-ADION and 7β-hydroxy-dehydroepiandrosterone (7βOH-DHEA) are important parameters as well, capable of increasing the specificity and efficiency of the screening. [18–22] As more and more anti-doping laboratories are equipped with the more sensitive GC-MS/MS instrument (compared to the single quadrupole GC-MS), the ability arises to quantify these low concentration minor metabolites with high reliability. [23] As a result, an increasing amount of anti-doping laboratories across different continents are willing to including them in their steroid profiling screening procedure and concentration thresholds need to be set up for effective screening. Concentrations beneath these thresholds would be considered as endogenous and higher concentrations would be regarded as suspicious and forwarded to IRMS to determine whether the elevated concentrations are caused by anabolic steroid abuse or due to an exceptional high endogenous excretion. The importance of such
minor metabolites has been recognised for some time, but setting up such thresholds is not that straightforward with a single quadrupole GC-MS because of the low concentrations and this is especially the case for 6αOH-ADION. [18–22]

In this work we focus on 6αOH-ADION, a minor metabolite with extremely low endogenous concentrations that can increase up to 100 times its basal concentrations after T administration. [21] Due to these very low basal concentrations, an elevation of the 6αOH-ADION concentration can be noticed fairly easy, making it a valuable steroid profile parameter. The main aim of this study was to propose a threshold above which the detected 6αOH-ADION is considered as being suspicious and IRMS is recommended. GC-MS/MS was used for this because of its higher sensitivity (and reliability for quantification of low concentration compounds).

When elevated 6αOH-ADION concentrations are observed, IRMS would normally be performed on the traditional TCs: A, Et, βαβ, ααβ and T. In this work we present an additional IRMS method for 6αOH-ADION. If a urine sample would have an elevated 6αOH-ADION concentration and normal CIR for the traditional TCs, we are still able to check the CIR of 6αOH-ADION. Analyzing 6αOH-ADION by IRMS provides both challenge and opportunity: a sensitive IRMS method is needed to be able to determine the CIR as low as the screening threshold; on the other hand the CIR endogenous dilution is very small, facilitating the differentiation between exogenous and endogenous.

3 Experimental

3.1 Reagents and chemicals

NaH₂PO₄·H₂O, Na₂HPO₄·2H₂O, K₂CO₃ and toluene were purchased from Merck (Darmstadt, Germany). Ethyl acetate, LCMS grade methanol and NaHCO₃ were obtained from Fisher Scientific (Leicestershire, UK) and methyl tert-butyl ether, n-
hexane, LCMS grade water and acetonitrile were purchased from Biosolve (Valkenswaard, The Netherlands). β-Glucuronidase (E. coli) was purchased from Roche Diagnostics (Mannheim, Germany); helium, carbon dioxide and oxygen from Air Liquide (Bornem, Belgium); acetic anhydride, pyridine, PD, PD-Ac₂, 11-oxoEt, 11oxo-Ac and 17β-trenbolone (β-Tren) were obtained from Sigma Aldrich (St. Louis, MO, USA); 17β-Trenbolone acetate (β-Tren-Ac), 5α-androstan-3β-ol acetate (5α-ol-Ac) and 6αOH-ADION from Steraloids (Newport, RI, USA). All steroid standards contained less than 1 % impurities. All standard solutions were prepared in methanol.

3.2 Reference populations and excretion studies

In 2013, 2128 routine doping control samples from athletes analyzed at our lab and not found suspicious for the intake of any doping substance were further investigated regarding their endogenous 6αOH-ADION concentrations. Informed consent was given by the athletes and their sport federations. GC-MS/MS, capable of reliable quantification of such low concentrations, was used for this purpose. The population, consisting of 1704 male and 424 female samples, was used to determine endogenous concentration distributions in males and females and to determine endogenous CIR for 6αOH-ADION. Out of the 2128 samples, 4 had a sufficiently high concentration for GC-C-IRMS analysis of 6αOH-ADION.

Four healthy male volunteers (23-26 years, 64-94 kg) were administered with 40 mg of oral testosterone undecanoate (TU, Testocaps, Organon, The Netherlands). One healthy male volunteer (41 years, 95 kg) ingested 50 mg ADION. One other healthy male volunteer (32 years, 75 kg) ingested 300 mg 4-androstene-3,6,17-trione (6-oxo). For the TU excretion study, a urine sample was collected at 0, 2, 4, 6, 8, 10 and 12 h post-administration. For the ADION excretion study, urine samples were collected at 0, 3, 5, 7, 8.5, 9, 10 and 12 h post-administration and for the 6-oxo administration, urine was collected at 0, 2, 4, 6, 8, 10, 12, 24 and 48 h post-administration. All samples were stored at -20°C pending analysis. Blood tests were
performed to confirm that none of the participants suffered from any liver problems or had an androgen insufficiency. The Ethical Committee of the Ghent University Hospital (B67020064707) approved the study and all volunteers gave informed consent. The steroid profile of all samples was gathered by GC-MS/MS using the procedure explained in previous publications. [23,24]

3.3 Steroid profiling with GC-MS/MS and pre-analytical steps

An aliquot of each urine sample was prepared to check the steroid profile according to routine sample preparation procedures. [23,24] The concentration of the TC allowed us to calculate the necessary sample volume using the following formula.

\[ V_{IRMS} = \frac{250 \text{ ng}}{C_x} \]

A detailed description of the sample preparation procedure was published earlier. [25] In brief: urine was loaded on a conditioned C18 solid phase extraction cartridge, washed with 5 mL of H2O and 5 mL of 10/90 CH3OH/ H2O; the compounds of interest were eluted with 5 mL CH3OH; the dried residue was reconstituted in 1 mL of a 0.1 M pH 7 phosphate buffer and 50 µL of β-glucuronidase enzyme was added for the hydrolysis; afterwards 1 mL NaHCO3/K2CO3 buffer (pH 9.5) was added. To increase the 6αOH-ADION recovery 5 mL of methyl tert-butyl ether was added instead of n-pentane for the extraction; the organic phase was transferred to a new tube and evaporated. The residue was reconstituted in 50 µL of β-Tren-Ac (100 µg/mL) and 50 µL of 50/50 CH3OH/H2O and transferred to an LC vial. The retention time of β-Tren-Ac was used to verify that for every sample the correct fractions were collected.
3.4 High performance liquid chromatography fraction collection

To purify the sample, two semi-preparative HPLC fraction collections were performed on a Thermo Scientific Surveyor (Bremen, Germany) with an injection volume of 100 µL. The first separation was achieved using a Zorbax Extend-C18 (250 mm x 4.6 mm x 5 µm) column (Palo Alto, CA, USA) maintained at 35°C. The mobile phase flow rate was 1 mL/min using an CH₃CN/H₂O gradient (solvent A: H₂O; solvent B: CH₃CN) that started with 10 % of solvent B, then 100 % B at 24 min, held until 28 min, 10 % B at 29 min and held until 35 min. Photodiode array detection was performed at 195, 245 and 345 nm. β-Tren-Ac was used as retention time marker (19.4 min) to check the collection windows. Three fractions were automatically collected with a Gilson FC 204 (Gilson, Middleton, WI, USA); fraction A (FA: 10.9 – 12.2 min) contained 6αOH-ADION, fraction B (FB: 14.1 – 15.3 min) contained 11-oxoEt and fraction C (FC: 18.7 – 20.7) contained PD. The collection windows were set sufficiently wide to collect the entire peak of the target compounds. Each batch started with a retention time check of β-Tren-Ac. Afterwards, 50 µL of β-Tren (100 µg/mL) was added to FA. All fractions were dried under nitrogen at 60°C.

Acetylation took place by adding 50 µL of acetic anhydride and 50 µL of pyridine (60 min at 80°C). The acetylation reagents were evaporated under nitrogen at 60°C and the residue of FA was reconstituted in 100 µL of 75/25 CH₃OH/H₂O.

A second HPLC fraction collection using a Phenomenex Gemini C6-Phenyl (150 mm x 4.6 mm x 5 µm) column (Torrance, California, USA) was conducted on the acetylated 6αOH-ADION. The mobile phase flow was 1 mL/min using an CH₃OH/H₂O gradient (solvent A: H₂O; solvent B: CH₃OH) that started with 10 % of solvent B, 50 % B at 1 min, then 75 % B at 19 min, 100 % B at 20 min and held until 24 min, 10 % B at 25 min and held until 30 min. Photodiode array detection was performed at 195, 245 and 345 nm. β-Tren-Ac was used as retention time marker (19.9 min) to check the collection windows. One fraction containing 6αOH-ADION -Ac (13.0 – 14.0 min) was automatically collected with a Gilson FC 204 (Gilson, Middleton, WI, USA). Each
batch of analyses started with a retention time check of β-Tren-Ac. All fractions were dried under nitrogen at 60°C. All residues were quantitatively transferred (2 x 150 µL ethyl acetate) to a GC-MS vial, dried under nitrogen at 40°C and reconstituted in an appropriate volume of internal standard solution 5α-ol-Ac (1.66 µg/mL, in 1/1 n-hexane/toluene) for the GC-MS and GC-C-IRMS analysis.

3.5 GC-MS

An Agilent 6890 gas chromatograph coupled to a 5975B VI MSD from Agilent Technologies (Palo Alto, CA, USA) was used. Helium was used as carrier gas. 13 µL solvent vent injections on a Gerstel PTV injector (Mülheim an der Ruhr, Germany) were used. Here, the compounds were dissolved in a n-hexane-toluene (1:1) solution and an injection speed of 3 µL/s was used. PTV injector settings: 70°C for 0.35 min, then the temperature was increased at 720°C/min to 330°C and hold for 0.8 min, followed by a temperature gradient of 720°C/min to 400°C. The solvent vent lasted for 0.3 min (15 mL/min at 5 psi). At 1 min, a purge vent of 300 mL/min was applied. The GC column (30 m x 0.25 mm x 0.25 µm) was an Agilent J&W DB-17MS. The column temperature was held at 70°C for 1 minute, increased until 250°C with a rate of 30°C/min and then increased until 280°C with a rate of 2°C/min. The final temperature gradient consisted of a rate of 5°C/min until 310°C. The MSD acquired data in full scan mode from m/z 57 to 645 using Chemstation software (Agilent, Waldbronn, Germany).

Each fraction was analyzed by full scan GC-MS to determine the relative retention of the acetylated steroids versus IS 5α-ol-Ac according to WADA criteria. [3] The acetylated steroids were identified, the peak purity checked and the concentration was assessed using one-point calibration in order to estimate the ideal sample volume for GC-C-IRMS analysis.
3.6 GC-C-IRMS

An Agilent 7890A gas chromatograph (Palo Alto, CA, USA) was coupled to a Thermo GC-Isolink, a Thermo ConfloIV interface and a Thermo Scientific MAT253 IRMS (Bremen, Germany) to determine the $\delta^{13}C$ value of the compounds of interest. The GC conditions were exactly the same as those described for full-scan GC-MS analysis. Data was acquired using ISODAT NT 3.0 software (Thermo Scientific, Bremen, Germany). The IRMS was calibrated as reported previously. [26]

3.7 Correction for acetate adducts

In the acetylation step, additional carbon atoms from the acetate moiety are incorporated into the steroidal skeletal. Therefore the measured $\delta^{13}C$ value has to be corrected using the following formula [3]

$$
\delta^{13}C_{\text{sterol}} = \frac{(n_{sa} \delta^{13}C_{sa}) - (n_a \delta^{13}C_{acorr})}{n_{\text{sterol}}}
$$

Here, ‘n’ is the number of moles of carbon, ‘sa’ stands for steroid acetate and ‘a’ for the acetate derivative group. $\delta^{13}C_{acorr}$ is indirectly calculated by measuring the $\delta^{13}C$ value of the reference material of each relevant analyte in the free and derivatised fractions.

4 Results and discussion

4.1 Method validation

Precision (repeatability and reproducibility)

The within-batch and between-batch precision of the method for the determination of raw $\delta^{13}C$ value was tested by the analysis of steroid stripped urines spiked at three concentration levels as specified in Table 8.1 and analysed by three different
operators (A, B and C). Each batch contained 6 samples. The within-batch precision (repeatability) ranged from 0.39 ‰ – 0.43 ‰, 0.24 ‰ – 0.36 ‰ and 0.14 ‰ – 0.41 ‰ for 6αOH-ADION-Ac, 11-oxoEt-Ac and PD-Ac₂, respectively (Table 8.2). The between batch precision (reproducibility) was 0.48 ‰, 0.43 ‰ and 0.37 ‰ for 6αOH-ADION-Ac, 11-oxoEt-Ac and PD-Ac₂ respectively.

Table 8.1: Spiked concentrations of the steroid stripped urines

<table>
<thead>
<tr>
<th>operator</th>
<th>batch</th>
<th>6αOH-ADION</th>
<th>11-oxoEt</th>
<th>PD</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1</td>
<td>5</td>
<td>500</td>
<td>100</td>
</tr>
<tr>
<td>B</td>
<td>2</td>
<td>25</td>
<td>1250</td>
<td>250</td>
</tr>
<tr>
<td>C</td>
<td>3</td>
<td>250</td>
<td>5000</td>
<td>1000</td>
</tr>
</tbody>
</table>

Table 8.2: Repeatability and reproducibility of 6αOH-ADION-Ac, 11-oxoEt-Ac and PD-Ac₂

<table>
<thead>
<tr>
<th>δ¹³C value (‰)</th>
<th>6αOH-ADION-Ac</th>
<th>11-oxoEt-Ac</th>
<th>PD-Ac₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>batch 1 average</td>
<td>-34.71</td>
<td>-19.53</td>
<td>-33.4</td>
</tr>
<tr>
<td>SD</td>
<td>0.42</td>
<td>0.34</td>
<td>0.31</td>
</tr>
<tr>
<td>batch 2 average</td>
<td>-34.08</td>
<td>-18.86</td>
<td>-33.48</td>
</tr>
<tr>
<td>SD</td>
<td>0.43</td>
<td>0.36</td>
<td>0.14</td>
</tr>
<tr>
<td>batch 3 average</td>
<td>-34.12</td>
<td>-19.06</td>
<td>-33.47</td>
</tr>
<tr>
<td>SD</td>
<td>0.39</td>
<td>0.24</td>
<td>0.41</td>
</tr>
<tr>
<td>total average</td>
<td>-34.29</td>
<td>-19.21</td>
<td>-33.56</td>
</tr>
<tr>
<td>SD</td>
<td>0.48</td>
<td>0.43</td>
<td>0.37</td>
</tr>
</tbody>
</table>
Chapter 8: Steroid Profile Threshold for 6αOH-ADION

Limit of detection

The $\delta^{13}$C value of 6αOH-ADION can be determined at a urinary concentration of 5 ng/mL starting from 25 mL of urine. At this concentration the peak height is within the linear range of our IRMS (300 – 5000 mV) and the background is sufficiently low for accurate measurements. This 5 ng/mL limit is chosen quite conservative because the 6αOH-ADION sample preparation recovery tends to vary from urine sample to urine sample. This means that for some urine samples with concentrations beneath 5 ng/mL, a signal intensity above 300 mV and a sufficiently low background is still obtained. All reported CIR of samples with concentrations beneath this threshold were still within the linear range of the IRMS and had a sufficiently low background (Figure 8.1).

![IRMS analysis of a urine sample containing 4.7 ng/mL of 6αOH-ADION](image)

Figure 8.1: IRMS analysis of a urine sample containing 4.7 ng/mL of 6αOH-ADION
4.2 Reference population

The 6αOH-ADION concentration in all 2128 urine samples was determined by GC-MS/MS. A right skewed distribution (Figure 8.2) was obtained as expected for urinary steroid concentrations (mean = 1.03 ng/mL, standard deviation = 0.76 ng/mL). The program REFVAL was used for the determination of the upper reference limit as recommended by the Expert Panel on Theory of Reference values of the International Federation of Clinical Chemistry. [27,28] The calculated 99.9% reference limit (95% confidence interval) was 4.9 ng/mL. No significant difference between the male and female population could be found (Figure 8.3). The means were compared using the Mann-Whitney test at p = 0.01 and did not differ significantly (p = 0.086). Based upon these data, urinary 6αOH-ADION concentrations above 5 ng/mL can be classified as suspicious and will be forwarded to IRMS in routine practice.

Figure 8.2: Density plot of the urinary 6αOH-ADION concentrations (reference population n = 2128)
Only 4 samples (3 male, 1 female) contained sufficiently high 6αOH-ADION concentrations for IRMS analysis of 6αOH-ADION and were subjected to a CIR determination (Table 8.3). All 4 investigated samples had comparable CIR Δ values for PD – 6αOH-ADION and 11-oxoEt – 6αOH-ADION and were in line with previously reported ranges for most endogenous steroids and the WADA 3 ‰ rule. [10,15–17,29–33] There are endogenous steroids exhibiting Δδ^{13}C values above 3 ‰, but based upon the limited data we have, it appears not to be the case for 6αOH-ADION. [13,34–36] Obviously, further data will be required to collect a statistically manageable population to confirm this statement.

Figure 8.3: Box plot of the urinary 6αOH-ADION concentrations in females (n = 424) and males (n = 1704)
**Chapter 8: Steroid Profile Threshold for 6αOH-ADION**

**Table 8.3: Endogenous CIR values in 4 urine samples**

<table>
<thead>
<tr>
<th>sample</th>
<th>sex</th>
<th>concentration 6α-ADION (ng/mL)</th>
<th>δ(^{13})C value (%)</th>
<th>Δδ(^{13})C value (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>F</td>
<td>5.7</td>
<td>-24.10</td>
<td>-24.83</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>-24.16</td>
<td>-0.73</td>
</tr>
<tr>
<td>B</td>
<td>M</td>
<td>4.9</td>
<td>-22.52</td>
<td>-23.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>-22.68</td>
<td>-0.53</td>
</tr>
<tr>
<td>C</td>
<td>M</td>
<td>4.6</td>
<td>-23.97</td>
<td>-23.08</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>-23.39</td>
<td>0.90</td>
</tr>
<tr>
<td>D</td>
<td>M</td>
<td>4.4</td>
<td>-23.61</td>
<td>-23.55</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>-22.73</td>
<td>0.06</td>
</tr>
</tbody>
</table>

|       |     | mean                          | -0.07                   |
|       |     | SD                            | 0.73                    |

**4.3 Excretion studies**

Figure 8.4 visualizes the concentration and δ\(^{13}\)C value time profiles of the ADION administration study. The urinary 6αOH-ADION concentrations rise very rapidly (from 0.9 ng/mL at 0 h to 120 ng/mL at 5 h), but also drop again very fast (4.5 ng/mL at 8.5 h). This demonstrates that by administering ADION, the metabolic system is very briefly overloaded, pushing the excess of administered steroid to less common metabolic pathways, resulting in elevated concentrations of minor metabolites. Similar behaviour has been noticed for example with F. [19,21,32] The 6αOH-ADION metabolic pathway has very limited activity under normal circumstances (very low endogenous concentrations), shows intensified activity immediately after the administration and quenches very fast as soon as the metabolic equilibrium starts to restore again. Due to these low endogenous concentrations and the very steep rise and fall after administration, only a limited number of urine samples had a sufficiently high amount of 6αOH-ADION present for δ\(^{13}\)C value determination. The
Δδ^{13}C values reached a maximum at 7 h (8.46 ‰ for PD - 6αOH-ADION and 8.08 ‰ for 11-oxoEt - 6αOH-ADION).

By using 5 ng/mL as limit in the steroid profile, the detection time is set at 7 h (30 ng/mL). By coincidence 5 ng/mL is also the detection limit of our IRMS method. However, some urine samples exhibit higher recoveries during the sample preparation which makes it possible to determine the CIR of some of the samples with a concentration beneath 5 ng/mL. The urine sample at 8.5 h for example is still detectable (4.5 ng/mL), making 8.5 h the detection time for the IRMS for this urine sample.

Figure 8.4: Time plot after administration of 50 mg ADION at t = 0 h. The bold line shows the urinary 6αOH-ADION concentrations, the dashed lines represent the δ^{13}C values of 6αOH-ADION (triangles), PD (circles) and 11-oxoEt (squares)
Chapter 8: Steroid Profile Threshold for 6αOH-ADION

The concentration and δ^{13}C value time profiles of the TU administrations are displayed in Figure 8.5. In general, the same phenomena as with the ADION administration occurs: a very steep concentration rise and fall after administration. For TU subject 1 however this seems to be less the case: at 2 h post-administration the concentration has only slightly increased to 2.7 ng/mL (basal concentration at 0 h = 1.0 ng/mL) and the steep rise takes place between 2 and 4 h post-administration with a prolonged detection time in comparison with the other three TU subjects. By using 5 ng/mL as steroid profile limit, the detection time varies between 4 – 8 h. The same detection time is valid for the IRMS.

Figure 8.5: Time plots after administration of 40 mg TU at t = 0 h. The bold line shows the urinary 6αOH-ADION concentrations, the dashed lines represent the δ^{13}C values of 6αOH-ADION (triangles), PD (circles) and 11-oxoEt (squares)
Chapter 8: Steroid Profile Threshold for 6αOH-ADION

The concentration and δ\(^{13}\)C value time profiles of the 6-oxo administration are shown in Figure 8.6. The observed urinary concentrations are a lot higher (maximum of 190 µg/mL at 2 h) in comparison with the other administration studies because 6αOH-ADION is a main metabolite of 6-oxo. [37] The concentrations remained above 5 ng/mL for 24 h (26 ng/mL at 24h, 2 ng/mL at 48 h) and the Δδ\(^{13}\)C values reached a maximum at 4 h (11.11 ‰ for PD - 6αOH-ADION and 11.19 ‰ for 11-oxoEt - 6αOH-ADION).

![Figure 8.6: Time plot after administration of 300 mg 6-oxo at t = 0 h. The bold line shows the urinary 6αOH-ADION concentrations, the dashed lines represent the δ\(^{13}\)C values of 6αOH-ADION (triangles), PD (circles) and 11-oxoEt (squares)
Chapter 8: Steroid Profile Threshold for 6αOH-ADION

In all 6 excretion studies, 6αOH-ADION had shorter detection times than at least one of the other markers that can trigger on IRMS confirmation (an elevated T/EpiT ratio, elevated concentration of A, E,...). This means that all samples that had 6αOH-ADION concentrations above 5 ng/mL, would have been labelled “suspicious” and forwarded to IRMS anyway because at least one other marker had a suspicious value. Nevertheless, when dealing with steroid profiles, one must always keep the large inter-individual differences in mind, meaning that this observation is not necessarily applicable for every individual. In 2013, our lab received a sample with perfectly normal values for the traditional steroid profile parameters according to population based reference intervals (Table 8.4). However, the 6αOH-ADION concentration was elevated (17 ng/mL), leading to an IRMS confirmation and consequently an adverse analytical finding was revealed. Based on the traditional parameters alone there would not have been an IRMS confirmation, illustrating the usefulness of monitoring 6αOH-ADION in the steroid profile.

Table 8.4: Steroid profile and δ¹³C values of a urine sample with a suspicious 6αOH-ADION concentration

<table>
<thead>
<tr>
<th>steroid profile (concentrations in ng/mL)</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Et</td>
<td>ααβ</td>
<td>βαβ</td>
<td>T</td>
<td>EpiT</td>
<td></td>
</tr>
<tr>
<td>3200</td>
<td>1300</td>
<td>58</td>
<td>13</td>
<td>3.4</td>
<td>8.7</td>
<td></td>
</tr>
<tr>
<td>DHEA</td>
<td>DHT</td>
<td>ADION</td>
<td>F</td>
<td>7βOH-DHEA</td>
<td>6αOH-ADION</td>
<td></td>
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<tr>
<td>12</td>
<td>2.8</td>
<td>0.63</td>
<td>8.5</td>
<td>4.9</td>
<td>17</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>δ¹³C value (‰)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
</tr>
<tr>
<td>6αOH-ADION</td>
</tr>
<tr>
<td>-29.5</td>
</tr>
</tbody>
</table>
Chapter 8: Steroid Profile Threshold for 6αOH-ADION

5 Conclusions

6αOH-ADION reference population data (n = 2128) was gathered using GC-MS/MS. Based upon these data the use of a urinary concentration threshold of 5 ng/mL is proposed. Concentrations above this threshold are considered as suspicious and are forwarded to IRMS for confirmation in routine practice. To stress the applicability of this threshold, 6 excretion studies were executed by visualising the concentration and δ^{13}C value time profiles of 6αOH-ADION.

The data illustrates that the 6αOH-ADION concentration is a valuable parameter and an asset to the steroid profile. It is capable of detecting steroid abuse in cases where the traditional steroid profile parameters cannot. This combined approach of GC-MS/MS and GC-C-IRMS provides specific detection of endogenous steroid misuse for improved doping analysis.

6 Acknowledgements

The World Anti-Doping Agency is gratefully acknowledged for its financial support.
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7 References


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Chapter 8: Steroid Profile Threshold for 6αOH-ADION


Chapter 8: Steroid Profile Threshold for 6αOH-ADION


Chapter 8: Steroid Profile Threshold for 6αOH-ADION


[37] K. Deventer, P. Van Eenoo, P. Mikulciková, W. Van Thuyne, F.T. Delbeke, Quantitative analysis of androst-4-ene-3,6,17-trione and metabolites in human urine after the administration of a food supplement by liquid
Part 4

Confirmation

Method for AICAR
Chapter 9: Confirmation Method for AICAR
Chapter 9: Confirmation Method for AICAR

1 Introduction

Ribonucleotides can be synthesized in organisms through the *de novo* pathway or recycled through the salvage pathway. *De novo* biosynthesis of purine nucleotides is fairly complex and consists of several enzymatic reactions in which the purine ring is built a few atoms at a time. [1] The nitrogen atoms in the purine ring originate from the amino acids aspartic acid, glutamine and glutamic acid. 5-amino-4-imidazolecarboxyamide ribonucleoside (acadesine, AICAR) is a natural metabolic intermediate of the *de novo* purine biosynthesis. Adenosine kinase can phosphorylate AICAR to 5-amino-4-imidazolecarboxyamide-1-β-ribofuranosyl 5'-monophosphate (ZMP) which is further metabolized to inosine 5'-monophosphate (IMP) (Figure 9.1). [2] IMP is further converted into the purine nucleotides required for nucleic acid synthesis. [1] In humans, pyrimidine rings can be degraded completely to CO2 and urea which is excreted in urine. Purine rings cannot. They are degraded to uric acid. The ribose unit from IMP is removed and hypoxanthine is obtained. Hypoxanthine is further oxidized to uric acid and excreted in urine.

Recent studies however indicate that AICAR is much more than just a metabolic intermediate. It's a key molecule showing various complex effects on multiple functions. [2] In the early 90's, ZMP was found to activate the human AMP-activated protein kinase (AMPK) activity *in vitro*. [3] Later on, AICAR was used as ZMP precursor to activate AMPK. [4] Recent publications have shown that AICAR targets other proteins as well, where the effects are totally or partially independent of AMPK. [5–8] A paper of 2008 attracted particular attention of the sports drug testing community. The use of AICAR caused an improved endurance of untrained mice by 23 - 44 % by upregulating genes associated with oxidative metabolism without exercise. [9] Hence, AICAR could potentially improve the athletic performance in humans as well, but until this day there is no conclusive scientific proof to support this statement. Nonetheless, misuse of AICAR by athletes is conceivable, despite the known (and unknown) health issues. [10,11] As such, AICAR has been added to the WADA prohibited list in 2009. [12]
A study of 2010 presented data of urinary AICAR concentrations of elite athletes. According to this paper, urinary AICAR concentrations above 20 µg/mL are inconsistent with an endogenous production in healthy humans. [13] However, if AICAR concentrations above 20 µg/mL are detected in an athlete’s urine, there is need for a method that can disclose AICAR abuse indisputably, because the exogenous origin of the administered AICAR cannot be verified. It is possible that an endogenous concentration above this threshold is reached, because of a physical disorder or emerging illness or condition of which the athlete might or might not be aware of or simply because he or she has exceptional high natural values. For
example, it has happened in the past that an athlete had very suspicious urinary steroid concentrations and ratios which, under normal circumstances, indicate anabolic steroid abuse. Further investigation showed the steroids were endogenous and that the athlete was in an early stage of a serious infirmity. Although these cases are very rare, they are sufficient to dismiss a doping violation in court.

In analogy with endogenous steroids, there is need of an IRMS method to unambiguously prove the exogenous nature of the detected AICAR. In doping control, when suspicious endogenous steroid concentrations and/or ratios are found in an athlete’s urine, a second confirmatory analysis is conducted on IRMS. [14–21] This technique exploits the difference in carbon isotope-ratio between endogenous and exogenous steroids. Figure 9.2 gives an overview of the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values documented in recent literature. [16,22–25] Commercially produced synthetic steroids are derived from soy, a C3 plant that has a lower CIR than human biomolecules which arise from a mix of C3 and C4 precursors. To compensate for the inter-individual variation in CIR of a particular steroid due to differences in diet, ERCs are used. ERCs are compounds of which the CIR does not change after synthetic steroid administration because they are for example situated higher in the metabolic chain. According to WADA regulations, the administration of an endogenous steroid is demonstrated when a difference of more than 3 ‰ (or 4 ‰, depending on the steroid) between the CIR of the ERC and the administered steroid (or a metabolite) is obtained. [21]

IRMS remains a very laborious and expensive technique because one can only determine the CIR of a pure compound. This means that a lot of purification steps have to be conducted (a solid phase extraction, a liquid liquid extraction and one or two liquid chromatography fraction collections) to isolate the TCs while isotope fractionation caused by one of these steps is unacceptable. After clean-up, the steroids of interest are analyzed with a GC-C-IRMS in which the steroids are separated from the remaining impurities in the GC part, prior to CIR determination.
in the IRMS detector. In general, the same analysis (with identical GC parameters) is also conducted on a GC-MS to verify that the detected steroid peak is indeed pure.

Figure 9.2: $\delta^{13}$C and $\delta^{15}$N values for fauna and flora

Developing a GC-C-IRMS method for AICAR provides an even greater analytical challenge because of its polarity. It is a very polar compound with multiple polar functions: three alcohol functions, one amine and one amide for only nine carbon atoms. In order to be able to analyze and introduce such a compound on a GC, the polar functions have to be derivatized and converted to apolar groups. For IRMS, this conversion has to be as complete as possible (to avoid isotope fractionation), all polar functions have to be derivatized otherwise strong peak tailing will occur (which can severely influence the determined isotope-ratio) and only one
Chapter 9: Confirmation Method for AICAR

chromatographic peak should be obtained. If the derivatized AICAR results in multiple chromatographic peaks this would add an extra level of complexity to an already convoluted method. Generally, different types of polar groups require different specific derivatization reagents and converting carbohydrates into one single compound has always been very difficult. [26] Moreover, derivatizing means adding extra carbon atoms with a different isotope-ratio to the compound. If the $^{13}$C isotope-ratio is measured, as is the case with steroids, a correction has to be made and for AICAR this correction (and the additional variation) would be substantial as AICAR itself contains only nine carbon atoms. For steroids, the polar groups are derivatized by acetylation which means that only two extra carbon atoms are added per alcohol function. In addition, this correction factor is generally calculated by comparing the $^{13}$C isotope-ratio of the derivatized and non-derivatized compound, but due to its polarity, it seems impossible to analyse AICAR in the non-derivatized form on GC-C-IRMS.

It therefore seems more straightforward to measure the $^{15}$N isotope-ratio. AICAR contains 4 nitrogen atoms and classic GC derivatization agents have none. By using the enzyme purine nucleoside phosphorylase (PNP), the ribose can also be cleaved off fairly easily, removing three out of five polar functions, without lose of nitrogens. [27] The last couple of years, the $\delta^{13}$C values of more and more steroid preparations seem to shift towards endogenous $\delta^{13}$C values, making it more difficult to detect doping violations, certainly in populations with relatively low endogenous $\delta^{13}$C values, as is the case in (northern) European countries (cf. Figure 9.2). It is likely that a comparable trend is taking place for AICAR and the $^{15}$N isotope-ratio can provide a solution.

In parallel with the IRMS confirmation procedure for steroids, it is advisable to use an ERC for AICAR as well. The nitrogen present in the purine ring of AICAR originates from amino acids, the building blocks of proteins. Urea, the main carrier of nitrogenous waste (primarily originating from proteins), is present abundantly in urine and can be used as ERC. Urea represents about 87 % and uric acid 1 – 2 % of
all nitrogen in urine. In this regard, one should take into account the fairly complex $^{15}$N isotope fractionation processes that take place. [23,25,28] In general, the $\delta^{15}$N value increases with every trophic level as is visualized in Figure 9.2. This is an unexpected trend because every (bio)chemical process is associated with kinetic isotopic fractionation in which the end product is depleted (and not enriched) with the heavier isotope. However, the fractionation associated with the production of nitrogenous waste such as urea and uric acid appears to be greater than that associated with the absorption of nitrogen from diet. That is, consumers’ tissues tend to be enriched in $\delta^{15}$N values relative to the diet rather than depleted. As humans are high up the trophic chain, the $\delta^{15}$N values of urea should theoretically still be quite high as well. This is an important issue because exogenous AICAR will most likely be synthesized from plant precursors originating from low trophic levels. In addition every chemical synthesis step that is executed to obtain AICAR from its precursor should result in a further depletion in $^{15}$N, widening the gap between exogenous and endogenous origin. Ideally, there would not be any overlap between exogenous and endogenous values as is sometimes the case with the $^{13}$C isotope (cf. Figure 9.2).

2 Experimental

2.1 Reagents and chemicals

AICAR and AICAR ($^{13}$C$_2$$^{15}$N) were purchased from Toronto Research Chemicals (Toronto, Canada). A second AICAR standard was obtained from Sigma-Aldrich (St. Louis, MO, USA), together with hypoxanthine, diethoxymethyl acetate (DEMA), N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA), 1,1,3,3-tetramethoxypropane (MBDMA), N-N-dimethylformamide (DMF), acetic acid (HAc), hydrochloric acid, uric acid, androsterone and the enzyme purine nucleoside fosforylase (PNP). N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) was
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purchased from Karl Bucher (Waldstetten, Germany), 5-amino-4-imidazolecarboxamide (AICA) from Acros organics (Geel, Belgium). LC-MS grade acetonitril (ACN), methanol and water were from J.T. Baker (Deventer, Netherlands). IRMS standards urea ($\delta^{15}N = 20.17 \%o, \sigma = 0.08 \%o$) and caffeine ($\delta^{15}N = -2.90 \%o, \sigma = 0.03 \%o; \delta^{13}C = -35.05 \%o, \sigma = 0.02 \%o$) were obtained from Indiana University (Bloomington, IN, USA), ammonium formate and formic acid from Fisher Scientific (Leicestershire, UK). NaH$_2$PO$_4$·H$_2$O, Na$_2$HPO$_4$·2H$_2$O and phosphoric acid were purchased from Merck (Darmstadt, Germany).

2.2 Reference population

In 2013, 631 routine doping control samples from athletes analyzed in our lab and not found suspicious for the intake of any doping substance were further investigated regarding their endogenous AICAR concentrations. Informed consent was given by the athletes and their sport federations. In addition, 257 non-athlete samples obtained from the Ghent university hospital (of healthy staff members that underwent the yearly health check up) were analyzed. Both populations were used to establish endogenous AICAR concentration distributions and to determine endogenous $\delta^{15}N$ values for AICAR.

The exogenous AICAR $\delta^{15}N$ values of four preparations were determined. Two originated from Belgium customs seizures, one from Sigma-Aldrich and one from Toronto Research Chemicals.

2.3 AICAR quantification by LC-MS/MS

Aliquots of 50 µL urine were diluted with 450 µL ACN 0.1 % HAc and centrifuged. 50 µl of supernatant was transferred to a LC vial, 50 µL IS (20 µg/mL AICAR ($^{13}C_2^{15}N$) in ACN) was added and the solution was mixed. The calibration curve was obtained by preparing six calibration solutions (water fortified with appropriate amounts of AICAR (0.1, 0.3, 0.8, 1.5, 2.5, 4 µg/mL)) and applying the same sample preparation.
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LC-MS/MS was performed on a Surveyor Plus (Thermo Scientific, Bremen, Germany) coupled to a TSQ Quantum Discovery MAX triple stage quadrupole mass spectrometer (Thermo Scientific, Bremen, Germany). A Phenomenex Luna NH2 column (50 mm x 2.0 mm x 3 µm, Torrance, CA, USA) was used. 20 µl injections were applied. Mobile phase A consisted of H2O, 0.1 % HAc and phase B of ACN, 0.1 % HAc. With a flow rate of 300 µL/min, the gradient started at 5 % solvent A, held isocratic for 1 min, increased to 15 % A at 4 min, switched to 100 % A at 4.1 min, held until 6 min, switched back to 5 % A at 6.1 min and held until 8 min. The mass spectrometer was operated in positive mode with multiple reaction monitoring (MRM) and nitrogen was used as collision gas (1.5 mTorr). The main mass spectrometric parameters are tabulated in Table 9.1.

<table>
<thead>
<tr>
<th>Table 9.1: Mass spectrometric parameters</th>
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<td>Precursor ion</td>
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<tr>
<td>MH⁺ (m/z)</td>
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<tr>
<td>----------------</td>
</tr>
<tr>
<td>AICAR</td>
</tr>
<tr>
<td>259</td>
</tr>
<tr>
<td>259</td>
</tr>
<tr>
<td>259</td>
</tr>
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<td>262</td>
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</table>

2.4 AICAR confirmation by GC-C-IRMS

As mentioned in the general introduction, AICAR needs be converted to an apolar compound suitable for analysis on GC-C-IRMS, without loss of nitrogens. In short: ribose is cleaved off by using PNP and 5-amino-4-imidazolecarboxyamide (AICA) is obtained; a ring closure reaction is carried out on AICA which results in
hypoxanthine and finally hypoxanthine is derivatized by using MTBSTFA. Urea needs to be converted as well. In short: urea is reformed to 2-hydroxypyrimidine by using MBDMA and afterwards derivatized with MTBSTFA. In addition, uric acid and hypoxanthine are included in the method as metabolites of AICAR. Uric acid is also derivatized using MTBSTFA. An overview is given in Figure 9.3.

Figure 9.3: Sample preparation procedure
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Pre-analytical steps

A maximum of 5 mL of urine is aliquoted. The total aliquoted amount of urine should comprise around 10 µg of AICAR or more for analysis on IRMS. The urine is filtered with a 0.2 µm steriflip (Millipore, Darmstadt, Germany) and a 10 K filter (Millipore, Darmstadt, Germany). The urine is dried under oxygen free nitrogen (OFN) at 60°C, 200 – 350 µL of 5 mM ammonium formate, 0.05 % formic acid is added and filtered again with a 0.45 µm microfilter (Millipore, Darmstadt, Germany). After filtration, 5 mM ammonium formate, 0.05 % formic acid is added until a 500 µL solution is obtained.

For the analysis of urea, hypoxanthine and uric acid, 100 µL of urine is used. 400 µL of 5 mM ammonium formate, 0.05 % formic acid is added and filtered with the 0.45 µm microfilter.

HPLC fraction collection and derivatization

To purify the sample, a semi-preparative HPLC fraction collection was performed on a Thermo Scientific Surveyor (Bremen, Germany) with an injection volume of 500 µL. Separation was achieved using a Phenomenex Gemini C18 (150 mm x 10 mm x 5 µm) column (Torrance, California, USA) maintained at 35°C. The mobile phase flow rate was 5 mL/min using an ACN/H2O gradient (solvent A: H2O 5 mM ammonium formate, 0.05 % formic acid; solvent B: 9/1 ACN/H2O 5 mM ammonium formate, 0.05 % formic acid) that started with 100 % of solvent A, held for 7 min then 100 % B at 8 min, held until 12 min, 100 % A at 13 min and held until 18 min. Photodiode array detection was performed at 245 and 285 nm. Standards of AICAR, hypoxanthine and uric acid were used as retention time markers to check the collection windows.

Four fractions were automatically collected with a Gilson FC 204 (Gilson, Middleton, WI, USA); F1 (1.4 – 3.4 min) contained urea, F2 (2.6 - 4.6 min) uric acid, F3 (4.6 – 7.0 min) hypoxanthine and F4 (7.4 – 9.0 min) contained AICAR. The collection windows were set sufficiently wide to collect the entire peak of the target compounds.
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F1: urea fraction

500 µL of the collected fraction was transferred to a tube and dried under OFN at 60°C. The ring closure was performed by adding 50 µL H₂O, 50 µL 0.3 M MBDMA and 80 µL 37% hydrochloric acid. The mixture was kept at room temperature for 1 h and dried under OFN at 40°C. 25 µL MTBSTFA was added and the vial was kept at 130°C for 20 min for derivatization.

F2 and F3: uric acid and hypoxanthine fraction

The fraction was dried, dissolved in 200 µL H₂O, transferred to a vial and dried under vacuum. 25 µL DMF – MTBSTFA (1:1) was added and the vial was kept at 130°C for 20 min for derivatization.

F4: AICAR fraction

Afterwards, the AICAR fraction was dried under OFN at 60°C, dissolved in 500 µL methanol, transferred to a vial and dried under OFN at 40°C. 90 µL 0.1M phosphate buffer and 10 µL PNP were added and kept overnight at 56°C in order to convert AICAR into AICA, followed by filtration on a 0.45 µm filter.

Following was a second semi-preparative HPLC fraction collection with an injection volume of 100 µL. This second HPLC fraction collection was mainly executed to remove the hypoxanthine that is present in the PNP enzyme solution. Removal of hypoxanthine is essential as AICA is transformed in hypoxanthine in the following steps before analysis on IRMS. Separation was achieved using a Phenomenex Gemini C18 (150 mm x 4.6 mm x 5 µm) column (Torrance, California, USA) maintained at 35°C. The mobile phase flow rate was 1 mL/min using an ACN/H₂O gradient (solvent A: H₂O 5 mM ammonium formate, 0.05 % formic acid; solvent B: 9/1 ACN/H₂O 5 mM ammonium formate, 0.05 % formic acid) that started with 100% of solvent A, held for 3 min then 100% B at 4 min, held until 8 min, 100% A at 9 min and held until 14 min. Photodiode array detection was performed at 245 and
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285 nm. AICA and hypoxanthine were used as retention time markers to check the collection window. One fraction containing AICA was collected (2.3 – 3.4 min).

The AICA fraction was dried under OFN at 60°C, dissolved in 200 µL H₂O, transferred to a vial and dried under vacuum. 100 µL DEMA was added and kept at 120°C for the ring closure reaction. Afterwards, the residue was dried under vacuum. 20 µL MTBSTFA was added and the vial was kept at 130°C for 20 min for derivatization.

**GC-MS**

An Agilent 6890 GC coupled to a 5975B VI MSD from Agilent Technologies (Palo Alto, CA, USA) was used with helium as carrier gas at a flow of 1.4 mL/min. 2 µL pulsed splitless injections (70°C, 0.2 min at 18 psi) on a Gerstel PTV injector (Mulheim an der Ruhr, Germany) were used. The temperature of the liner was increased at 720°C/min until 300°C, kept for 2 min and further increased until 400°C at 720°C/min. The GC column (30 m x 0.25 mm x 0.25 μm) was an Agilent J&W DB-17MS. The column temperature was held at 70°C for 0.1 min, increased until 160°C with a rate of 10°C/min and then increased until 310°C with a rate of 25°C/min. The total run time was 17.1 min. The MSD acquired data in full scan mode using Chemstation software (Agilent, Waldbronn, Germany).

**GC-C-IRMS**

An Agilent 7890A GC (Palo Alto, CA, USA) was coupled to a Thermo GC-Isolink, a Thermo Conflon interface and a Thermo Scientific MAT253 IRMS (Bremen, Germany) to determine the δ¹⁵N value of the compounds of interest. The GC conditions were exactly the same as those described for full-scan GC-MS analysis. Data was acquired using ISODAT NT 3.0 software (Thermo Scientific, Bremen, Germany). Figure 9.4 visualizes the chromatogram of such an IRMS analysis.
Figure 9.4: Chromatogram of a GC-C-IRMS analysis

The IRMS was calibrated against the IRMS standards urea ($\delta^{15}N = 20.17 \, \%\text{oo}, \sigma = 0.08 \, \%\text{oo}$) and caffeine ($\delta^{15}N = -2.90 \, \%\text{oo}, \sigma = 0.03 \, \%\text{oo}; \delta^{13}C = -35.05 \, \%\text{oo}, \sigma = 0.02 \, \%\text{oo}$) that were obtained from Indiana University. These cover the calibration range where natural $\delta^{15}N$ values are situated (cf. Figure 9.2). Ideally, the $N_2$ reference pulse is calibrated against an isotopic standard with a chemical structure that is very similar or equal to that of the analytical compounds that need to be analyzed on IRMS. It is important that reference materials have similar chemical properties because the combustion efficiency may vary, changing the isotopic composition of the gases. Using caffeine and urea as isotopic standards fulfils this requirement (cf. Figure 9.5).
3 Results and discussion

3.1 AICAR quantification by LC-MSMS

Method validation

Due to the presence of endogenous AICAR in urine, it is unsuitable to fortify urine with AICAR for the preparation of the calibration curve and fortified water was used instead. To verify that the same response is generated in water and urine, three calibration curves were prepared in water and three in urine. The unpaired t-test (p = 0.261, 95 % confidence interval) was used to confirm that the mean slope of the calibration curves in water and urine is identical, illustrating that if matrix effects occur in urine, they are compensated by the IS and water can be used for the calibration curve. The validation results are specified in Table 9.2. The linearity of the method was assessed in the range 0.1 – 4 µg/mL by spiking water with respective amounts of AICAR (0.1, 0.3, 0.8, 1.5, 2.5, 4 µg/mL). The accuracy and repeatability was tested by the analysis of spiked water at three concentration levels (0.1, 1.5, 4 µg/mL) with three replicates at each concentration level. Two additional batches were analyzed on another day by two different operators in order to determine the reproducibility. Matrix effects (ion suppression/enhancement) were examined by analysing 20 urine samples (and determining the endogenous concentration), fortifying those 20 urine samples with
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a known amount of AICAR and reanalyzing the samples. As expected, any occurring matrix effects were compensated by the IS.

Table 9.2: LC-MS/MS validation results

<table>
<thead>
<tr>
<th></th>
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<th>1.5 μg/mL</th>
<th>4 μg/mL</th>
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<td><strong>Accuracy</strong></td>
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<td></td>
<td></td>
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<tr>
<td>n = 3+3+3</td>
<td>95.0 %</td>
<td>93.4 %</td>
<td>99.9 %</td>
</tr>
<tr>
<td><strong>Precision</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Repeatability</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 3+3+3</td>
<td>2.1 %</td>
<td>3.3 %</td>
<td>7.2 %</td>
</tr>
<tr>
<td><strong>Reproducibility</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 9+9+9</td>
<td>8.4 %</td>
<td>6.5 %</td>
<td>8.1 %</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Linearity (0.1 – 4 μg/mL)</th>
<th>Correlation coefficient ($r^2$)</th>
<th>Slope</th>
<th>Intercept</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.9977</td>
<td>3.1497</td>
<td>-0.191855</td>
</tr>
</tbody>
</table>

Reference population

888 urine samples (631 athletes, 257 non-athletes) were analyzed according to the described method. None of them had interfering peaks in the MRM chromatograms at the respective retention time (2.71 min). Urine samples with concentrations outside the linear range (0.1 – 4 μg/mL) were diluted and re-analyzed.

A right skewed distribution was obtained for both populations (athletes and non-athletes) (Figure 9.6). The program REFVAL was used for the determination of the upper reference limits as recommended by the Expert Panel on Theory of Reference values of the International Federation of Clinical Chemistry. [29,30] The calculated 99 % reference limit (95 % confidence interval) was 1.7 μg/mL for the non-athlete
population and 3.1 µg/mL for the athlete population. The samples of the athlete population were randomly selected routine doping analysis urine samples (from amateur and elite athletes) and are therefore a direct representation of the kind of samples we are dealing with in doping control. Based upon this reference limit, all routine urine samples that contain AICAR concentrations above 5 µg/mL should be forwarded to an IRMS conformational procedure. A threshold of 5 µg/mL (slightly above the calculated reference limit of 3.1 µg/mL) was chosen in order to avoid having to reanalyze and confirm too many samples that are in fact negative with a time consuming IRMS method, taking into account that the expected AICAR concentration after AICAR administration will peak at more than 100 µg/mL, at least for a time period of approximately 10 h after administration as stated in a previous publication. [13] In addition, this study calculated a 99 % reference limit of 6.9 µg/mL, but by using a population of elite athletes (n = 459).
Figure 9.6: AICAR concentrations for an athlete and non-athlete population
Chapter 9: Confirmation Method for AICAR

3.2 AICAR confirmation by IRMS

The exogenous $\delta^{15}$N values of four AICAR preparations were determined and are given in Table 9.3. The values ranged from -4.40 until -2.26 ‰ which was within our expectations.

Table 9.3: Exogenous $\delta^{15}$N values for AICAR

<table>
<thead>
<tr>
<th>Exogenous AICAR</th>
<th>$\delta^{15}$N values AICAR (‰)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AICAR TRC</td>
<td>-4.40</td>
</tr>
<tr>
<td>AICAR Sigma</td>
<td>-2.26</td>
</tr>
<tr>
<td>AICAR customs seizure 1</td>
<td>-3.88</td>
</tr>
<tr>
<td>AICAR customs seizure 2</td>
<td>-3.95</td>
</tr>
</tbody>
</table>

Endogenous $\delta^{15}$N values for urea, uric acid, hypoxanthine and AICAR were determined in 14 urine samples (Table 9.4). The $\delta^{15}$N values for urea ranged from 4.40 until 7.01 ‰. These values were as expected and according to literature (cf. Figure 9.2). [23,25] However, the $\delta^{15}$N values for AICAR were depleted to a great extent (-6.85 until -3.23 ‰), making it impossible to differentiate between endogenous and exogenous AICAR. Hypoxanthine is systematically less depleted than AICAR and uric acid is systematically less depleted than hypoxanthine. This suggests that endogenous synthesis of AICAR itself is accompanied with a very large kinetic isotopic effect, resulting in $\delta^{15}$N values that are a lot lower than those found in literature. The presented data in those publications is generally obtained by calculating the total $\delta^{15}$N value of urine or tissue, and not of separate compounds. In urine for example, urea represents about 90 % of all nitrogen atoms and uric acid contributes only 1 – 2 ‰.
Chapter 9: Confirmation Method for AICAR

As it became clear that the nitrogen isotope is incapable of differentiating between endogenous and exogenous AICAR and because an IRMS confirmation method for AICAR had become available, all further research concerning AICAR was suspended.

[31]

Table 9.4: Endogenous $\delta^{15}$N values

<table>
<thead>
<tr>
<th>Sample</th>
<th>Urea</th>
<th>Uric acid</th>
<th>Hypoxanthine</th>
<th>AICAR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.09</td>
<td>-0.53</td>
<td>-</td>
<td>-4.96</td>
</tr>
<tr>
<td>2</td>
<td>6.60</td>
<td>-1.14</td>
<td>-</td>
<td>-3.23</td>
</tr>
<tr>
<td>3</td>
<td>4.40</td>
<td>-1.52</td>
<td>-</td>
<td>-5.77</td>
</tr>
<tr>
<td>4</td>
<td>5.56</td>
<td>-1.38</td>
<td>-2.32</td>
<td>-5.89</td>
</tr>
<tr>
<td>5</td>
<td>5.40</td>
<td>-1.40</td>
<td>-</td>
<td>-6.85</td>
</tr>
<tr>
<td>6</td>
<td>6.02</td>
<td>-1.83</td>
<td>-2.63</td>
<td>-3.72</td>
</tr>
<tr>
<td>7</td>
<td>5.93</td>
<td>-1.48</td>
<td>-1.69</td>
<td>-6.69</td>
</tr>
<tr>
<td>8</td>
<td>7.01</td>
<td>0.14</td>
<td>-</td>
<td>-5.56</td>
</tr>
<tr>
<td>9</td>
<td>5.14</td>
<td>-2.24</td>
<td>-3.06</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>5.39</td>
<td>-1.91</td>
<td>-2.63</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-3.40</td>
</tr>
<tr>
<td>12</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-4.03</td>
</tr>
<tr>
<td>13</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-4.08</td>
</tr>
<tr>
<td>14</td>
<td>-</td>
<td>-2.19</td>
<td>-3.33</td>
<td>-</td>
</tr>
<tr>
<td>average</td>
<td>5.78</td>
<td>-1.39</td>
<td>-2.67</td>
<td>-4.98</td>
</tr>
<tr>
<td>SD</td>
<td>0.77</td>
<td>0.80</td>
<td>0.51</td>
<td>1.26</td>
</tr>
</tbody>
</table>
4 Conclusions

Based upon a reference population of 888 urine samples, a threshold of 5 µg/mL was set for AICAR. Higher concentrations are recommended for confirmation by IRMS.

Our data shows that it is impossible to differentiate between endogenous and exogenous AICAR by using the nitrogen isotope. Very recently however, a GC-C-IRMS method has been developed which is capable of detecting exogenous AICAR abuse by using the carbon isotope. [31]
5 References


Chapter 9: Confirmation Method for AICAR


Chapter 9: Confirmation Method for AICAR

Postlude
Chapter 10: General Discussion and Future Perspectives
Endogenous compounds, especially endogenous AAS are widely misused in sports and their detection remains difficult. These substances are naturally produced by the human body and a distinction between a synthetic and an endogenous origin needs to be made. Differentiation between both is possible with IRMS, but this remains a complicated, time consuming and costly technique. Optimization of IRMS as a confirmation technique is required on two different levels. On a first level, the IRMS technique itself needs to be exploited to its full potential. On a second level, the diagnostic accuracy and efficiency of the screening needs to be increased, leading to a reduced amount of samples that need confirmation by IRMS.

One of the mayor drawbacks of IRMS is its lack of sensitivity. This forces the analyst to use a substantial amount of urine in order to obtain a sufficient amount of compound for adequate IRMS analysis. Higher amounts of urine induce a higher background and require a more extensive sample clean up which prolongs the method. By using solvent vent injections on a PTV, an IRMS method with increased sensitivity was developed for the detection of endogenous AAS abuse. This allowed a reduction of the required amount of urine by a factor of five, simplifying the sample preparation procedure.

WADA publishes and imposes technical documents that describe how doping control laboratories have to perform their analyses and this is also the case for GC-C-IRMS. GC-C-IRMS and its application to doping control have evolved a lot during the last decade and so have the WADA technical documents that describe how GC-C-IRMS should be applied. In 2014, WADA introduced a new technical document (TD2014IRMS) with a great deal of novelties such as a number of obligatory QCs and a fixed analytical methodology that should be used, along with new calibration standards. [1] This document imposed more uniform methods between the different doping control laboratories in order to decrease the inter laboratory SD and acquire similar results for the analysis of the same urine samples. All these new developments were implemented and comparison between data collected before and after the implementation of TD2014IRMS did not show a significant shift in CIR
values, confirming the reliability and validity of the DoCoLab GC-C-IRMS confirmation protocol since its launch.

An IRMS confirmation procedure contains many steps (SPE, LLE, hydrolysis, acetylation,..) which complicates troubleshooting to a great extend. When deviating CIR are obtained it can be difficult to locate the cause. The problem can be situated in one or multiple sample preparation steps or originate from a malfunctioning GC-C-IRMS instrument. In this light, the importance of the different QCs that are present in the IRMS method cannot be stressed enough.

The switch from splitless injections to solvent vent injections on the GC-C-IRMS instrument also opened other possibilities and new opportunities for doping control purposes. NA and B are AAS that can be abused by athletes. In some rare cases however, they can also be produced endogenously in very low amounts. [2,3] Here the necessity arises for a GC-C-IRMS method that can measure the CIR at such a low concentrations and verify if a doping violation occurred. Due to the modification in the injection mechanism and enhanced sensitivity, GC-C-IRMS confirmation methods for NA and B could be developed, validated and put into practice for routine purposes. As CIR determination of NA and/or B requires a pure compound without contaminations, an extensive sample clean up with two HPLC-FCs was essential.

Likewise, the increased sensitivity allowed CIR determination of the minor metabolites F and 6αOH-ADION. Minor steroid metabolites had already proven their relevance and importance for specific detection of endogenous AAS abuse in the past. [4,5] Recently however, the introduction of the GC-MS/MS provided opportunity for a more sensitive screening and allowed a reliable quantification of these minor metabolites, even at very low concentrations. [6] As minor metabolites could now be implemented in the routine screening as additional steroid profile parameters, appropriate thresholds were needed to establish a concentration range that should be regarded as suspicious and for which confirmation by IRMS is
needed. By analyzing reference population samples and samples from excretion studies on both GC-MS/MS and GC-C-IRMS and combining the data suitable thresholds could be set up. Because of the availability of a sensitive IRMS, urine samples with low concentrations of these minor metabolites could still be analyzed and their CIR could still be determined. Introduction of these new thresholds has been another step forward towards a more efficient screening and in extend a more efficient use of IRMS as confirmation technique. Minor metabolites occur in low concentrations and hence, are less prone to endogenous dilution which can be problematic for major metabolites like A and Et. After administration, the relative increase of minor metabolites is higher than that of major metabolites, resulting in a larger fraction of exogenous compound and facilitating the detection of doping abuse by IRMS. [7,8]

In doping control laboratories, IRMS is currently mainly applied for the analysis of endogenous AAS and their metabolites. There are other endogenous compounds however that can also be used as doping such as AICAR and glycerol. Developing IRMS confirmation methods for this type of compounds is not straightforward because of their polarity. Such compounds cannot be analyzed and introduced on a GC-C-IRMS in their free form and require (extensive) derivatization. This means adding extra carbon atoms with a different isotope ratio to the compound. If the $^{13}$C isotope ratio is measured, as is the case with steroids, a correction has to be made and this correction (and the additional variation) will be substantial as the compounds themselves contain only a limit amount of carbon atoms. Moreover, extracting polar compounds out of a polar matrix (i.e. urine) and purifying them is much more difficult than for apolar compounds like AAS. Progress is being made and for AICAR for example an IRMS confirmation method has recently been developed. [9] The method uses the $^{13}$C isotope ratio as the $^{15}$N isotope ratio is incapable of differentiating between an endogenous and an exogenous origin.
Inter laboratory comparison experiments

Inter laboratory experiments might be useful to further decrease the inter laboratory SD and to map differences in reference populations between the different doping control laboratories. At this stage there are parallels and differences between the laboratories and their populations and it unclear how to clarify these in a consistent manner. The origin could be related to the population (different biology and physiology). There could also be an analytical cause, resulting in (small) deviations between laboratories. Analytical causes for observed dissimilarities have been reported in the past. [10] In this context, analyzing an identical small population by different laboratories should bring clarification.

H²/H¹ ratios of endogenous AAS

Sometimes the differences in CIR between endogenous and synthetic AAS are not always large enough to be able to distinguish them. There have been synthetic preparations reported with CIR values in (or very close to) the endogenous range. [11–13] Here, an IRMS analysis of the H²/H¹ ratio might offer a solution to avoid false negatives. For endogenous steroids, the HIR is largely dependent on drinking water whereas CIR is dependent on food intake. Thus, HIR and CIR should be independent of each other allowing detection of steroid abuse where CIR alone might fail. At this moment only a few doping control laboratories apply HIR analyses. [10,14] A lot remains to be investigated, but a combined CIR and HIR analysis has potential and in the future more laboratories might implement the technique.
Chapter 10: General Discussion and Future Perspectives

LC-IRMS

In 2014, a pilot study was published concerning the use of LC-IRMS for the CIR determination of endogenous AAS. [15] LC-IRMS is making progress, but at this stage it is clearly inferior to GC-C-IRMS. Step by step, the technical difficulties of LC-IRMS are being tackled but routine applications for doping control analyses have not yet been reported. LC-IRMS does have a lot of potential however for polar endogenous compounds like AICAR and glycerol as there is no need for derivatization. Polar compounds are very difficult to analyze by GC-C-IRMS and LC-IRMS could very well be the solution.

Athlete biological passport

For a long time, population based references limits have been the sole manner to establish decision limits. Unfortunately, these reference limits are set up by using population data which are characterized by large inter individual variation. These population based thresholds are often not effective enough leading to false negatives, especially for individuals with naturally low basal values. In addition these population based reference limits invoke a high amount of samples that need to be confirmed by IRMS. In the mean time, a new evolution with the potential to drastically improve the detection efficiency of endogenous steroid abuse has been introduced. Sottas et al. used an adaptive algorithm that implemented population based and subject based information for the T/EpiT ratio to set up reference ranges for an individual athlete at each time of the build-up of a longitudinal data series. [16] This approach has now been implemented in the steroidal module of the ABP by WADA. [17] This ABP stores the collected results of an athlete by doping control laboratories world-wide and allows the systematic follow up of longitudinal data. With each measurement, the ABP progressively adapts the calculated upper and lower reference limits, evolving from a population based to an individual based
threshold. As the number of test records increases, the calculated reference limits adapt from population thresholds (i.e. T/EpiT > 4) towards individual thresholds leading to increased sensitivity and specificity. Over time, the use of the ABP will lead to a change in the types of samples submitted to IRMS analysis. More T/EpiT < 4 samples will undergo an IRMS analysis, while other ones with T/EpiT > 4 will no longer require testing. This drastic and fundamental change will be a big factor for IRMS analysis moving forward and should result in a more selective and efficient use of a costly and time consuming IRMS confirmation. In effect, a more efficient screening for suspicious samples should reduce the financial cost of over confirming too many (false) positive screening results. At this stage, the number of steroid profile parameters that are being monitored by the ABP is limited (T/EpiT, A/T, A/Et and ααβ/βαβ) but in the future others might also be included (e.g. minor metabolites), further enhancing the sensitivity and specificity.
Chapter 10: General Discussion and Future Perspectives

References


Chapter 10: General Discussion and Future Perspectives


Chapter 10: General Discussion and Future Perspectives


Chapter 11: Summary

In order to detect the abuse of endogenous substances, doping control laboratories require methods that are capable of differentiating between endogenous compounds and their synthetic copies. GC-C-IRMS is able to differentiate between both of them by measuring their isotope ratio. GC-C-IRMS and its application to doping control has evolved a lot during the last years and so have the WADA technical documents that describe how GC-C-IRMS should be applied. The main goal of this thesis was to develop, extend, update and validate GC-C-IRMS methods for various compounds (according to WADA requirements) and implement them at DoCoLab.

The thesis is subdivided in four parts, each coping with different aspects of GC-C-IRMS as a doping control technique.

Part 1: improved confirmation method for testosterone

In 2010, DoCoLab’s first GC-C-IRMS method was developed and validated. The method aimed at detection of T (or T prohormones) abuse by CIR analysis of the main T metabolites (A, Et, ααβ and βαβ) as TCs and PD as ERC. [1] Starting from January 2011 the method was implemented in the routine procedure of the laboratory and subsequently the first samples from sport federations were analyzed.

In the following years this method has been expanded and improved. In September 2011, T and EpiT were added to the method as additional TCs and diethyl ether was replaced by n-pentane in the LLE procedure. By adding T to the method, it was now possible to measure the CIR of T itself (and not only its main metabolites), enhancing the efficiency of the IRMS confirmation procedure. Since February 2012, splitless injections were replaced by solvent vent injections on a PTV (chapter 3). These solvent vent injections were a big step forward as they drastically increased the sensitivity of our IRMS and allowed us to simplify our sample preparation.
procedure by reducing the required urine volume for IRMS analysis by a factor of 5. For a TC with a urinary concentration of 50 ng/mL we now only needed 5 mL of urine instead of 25 mL in the past.

In 2013, 11-oxoEt was introduced as secondary ERC. A supplementary ERC has its value in cases were the primary ERC (PD) is suppressed, suffers from poor chromatography or if pregnenolone has been administered. In addition, new QCs were added and the calibration procedure was altered in order to comply with WADA regulations. [2] The n-alkane calibration mixture was replaced by CU-USADA 33-1 but this did not induce a shift in the $\delta^{13}$C value of the reference gas (or any of the QCs) (chapter 4).

From January 2011 until December 2014 907 routine samples from athletes have been analyzed with this method; 12 samples were found positive (Table 10.1). The collected data was investigated and interpreted, covering different areas relevant to doping control with a special emphasis on calibration, quality control and measurement uncertainty.
<table>
<thead>
<tr>
<th>Year</th>
<th>Samples</th>
<th>Positive</th>
<th>T/EpiT</th>
<th>Sport</th>
</tr>
</thead>
<tbody>
<tr>
<td>2011</td>
<td>209</td>
<td>3 (1.4%)</td>
<td>21.8</td>
<td>autoracing</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10.2</td>
<td>fitness</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>22.6</td>
<td>fitness</td>
</tr>
<tr>
<td>2012</td>
<td>171</td>
<td>1 (0.6%)</td>
<td>12.6</td>
<td>shooting</td>
</tr>
<tr>
<td>2013</td>
<td>158</td>
<td>4 (2.5%)</td>
<td>30.0</td>
<td>fitness</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>82.2</td>
<td>fitness</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.4</td>
<td>cycling</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.4*</td>
<td>cycling</td>
</tr>
<tr>
<td>2014</td>
<td>369</td>
<td>4 (1.1%)</td>
<td>81.1</td>
<td>powerlifting</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>57.3</td>
<td>fitness</td>
</tr>
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<td></td>
<td></td>
<td>25.8</td>
<td>cycling</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>9.4</td>
<td>cycling</td>
</tr>
<tr>
<td>total</td>
<td>907</td>
<td>12 (1.3%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Samples Type</th>
<th>Samples</th>
<th>Positive</th>
<th>Sport</th>
</tr>
</thead>
<tbody>
<tr>
<td>B/BM samples (since 2012)</td>
<td>16</td>
<td>5 (31.3%)</td>
<td>kick boxing</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>athletics</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>fitness</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>fitness</td>
</tr>
<tr>
<td>NA samples (since 2013)</td>
<td>9</td>
<td>3 (33.3%)</td>
<td>powerlifting</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>weightlifting</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>fitness</td>
</tr>
</tbody>
</table>

Table 10.1: Overview of routine samples confirmed by GC-C-IRMS

*sample with a suspicious 6αOH-ADION concentration (cf. p199)
Part 2: confirmation methods for NA, B and F

NA and B are AAS that are normally not detected in urine from athletes that are not using doping. However, in some rare cases they can be present endogenously in low concentrations. Endogenous F is always present in every urine sample, but in (very) low amounts. Due to the development of more sensitive screening methods, a higher number of urine samples with NA, B or F present are being reported and these samples need an IRMS confirmation analysis to determine whether the detected NA, B or F has an endogenous or exogenous origin. This implies that a GC-C-IRMS method is required that can determine the CIR at such a low concentrations. Intrinsically, IRMS is a very precise technique, but it is not sensitive at all, meaning that these compounds provide a substantial challenge. However, due to the use of solvent vent injections, the sensitivity of our IRMS was drastically increased and confirmation methods could be developed, validated and implemented in the laboratory in accordance with WADA specifications. [2,3]

To comply with these stringing demands, a separated second IRMS method was put into effect. CIR determination of NA and/or F concentrations as low as 2 ng/ml could be achieved by using 25 mL of urine. By using two HPLC fraction collections, the sample clean up proved to be sufficient and a good repeatability and reproducibility were obtained (chapter 5). Two ERCs were included in the method: PD and 11oxo-Et.

A third GC-C-IRMS method was developed for B. The method used two HPLC fraction collections for sample clean up and the CIR of both B and BM could be determined at concentrations as low as 2 ng/mL (chapter 6).
Chapter 11: Summary

Part 3: steroid profile thresholds for minor metabolites

The introduction of more sensitive screening methods in doping control, allows a reliable quantification of minor metabolites. The value of these minor metabolites as additional steroid profile parameters had been recognized for some time, but without reliable quantification they could not be implemented. Nowadays, most laboratories have switched to the more sensitive GC-MS/MS for their screening and appropriate thresholds were needed in order to decide which concentration range should be regarded as suspicious and needs confirmation by IRMS.

The two most important minor metabolites are F and 6αOH-ADION. These compounds are present in urine in low to very low amounts and after administration of T there is a sharp increase of their urinary concentrations. The combination of a low endogenous concentration and a sharp increase after administration makes it easier to spot T abuse in the steroid profile.

Because of the availability of a sensitive IRMS, urine samples with low concentrations of these minor metabolites could still be analyzed and their CIR could be determined. As both instruments, the GC-MS/MS and GC-C-IRMS, were sensitive enough, data from both could be collected and combined and suitable thresholds could be established (chapter 7 and 8 for F and 6αOH-ADION respectively).

Part 4: confirmation method for AICAR

According to a paper of 2008 the use of AICAR caused an improved endurance of untrained mice by 23 - 44 %. [4] This attracted particularly attention of the sports drug testing community and in 2009 AICAR was added to the WADA prohibited list. [5] AICAR is an endogenous substance and in analogy with endogenous steroids, there was need for an IRMS method to unambiguously prove the exogenous nature
of the detected AICAR. Developing a GC-C-IRMS method for AICAR provides a substantial analytical challenge because of its polarity. It is a very polar compound with multiple polar functions: three alcohol functions, one amine and one amide for only nine carbon atoms. In order to be able to analyze and introduce such a compound on a GC, the polar functions have to be derivatized and converted to apolar groups.

AICAR contains 4 nitrogen atoms and in chapter 9 the potential of using the nitrogen isotope for an IRMS confirmatory method was explored. Unfortunately, our data shows that it is impossible to differentiate between endogenous and exogenous AICAR by using this isotope.
Chapter 11: Summary

References


Chapter 12: Samenvatting
Om misbruik van endogene substanties te kunnen opsporen, vereisen dopingcontrole laboratoria methodes die in staat zijn om te differentiëren tussen endogene componenten en hun synthetische kopieën. GC-C-IRMS kan beide onderscheiden door de isotopen ratio te meten. GC-C-IRMS en zijn toepassingen bij dopingcontrole hebben een intense evolutie ondergaan de laatste jaren, alsook de technische documenten van WADA die beschrijven hoe GC-C-IRMS dient toegepast te worden. De primaire focus van deze thesis was de ontwikkeling, uitbreiding en validatie van GC-C-IRMS methodes voor verscheidene componenten (in overeenstemming met de WADA vereisten) en de implementatie van deze methodes in DoCoLab.

De thesis is onderverdeeld in vier delen waarbij elk deel zich richt op een verschillend aspect van GC-C-IRMS als dopingcontrole techniek.

**Deel 1: confirmatiemethode voor testosteron**

DoCoLab’s eerste GC-C-IRMS methode werd ontwikkeld en gevalideerd in 2010. De methode is in staat om T misbruik op te sporen aan de hand van CIR analyse van de hoofdmetabolieten van T (A, Et, ααβ en βαβ) als TCs en PD als ERC. [1] De methode werd geïmplementeerd in de routineprocedure van het laboratorium vanaf januari 2011 en vervolgens werden de eerste stalen van sportfederaties geanalyseerd.

Sindsdien werd in de loop der jaren de methode uitgebreid en geoptimaliseerd. In september 2011 werden T en EPIT toegevoegd als bijkomende TCs en diethyl ether werd vervangen door n-pentaan bij de LLE procedure. Nu was het mogelijk om de CIR van T zelf (en niet enkel zijn hoofdmetabolieten) te bepalen wat de efficiëntie van de IRMS confirmatiemethode ten goede kwam. Sinds februari 2012 werden de “splitless” injecties vervangen door “solvent vent” injecties op een PTV (**hoofdstuk 3**). De introductie van deze “solvent vent” injecties was een grote stap vooruit omdat ze de gevoeligheid van het IRMS toestel drastig verhogen en toelieten om de staalvoorbereiding te vereenvoudigen door de vereiste hoeveelheid urine voor
Chapter 12: Samenvatting

IRMS analyse met een factor 5 te reduceren. Voor een TC met een concentratie van 50 ng/mL is er nu slechts 5 mL nodig, tegenover 25 mL in het verleden.

In 2013 werd 11-oxoEt geïntroduceerd als secundaire ERC. Een bijkomende ERC heeft zijn nut in gevallen waar de primaire ERC (PD) onderdrukt is, bij slechte chromatografie of als pregnenolone werd ingenomen. Daarnaast werden nieuwe QCs toegevoegd aan de methode en werd de calibratieprocedure aangepast om te voldoen aan de WADA reglementeringen. [2] Het n-alkaan calibratiemengsel werd vervangen door CU-USADA 33-1, maar dit veroorzaakte geen verschuiving in de $\delta^{13}$C waarde van het referentiegas (of één van de QCs) (Hoofdstuk 4).

Vanaf januari 2011 tot december 2014 werden 907 routinestalen van atleten geanalyseerd met deze methode; 12 stalen waren positief (Tabel 11.1). De gecollecteerde data werden onderzocht en geïnterpreteerd met nadruk op calibratie, kwaliteitscontrole en meetonzekerheid.
## Chapter 12: Samenvatting

<table>
<thead>
<tr>
<th></th>
<th>Aantal stalen</th>
<th>Positieve stalen</th>
<th>T/EpiT</th>
<th>sport</th>
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<td>2011</td>
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<td>3 (1.4 %)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>10.2</td>
<td>fitness</td>
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<td></td>
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<td></td>
<td>22.6</td>
<td>fitness</td>
</tr>
<tr>
<td>2012</td>
<td>171</td>
<td>1 (0.6 %)</td>
<td>12.6</td>
<td>schieten</td>
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<tr>
<td>2013</td>
<td>158</td>
<td>4 (2.5 %)</td>
<td>30.0</td>
<td>fitness</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>82.2</td>
<td>fitness</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>2.4</td>
<td>wielersport</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>0.4*</td>
<td>wielersport</td>
</tr>
<tr>
<td>2014</td>
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<td>4 (1.1 %)</td>
<td>81.1</td>
<td>powerlifting</td>
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<td></td>
<td></td>
<td>57.3</td>
<td>fitness</td>
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<td>9.4</td>
<td>wielersport</td>
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<tr>
<td>totaal</td>
<td>907</td>
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</tr>
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</table>

<table>
<thead>
<tr>
<th></th>
<th>Aantal stalen</th>
<th>Positieve stalen</th>
<th>T/EpiT</th>
<th>sport</th>
</tr>
</thead>
<tbody>
<tr>
<td>B/BM stalen (vanaf 2012)</td>
<td>16</td>
<td>5 (31.3 %)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>fitness</td>
</tr>
<tr>
<td>NA stalen (vanaf 2013)</td>
<td>9</td>
<td>3 (33.3 %)</td>
<td></td>
<td>powerlifting</td>
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<tr>
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<td>gewichtheffen</td>
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<td></td>
<td></td>
<td>fitness</td>
</tr>
</tbody>
</table>

Tabel 12.1: Overzicht van de routinestalen die werden geconfirmeerd met GC-C-IRMS

*staal met een verdachte 6αOH-ADION concentratie (cf. p199)
Deel 2: confirmatiemethodes voor NA, B and F

NA en B zijn androgene anabole steroïden die onder normale omstandigheden niet voorkomen in humane urine. In uitzonderlijke gevallen kunnen ze echter endogeen voorkomen in lage concentraties. Endogeen F is aanwezig in elk urinestaal, maar ook in (zeer) lage concentraties. Door de ontwikkeling van screeningsmethodes met een hogere gevoeligheid wordt er een hoger aantal stalen met gedetecteerd NA, B of F gerapporteerd. Deze stalen moeten worden geanalyseerd met een IRMS confirmatieprocedure om te bepalen of de gedetecteerde NA, B of F endogeen of exogeen is. Dit impliceert dat er nood is aan een GC-C-IRMS methode die de CIR kan bepalen bij dergelijke lage concentraties. IRMS is een zeer precieze techniek, maar ze is helemaal niet gevoelig waardoor deze componenten een substantiële uitdaging vormen. Echter, door gebruik te maken van “solvent vent” injecties kon de gevoeligheid van de IRMS drastisch worden opgedreven en konden confirmatiemethodes worden ontwikkeld, gevalideerd en geïmplementeerd in overeenstemming met de WADA specificaties. [2,3]

Om aan deze strenge voorwaarden te kunnen voldoen werd er een tweede aparte IRMS methode in gebruik genomen voor NA en F (hoofdstuk 5). Door gebruik te maken van maximum 25 mL urine is CIR bepaling van NA en/of F concentraties tot 2 ng/mL mogelijk. Twee HPLC fractiecollecties zorgen ervoor dat de componenten voldoende worden opgezuiverd. Twee ERC’s werden in de methode opgenomen: PD en 11oxo-Et.

Een derde GC-C-IRMS methode werd ontwikkeld voor B. Ook deze methode gebruikt twee HPLC fractiecollecties voor de staalopzuivering en de CIR van zowel B als BM kan worden bepaald tot concentraties van 2 ng/mL (hoofdstuk 6).
Deel 3: steroïdprofiel drempelwaarden voor “minor” metabolieten

De introductie van gevoeligere screeningsmethodes in dopingcontrole laat een betrouwbare kwantificering toe van “minor” metabolieten. De toegevoegde waarde van deze metabolieten aan het steroïdprofiel werd al enige tijd erkend, maar zonder betrouwbare kwantificering konden ze niet worden geïmplementeerd. Nu maken de meeste dopingcontrole laboratoria gebruik van de gevoeligere GC-MS/MS voor hun screening waardoor er geschikte drempelwaarden nodig zijn om te kunnen beslissen welke concentraties als verdacht dienen beschouwd te worden.

De twee belangrijkste “minor” metabolieten zijn F en 6αOH-ADION. Deze componenten zijn aanwezig in urine in lage tot zeer lage concentraties en na T administratie neemt hun concentratie zeer sterk toe. De combinatie van zeer lage endogene concentraties en sterke toename na administratie maakt het makkelijker om T misbruik te detecteren in het steroïdprofiel.

Door de beschikbaarheid van een gevoelige IRMS kunnen urinestalen met lage concentraties aan “minor” metabolieten geanalyseerd worden om hun CIR te bepalen. Doordat zowel de GC-MS/MS als de GC-C-IRMS gevoelig genoeg waren, konden data van beide instrumenten gecombineerd worden en geschikte drempelwaarden worden opgesteld (hoofdstuk 7 en 8 voor F en 6αOH-ADION respectievelijk).

Deel 4: confirmatiemethode voor AICAR

Volgens een publicatie van 2008 verhoogt het gebruik van AICAR het uithoudingsvermogen bij ongetrainde muizen met 23 – 44 %. [4] Dit zorgde voor een bijzondere aandacht voor AICAR bij de anti-doping gemeenschap en in 2009 werd AICAR toegevoegd aan de verboden lijst van het WADA. [5] AICAR is een endogene substantie en, in analogie met endogene steroïden, was er een IRMS
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methode nodig om de exogene oorsprong van het gedetecteerd AICAR ondubbelzinnig te kunnen aanduiden. Een GC-C-IRMS methode ontwikkelen voor AICAR vormt een substantiële analytische uitdaging omwille van zijn polariteit. AICAR is namelijk een zeer polaire component met meerdere polaire functies: drie alcoholfuncties, één amine en één amide voor slechts negen koolstofatomen. Om een dergelijke component te kunnen analyseren op een GC moeten de polaire functies worden gederivatiseerd tot apolare groepen.

AICAR bevat vier stikstofatomen en in hoofdstuk 9 werd het potentieel van het gebruik van het stikstofisotoop voor een IRMS confirmatiemethode onderzocht. Onze data tonen helaas aan dat het onmogelijk is om te differentiëren tussen endogeen en exogeen AICAR aan de hand van dit isotoop.
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Referenties


Postlude

Curriculum Vitae
1 Personal information

Michaël Polet

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Belgian nationality

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2 Education and work experience

February 2011 – February 2015

PhD studies were commenced at the Doping Control Laboratory, a part of Ghent University under supervision of Prof. Dr. Ir. P. Van Eenoo.

Responsible for the development, validation and accreditation of the anti-doping control methods on GC-C-IRMS, the execution of the routine IRMS analyses and the technical interpretation of the data.

26/07/2012 – 12/08/2012

Appointed as foreign expert during the Olympic Games at ADSC/GSK, Harlow, London.

18/09/2012 – 28/09/2012

Assisted and advised during the set up and validation of a GC-C-IRMS method for testosterone in the South African Doping Control Laboratory.

Since 01/2013

Appointed as Certifying Scientist

September 2009 – December 2010

PhD studies were commenced at Pfizer Analytical Research Park, a part of Ghent University under supervision of Prof. Dr. P. Sandra.

Research focussed on the development and evaluation of innovative column technologies for fast and high efficient HPLC.

2004 –2009

A master’s degree in Chemistry (magna cum laude) was obtained at Ghent University. The master thesis “Evaluation of unconventional microspheres in
Curriculum Vitae – February 2015

miniaturized liquid chromatography” was accomplished under supervision of Prof. Dr. P. Sandra.

1998 – 2004

A degree in secondary school was obtained in the Heilig-Hart college in Waregem. The secondary education focussed on Latin, Mathematics and Sciences.

3 Scientific contributions

3.1 Publications

P. Van Renterghem, M. Polet, L. Brooker, W. Van Gansbeke, P. Van Eenoo

Development of a GC/C/IRMS method – Confirmation of a novel steroid profiling approach in doping control

Steroids, 77, 11 (2012), p 1050-60

M. Polet, W. Van Gansbeke, K. Deventer, P. Van Eenoo

Development of a sensitive GC-C-IRMS method for the analysis of androgens


M. Polet, P. Van Renterghem, W. Van Gansbeke, P. Van Eenoo

Profiling of urinary formestane and confirmation by isotope ratio mass spectrometry


Metabolism of methylstenbolone studied with human liver microsomes and the uPA+/+ - SCID chimeric mouse model


M. Polet, P. Van Renterghem, W. Van Gansbeke, P. Van Eenoo

Studies on the minor metabolite 6a-hydroxy-androstenedione for doping control purposes and its contribution to the steroid profile

Drug Testing and Analysis, 6, 10 (2014), p 978-84.

M. Polet, P. Van Eenoo

GC-C-IRMS in routine doping control practice: three years of drug testing data, quality control and evolution of the method

Analytical and Bioanalytical Chemistry, in press, DOI: 10.007/s00216-014-8374-7
3.2 Proceedings

Development of a sensitive GC-C-IRMS method for the analysis of androgens

**M. Polet, W. Van Gansbeke, K. Deventer and P. Van Eenoo**

Editors: W. Schänzer, H. Geyer, A. Gotzman and U. Mareck


Sportverlag Strauss, Cologne

Profiling of urinary formestane and confirmation by isotope ratio mass spectrometry

**M. Polet, W. Van Gansbeke, P. Van Renterghem and P. Van Eenoo**

Editors: W. Schänzer, M. Thevis, H. Geyer and U. Mareck


Sportverlag Strauss, Cologne

Studies on the minor metabolite 6a-hydroxy-androstenedione for doping control purposes and its contribution to the steroid profile

**M. Polet, P. Van Renterghem, W. Van Gansbeke and P. Van Eenoo**

Editors: W. Schänzer, M. Thevis, H. Geyer and U. Mareck

Proceedings of the 32nd Cologne workshop on Dope Analysis, 2014.

Sportverlag Strauss, Cologne
3.3 Oral presentation

Development of a sensitive GC-C-IRMS method for the analysis of androgens

**M. Polet**, W. Van Gansbeke, K. Deventer and P. Van Eenoo

30th Cologne workshop on Dope Analysis

26/02/2012 – 02/03/2012

Development of a sensitive GC-C-IRMS method for the analysis of androgens in doping control

**M. Polet**, W. Van Gansbeke and P. Van Eenoo

Benelux Association of Stable Isotope Scientists annual meeting 2012

12/04/2012 – 13/04/2012

Profiling of urinary formestane and confirmation by isotope ratio mass spectrometry

**M. Polet**, W. Van Gansbeke, P. Van Renterghem and P. Van Eenoo

31th Cologne workshop on Dope Analysis

24/02/2013 – 01/03/2013

Studies on the minor metabolite 6a-hydroxy-androstenedione for doping control purposes and its contribution to the steroid profile

**M. Polet**, P. Van Renterghem, W. Van Gansbeke and P. Van Eenoo

32nd Cologne workshop on Dope Analysis
Confirmation of endogenous substance abuse in doping control by isotope ratio mass spectrometry

M. Polet, W. Van Gansbeke and P. Van Eenoo

7th International Symposium on Hormone and Veterinary Drug Residue Analysis

02/06/2014 – 05/06/2014

3.4 Posters

Summary of the Alternative Steroid Profiling WADA project


Presented at the 30th Cologne workshop on Dope Analysis

26/02/2012 – 02/03/2012

Advances in detection of endogenous steroids in doping analysis

P. Van Renterghem, M. Polet, W. Van Gansbeke and P. Van Eenoo

Presented at the 7th International Symposium on Hormone and Veterinary Drug Residue Analysis

02/06/2014 – 05/06/2014
4 Courses and seminars

Forensic Solutions Summit

Agilent Headquarters, Waldbronn, Germany

2011

Research Institute of Chromatography Seminar 2013 (Chemical Analysis)

Kortrijk, Belgium

22/01/2013