Forensic application of isotope ratio mass spectrometry: development and validation of GC-C-IRMS based methods in doping analysis

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List of abbreviations

11-Keto-Etio	11-keto-etiocholanolone
11β-OH-A	11β-hydroxy-androsterone
11β-HSD	11β-hydroxysteroids dehydrogenase
β-Bold	boldenone
19-NA	19-norandrosterone
19-NE	19-noretiocholanolone
3β-HSD	3β-hydroxysteroid dehydrogenase
5αAdiol	5α-androstane-3α,17β-diol
5βAdiol	5β-androstane-3α,17β-diol
5α-R	5α-reductase
5-ARI	5α-reductase inhibitor
Δ^1-SDH	Δ1-steroid-dehydrogenation
A	Androsterone
AAF	Adverse analytical finding
AAS	Anabolic androgenic steroids
ABP	Athlete Biological Passport
ACTH	Adrenocorticotropic hormone
ADD	Boldione
allo-THE	allo-tetrahydrocortisone
allo-THF	allo-tetrahydrocortisol
APMU	Athlete Passport Management Unit
AR	Androgen receptor
AREs	Androgen response elements
ATF	Atypical Finding
ATPF	Atypical Passport Finding
BHP	Benign prostatic hyperplasia
BM2	Boldenone metabolite
BUR	Blank urine sample
CAM	Crassulacean acid metabolism
CIR	Carbon isotope ratio
CHR	Corticotropin-releasing hormone
COPD	Chronic obstructive pulmonary disease
CYP	Cytochrome P450
DBS	Dried blood spots
DESA	Dexamethasone
DHEA	Dehydroepiandrosterone
DHT	5α-dihydrotestosterone
DPD	DNA-binding domain
E	Epitestosterone

E. Coli	Escherichia Coli
EAAS	Endogenous anabolic androgenic steroids
ER	Smooth endoplasmic reticulum
ERα/β	Estrogen receptor alpha/beta
ERC	Endogenous reference compound
Etio	Etiocholanolone
FSH	Follicle-stimulating hormone
GC	Gas-chromatography
GCs	Glucocorticoids
GC-C-IRMS	Gas-chromatography-combustion-isotopic ratio mass spectrometry
GC-MS	Gas-chromatography coupled to mass spectrometry
GC-MS/MS	Gas-chromatography coupled to tandem mass spectrometry
GnRH	Gonadotropin-releasing hormone
GR	Glucocorticoid receptor
GRE	Glucocorticoids response elements
H6PDH	Hexose-6-phosphate dehydrogenase
HPG-axis	Hypothalamic-pituitary-gonadal axis
HPLC	High performance liquid chromatography
HSD	Hydroxysteroid dehydrogenase
Hsp	Heat shock protein
IAAF	International Association of Athletics Federations
IAEA	International Atomic Energy Agency
IOC	International Olympic Committee
IRMS	Isotopic ratio mass spectrometry
ISCCS	International Standard for Code Compliance by Signatories
ISL	International Standard for Laboratories
ISO	International Organization for Standardization
ISPPPI	International Standard for the Protection of Privacy and Personal Information
ISTDRI	Internal standard for IRMS measurements
ISTI	International Standard for Testing and Investigations
ISTUE	International Standard for Therapeutic Use Exemptions
LBD	Ligand-binding domain
LC	Liquid chromatography

LC-MS	Liquid chromatography coupled to mass spectrometry
LC-MS/MS	Liquid chromatography coupled to tandem mass spectrometry
LH	Luteinizing hormone
LOD	Limit of detection
LOQ	Limit of quantification
LVI	Large volume injection
MCR2	Melanocortin receptor
MRPL	Minimum Required Performance level
MR	Mineralocorticoid receptor
MS	Mass spectrometry
MT	17α-methyltestosterone
NADPH	Nicotinamide adenine dinucleotide phosphate
NET	5β-estran-17α-ethynyl-3α,17β-diol
NIST	National Institute of Standards and Technology
NTD	N-terminal domain
P450scc	Cytochrome P-450 side chain cleavage enzyme
P450c17	Steroid 17 alpha-hydroxylase/17,20 lyase
PAPS	3'–phosphoadenosine-5'-phosphosulfate
PBR	Peripheral benzodiazepine receptor
PD	Pregnanediol
POMC	Pro-opiomelanocortin
PR	Progesterone receptor
PRED	Prednisone
PLONE	Prednisolone
PT	Pregnanetriol
PTV	Programmed temperature vaporizer
SARM	Selective androgen receptor modulator
SG	Specific gravity
SHBG	Sex hormone binding globulin
SPE	Solid phase extraction
SRE	Steroid responsive element
SSP	Suspicious steroid profile
StAR	steroidogenic acute regulatory protein
SULT	Sulfotransferases
T	Testosterone
TBME	tert-butyl methyl ether
TC	Target compound

TD2019IRMS	Technical Document 2019 Isotopic Ratio Mass Spectrometry
TD2018EAAS	Technical Document 2018 Endogenous Anabolic Androgenic Steroids
TD2019MRPL	Technical Document 2019 Minimum required performance levels for the detection and identification of non-threshold substances
TD2019NA	Technical Document 2019 Harmonization of analysis and reporting of 19-norsteroids related to nandrolone
THE	tetrahydrocortisone
THF	tetrahydrocortisol
THS	tetrahydro-11-deoxycortisol
TL19	Technical Letter. In situ formation of prednisone and prednisolone
UGT	Uridine diphosphate glucuronosyltransferase
USP	Positive urine sample
VPA	Arginine vasopressin
VPDB	Vienna Pee Dee Belemnite
WADA	World Anti-Doping Agency
WADC	World Anti-Doping Code

General Introduction

Chapter 1

Introduction

1.1 General aspects of doping

1.1.1 Historical perspective

The origin of the word "doping" is still under a controversial etymological investigation but seems to derive from "dope", a stimulant drink consumed by African tribes in religious ceremonies but also an alcoholic beverage made from grapes skin and used by Zulu warriors during battles. In 1889, doping was first mentioned in the English dictionary to describe a narcotic potion enhancing the racehorse's performances[1]. Today, it refers to the occurrence of one or more of the anti-doping rules violations described from the article 2.1 to the article 2.8 of the Anti-Doping Code (WADC), that are: "(i) the presence of a prohibited substance or its metabolites or markers in an athlete sample; (ii) the use or attempted use by athlete of a prohibited substance or a prohibited method; (iii) refusing or falling without compelling justification to submit to sample collection after notification as authorized in applicable anti-doping rules, or otherwise evading sample collection; (iv) violation of applicable requirements regarding athlete availability of Out-of-Competition Testing, including failure to file required whereabouts information and missed tests which are declared based on rules which comply with the international standard for testing; (v) tampering or attempting to tamper, with any part of doping control; (vi) possession of prohibited substances and prohibited methods; (vii) trafficking or attempted trafficking in any prohibited substance or prohibited method and (viii) administration or attempted administration to any athlete of any prohibited method or prohibited substance"[2].

The practice of resorting dietary and medical help to be competitive and enhance performances, even under adverse conditions (injury or illness), is not a modern phenomenon[3–9]: extracts from the plant Ephedra[10], hallucinogenic mushrooms[11], cocoa leaves or various stimulant mixture (strychnine, cocaine, caffeine and alcohol)[12,13] were largely used in past during competitions, especially endurance events. From the 19th century, the misuse of drugs rapidly boosted thanks to the remarkable scientific results obtained in pharmacological research[14,15]. The first

doping-related fatality was reported in 1896, when the Welsh cyclist Arthur Linton died of a combination of caffeine and strychnine overdose during a 600 km race between Bordeaux and Paris[12,16]. Even if some restrictions were introduced in 1928 by the International Association of Athletics Federations (IAAF) on the use of stimulants and narcotics, appropriate official doping testing procedures had not yet been implemented. The death of the Danish cyclist Knut Jensen during the Rome's Olympic Game in 1960 and the first televising doping death of the English cyclist Tom Simpson during the Tour de France in 1967, highlighted the urgency of anti-doping policy, leading the International Olympic Committee (IOC) to institute its own Medical Commission and publish the first List of Prohibited Substances (1967)[8,17]. The List of "Banned Substances Classes and Methods" included five groups: sympathomimetic amines; stimulants of the central nervous system; analgesic narcotics; anti-depressants and major tranquillizers. The last two categories were removed only one year later; then the list remained practically unchanged until the introduction of anabolic steroids just before the Summer Olympiad in Montreal (1976). Subsequent remarkable changes concerned the prohibition of caffeine (1984)[9,18,19], peptide hormones, like human chorionic gonadotropin[20], adrenocorticotropic hormone, human growth hormone[12,21,22] (all in 1989) and erythropoietin (1990)[17,23]. During the World Conference on Doping in Sport held in 1999 in Lausanne, Switzerland, the institution of an international anti-doping agency was established in preparation for the imminent Games of the XXVII Olympiad in Sydney in 2000, resulting in the World Anti-Doping Agency (WADA) creation[24]. WADA has been founded on equal partnership between public governments and Olympic sport to coordinate the fight against doping and harmonize the Olympic antidoping policies in a single code applicable and acceptable for all the stakeholders[4,7,25,26]: the World Anti-Doping Code[27]. It is the core document of the antidoping community, first adopted in 2003 and subjected to regular reviews and updates over the years[28]; the last revised version will come into force on January 2021. It establishes universal anti-doping rules and programs valid for all athletes, regardless of the country in which they compete. The Code acts in conjunction with six International Standards:

I. The International Standard for the Prohibited List (The List)[29].

II. The International Standard for Testing and Investigations (ISTI, a practical directive that preserves the integrity and the identity of the samples from their collection to their analysis)[30].
III. The International Standard for Laboratories (ISL, that ensures that all the accredited WADA Laboratory report valid results based on reliable evidentiary tests)[31].
IV. The International Standard for Therapeutic Use Exemptions (ISTUE, that describes the conditions in which the possession or the administration of prohibited substances are allowed for therapeutic purposes)[32].
V. The International Standard for the Protection of Privacy and Personal Information (ISPPPI, for ensuring appropriate privacy standards to all parties involved in the anti-doping procedures)[33].
VI. The International Standard for Code Compliance by Signatories (ISCCS, that describes the rights and the responsibilities for all the Signatories of the Code)[34].

1.1.2 The Prohibited List

WADA published its first List of Prohibited Substances and Methods in 2004, following the 2nd World Conference in Doping in Sport (Copenhagen, 2003). Since then, it is yearly updated according to new doping or new drugs trends showing a potential doping effect[35].
In the current version, it is divided in the subsequent sections:

<u>Substances and Methods prohibited at all-times</u>:

Prohibited Substances

- S0: Non-Approved Substances
- S1: Anabolic Agents
- S2: Peptide Hormones, Growth Factors, Related Substances and Mimetics
- S3: Beta-2 Agonist
- S4: Hormone and Metabolic Modulators
- S5: Diuretics and Masking Agents

Prohibited Methods

- M1: Manipulation of Blood and Blood Components

- M2: Chemical and Physical Manipulation
- M3: Gene and Cell Doping

Substances and Methods prohibited in competition:

Prohibited Substances

- S6: Stimulants
- S7: Narcotics
- S8: Cannabinoids
- S9: Glucocorticoids

Substances and Methods prohibited in particular sports:

- P1: Beta-Blockers

For each class, a list of most representative examples is reported, but compounds with similar chemical or pharmacological activities are also prohibited. Instead, some substances are not yet included in the List, but closely monitored to investigate their pattern of use by athletes (in and/or out of competitions, by systemic or local routes) and verify if they beneficially affect the sportive performance[36].

Banned compounds are divided into two groups, depending on whether they may be simply detected and identified (non-threshold substances), or they must reach concentrations above certain cut-off (threshold substances) to provide adverse analytical findings (AAF). This is the case of substances for which is difficult to discriminate the exogenous administration from the physiological production or the pharmacological/social use from a doping violation. Minimum required performance levels have been established for the analysis of the non-threshold substances to harmonize the method sensitivity requirements and the results obtained from all the accredited WADA laboratories[37].

Compounds specifically considered in this work are included in the

- section S1

19-norandrostenediol (estr-4-ene-3,17-diol); 19-norandrostenedione (estr-4-ene-3,17-dione); nandrolone (17β-hydroxyestr-4-en-3-one); boldenone (17β-hydroxyandrosta-1,4-dien-3-one); dihydrotestosterone (17β-hydroxy-5α-

androstan-3-one); testosterone (17β-hydroxyandrost-4en-3-one) and their metabolites.

- section S9

prednisolone (11β,17α, 21-trihydroxypregna-1,4-diene-3,20-dione) and prednisone (17α,21-dihydroxypregna-1,4-diene-3,11,20-trione).

1.1.3 Analytical anti-doping strategies

The current analytical strategies rely on the detection and, in some cases the quantification, of the prohibited substances and methods in biological samples collected in and/or out of competition. The routine anti-doping controls are mainly performed on urinary matrices, because they can be obtained in relatively large volume with non-invasive procedure. Moreover, the concentration of most of the forbidden drugs and their metabolites is higher in urine than in blood. The use of hematological specimens (whole blood, serum and plasma) remains mandatory for some peptide hormones and in case no urinary markers may be detected[38]. The anti-doping control is a two-step procedure, in which an initial fast, selective and sensitive screening is followed by a confirmatory process. The first step is applied to all the collected samples to give indication about the presence or the absence of banned substances by limiting the risk of false-negative and false-positive results: the same method is typically applied to a wide number of compounds with similar physical-chemical properties (multitargeted approach). An additional confirmatory test is carried out on suspicious samples to specifically identify the analyte of interest and its metabolites.

Detection of the misuse of banned substances is preferably performed using gas (GC) and liquid (LC) chromatography coupled to mass spectrometry (MS or MS/MS)-based methods[39]. Some strategies exploit immunoassays and electrophoretic techniques[40]. Since many of the banned compounds have their endogenously produced counterparts, the application of the isotopic ratio mass spectrometry technique has been demanded.

Evidences of the continuous sophistication of the illicit practices and the rise of novel doping agents lead the anti-doping community to opportunely enhance the existing analytical methods and find new strategies[41]. The selection of alternative biological matrices, the use of micro-sampling, miniaturized (like dried blood spots,

DBS), faster and more automated pre-treatment techniques are all approaches recently considered[42,43].

1.2 Steroid hormones

Steroid hormones are secreted by adrenal cortex, testes, ovaries, and by placenta during pregnancy. Depending on the receptors to which they bind and consequently on their physiological functions, they can be divided into five groups: glucocorticoids, mineralocorticoids, androgens, estrogens and progestogens. They are all structurally characterized by a tetracyclic hydrocarbon ring, the tetracyclopentanoperhydrophenantrene or gonane core, in which 3 cyclohexane rings (A, B and C) are fused with a cyclopentane ring (D) (Figure 1.1).

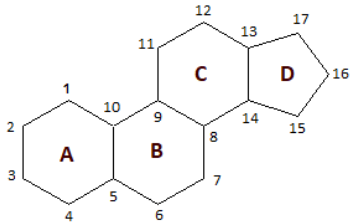

Figure 1.1 Gonane nucleus and conventional numbering of the rings and carbons

Steroid hormones are synthesized in the mitochondria and smooth endoplasmic reticulum (ER) from cholesterol, supplied from three different sources. It is provided by acetyl coenzyme A; esterified cholesterol deposits stored within the steroidogenic cells; and low-density lipoproteins derived from dietary cholesterol. The delivery of cholesterol from intracellular stores to the inner mitochondria membrane is the first rate-limiting step of steroidogenesis; the StAR (steroidogenic acute regulatory protein) and PBR (peripheral benzodiazepine receptor) are the two key proteins mainly involved in this process[44–47]. Cholesterol is then converted to pregnenolone via three different chemical reactions: two hydroxylations at C-20 and C-22 and a side-chain cleavage event, yielding pregnenolone and isocaproaldehyde. Pregnenolone, once left the mithocondria, may undergo 17α-hydroxylation by *P450c17* to obtain 17α-hydroxypregnenolone, or it may be converted to progesterone by *3β-hydroxysteroid dehydrogenase* (*3β-HSD*). A

subsequent sequence of hydroxylation and carbon-carbon bond cleavage reactions, mostly catalyzed by CYPs or HSDs, leads to the production of two categories of hormones: 21C steroids (progestogens, glucocorticoids and mineralocorticoids, characterized by 21 carbon atoms) and 19C steroids (androgens, that exhibit 19 carbon atoms and from which derive estrogens, 18C steroids). The overall diagram of the human steroidogenesis pathway is represented in Figure 1.2.

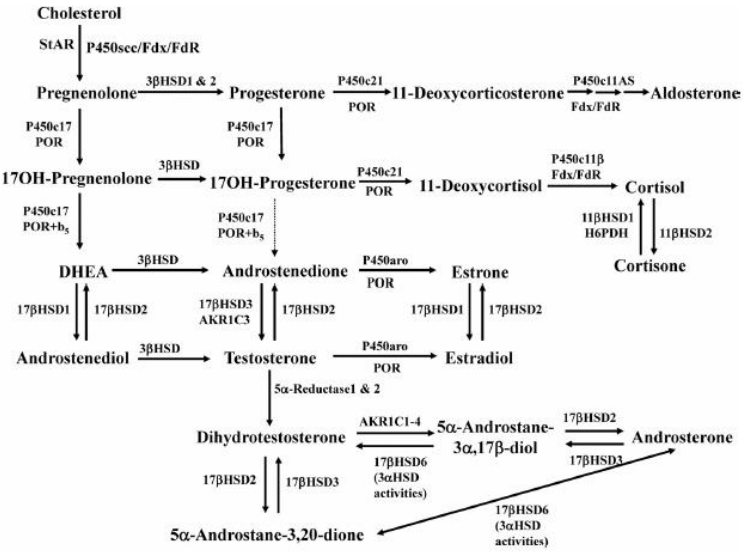

Figure 1.2 Steroidogenesis synthetic pathway[48]

The production of steroid hormones is regulated by complex reciprocal interactions among the hypothalamus, anterior pituitary and endocrine glands. Hypothalamic neurons in the supraoptic and paraventricular nuclei secrete hypothalamic releasing hormones that bind membrane receptors on specific subsets of pituitary cells and stimulate the secretion of the related pituitary hormones. The pituitary hormones flow to the target endocrine glands, where they activate the synthesis and the secretion of the target endocrine hormones. Constant negative feedback from the target glands to the anterior pituitary and hypothalamus ensures the body's homeostasis, turning off the cascade in case of high levels of circulating

endocrine hormones. Also the pituitary hormones can act back on the hypothalamus in the so-called short-loop feedback[49,50].

1.2.1 Androgens

1.2.1.1 Biosynthesis

Testosterone is the principal secreted androgen, mainly produced in Leydig cells by different pathways (see Figure 1.2). It is primarily derived from pregnenolone in a four steps process: the hydroxylation of the C-17 carbon atom (*P450c17)*; the cleavage of the C-17 and C-20 carbon-carbon (*17,20-lyase*) bond to form C19 compound DHEA; the conversion of the hydroxyl group at the C-3 carbon into a carbonyl group (*3β-HSD*) to form androstenedione; the reduction of the 17-oxo group (*17β-HSD*)[51]. Another minor biosynthetic precursor of testosterone is androstenediol. In women, testosterone is synthetized in corpus luteum and adrenal cortex by similar biosynthetic routes. In detail, it mainly derived from androstenedione conversion in peripheral tissues, while 30 % is produced by adrenal glands and 20 % by ovaries. The serum testosterone concentration ranges from 500 to 7000 ng/dL in men, compared to 30 to 50 ng/dL in women[52,53].

The luteinizing hormone (LH) and the follicle-stimulating hormone (FSH), secreted by the pituitary gonadotropes under the positive regulation of the hypothalamic peptide gonadotropin-releasing hormone (GnRH), stimulate the production of testosterone. The pulsatile secretion of GnRH and pituitary hormones results in a pulsatile daily secretion of testosterone, higher in the morning and lower in the evening. High testosterone and estradiol levels suppress, via negative feedback, the release of LH, FSH and GnRH, inducing the reduction of anabolic androgenic biosynthesis. In women, the secretion of LH is also inhibited by progesterone[51,54]. Moreover, in stress instances, glucocorticoids negatively modulate the steroidogenesis cascade at hypothalamic, pituitary and endocrine levels[55].

Once secreted, androgens circulate in blood mainly linked to albumin and sex-hormone binding globulin (SHBG), both produced by the liver; only a small portion of them remains unbounded.

1.2.1.2. Mechanism of action

Androgens are the key hormones responsible for the development, maturation and maintenance of male phenotype and sexual and reproductive characteristics and functions. Both in male and in female, they exert anabolic effects on skeletal muscle

and bone, by stimulating the linear growth and the protein synthesis[56,57]. In skeletal muscle, testosterone directly links the specific receptor and promotes the increase of muscle mass and strength, while, in reproductive tissues, it acts as prohormone of its more potent 5α-reduced derivative, 5α-dihydrotestosterone (DHT). In adipose tissues and parts of brain, testosterone is converted by aromatase to the estrogen, estradiol. In bone, both the direct effect of testosterone and the indirect action mediated by estradiol seem to be relevant (Figure 1.3)[54,58,59].

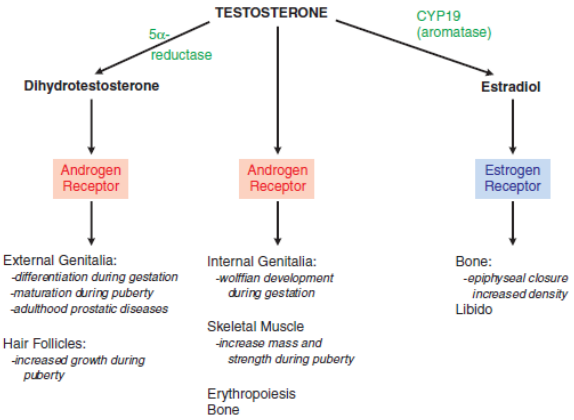

Figure 1.3 Physiological effects (direct and indirect) of testosterone[54]

At cellular level, the androgen action is mediated by the high affinity binding to the androgen receptor (AR) belonging to the nuclear receptor superfamily of ligand-activated transcription factors, as well as glucocorticoid (GR), mineralocorticoid (MR), progesterone (PR) and estrogen (ERα and ERβ) receptors. It consists of three domains: a N-terminal domain (NTD, containing polyglutamine repeats of different length), a DNA-binding domain (DBD, showing two Zn finger motifs) and a C-terminal ligand-binding domain (LBD). In the absence of ligand, the steroid receptor is located in the cytoplasm as an inactive oligomeric complex with molecular chaperone heat shock proteins Hsp90, Hsp70 and p23, and co-chaperones Hsp40 and Hop, essential to ensure the correct receptor conformation. The hormone binding induces the dissociation of the AR from heat-shock protein complex and its dimerization and translocation to the nucleus. The activated receptor interacts via DBD to androgen response elements (AREs) generally located at the promoter or enhancer regions of AR specific responsive genes, triggering the recruitment of a

cluster of gene transcription coregulators (co-activators and co-repressors). This leads to up or down-regulation of the target genes[60–64].

Besides their genomic activity, androgens can also influence directly the cells activity in a non-classic pathway, even with no translocation of the AR into the nucleus. The effects tend to be rapid (within seconds to few minutes) and may involve the binding to other membrane-bound androgen receptors, that make androgens no longer able to cross the plasma membrane[65–67].

1.2.1.3 Anabolic androgenic steroids

The anabolic androgenic steroids (AAS) are all testosterone derivatives properly synthetized to overcome the pharmacokinetic limitations (rapid first pass metabolism, short half-life) and the androgenic/feminizing side effects occurring after the administration of testosterone (Figure 1.4)[68].

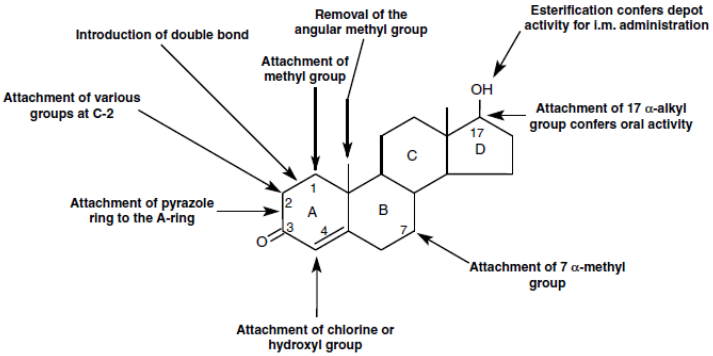

Figure 1.4 The main structural modifications of testosterone[59]

The main structural modifications include 17α-alkylation, 1-methylation, 17β-esterification, addition of a 19-normethyl group, and 7α-methylation[69,70]. The substitution of methyl (CH_3) or ethyl (C_2H_5) group for the H on the cyclopentane ring structure (position 17) hinders the first pass oxidation of the 17β-hydroxy group and confers oral availability. However, after prolonged use at high dosage, these 17α-derivatives (methyltestosterone, methandrostenolone, norethandrolone, fluoxymesterone, danazol, oxandrolone, oxymetholone, stanozol) may cause severe liver disfunction. The attachment of a methyl group on C-1 allows to obtain orally active derivates (methenolone or mesterolone), even if with a weaker pharmacological effect. The esterification of the 17β-hydroxy group

with acid moiety of different length leads to more fat-soluble steroids with a much longer half-life (testosterone enanthate, propionate, cypionate, decanoate, isocaproate, phenylpropionate and undecanoate; 19-nortestosterone cyclohexyl propionate, phenylpropionate, decanoate and laurate; methenolone enanthate, boldenone undecylenate, trenbolone acetate, dimeric testosterone). The length of the lateral chain influences the duration of action: more length ensures long-lasting release in the blood stream. Once adsorbed, the 17β-esters are rapidly hydrolyzed by the blood esterases to form the active compounds. They are parentally administered in an oil-based carrier, mainly a mixture of arachis/sesame seed oil and alcohol. Parental preparations show fewer negative effects on hepatic functions. Several other modifications on the ring A, such as junction with pyrazole ring, insertion of methyl group at position C-1, attachment of alkyl substituents or oxygen atom at position C-2, introduction of double bond at position C-1, C-2, and removal of the C-19 methyl group, enhance the anabolic activity relative to androgenic properties[71]. 19-Nortestosterone (nandrolone) was the first synthetic testosterone derivative showing an advantageous myotrophic-to-androgenic-ratio in animal experiments. It possesses strong affinity with the androgenic receptor, to which it directly binds in skeletal muscle. Whereas, in androgenic tissues, it is rapidly converted in its 5α-reduced metabolite, that acts on the androgen receptor with weak affinity compared to the parent compound. The insertion of a methyl substituent at the position C-7 of the base structure of 19-nortestosterone (trestolone) has been demonstrated favorable anabolic efficacy[68].

1.2.1.4 Metabolism

Anabolic steroids undergo an extensive phase I oxidoreductive reactions principally occurring in the liver[72–76]. They mainly involve the A and D-rings: C-4, C-5 double bond reduction (by *5α*- and *5β-reductase*); C-3 carbonyl group reduction (by *3α*- or *3β-hydroxysteroid dehydrogenase*); C-1, C-2 double bond reduction (in case of 1,4-diene steroids) and 17β-hydroxy group oxidation (by *17β-hydroxysteroid dehydrogenase*) are the most metabolic pathways. The human urine profile of testosterone includes androsterone, etiocholanolone, dihydrotestosterone, 5α-androstane-3α,17β-diol and 5β-androstane-3α,17β-diol as the more abundant phase I metabolites. The endogenous production of 1,2 dehydrogenated compounds (boldenone and metabolites) by the gut bacteria in humans has been also reported[77,78]. Metabolism on B-ring preferentially occurs for 17β-hydroxy-17α-methyl steroids on C-6 and C-7 positions to form 6β-hydroxy or 6,7-dehydro-

metabolites (minor pathway). The biotransformation reactions on C-ring are negligible; the hydroxylation at C-12 is the well-studied one. 16-Hydroxylated compounds have been identified for several AAS. Further metabolites can be identified depending on the chemical modifications inserted on the base steroid structure.

Most of endogenous and exogenous androgens are excreted as glucuro- or sulfo-conjugated metabolites[72–76,79,80]. Both the phase II reactions are enzymatically controlled. The glucuronidation is catalyzed by uridine diphosphate glucuronosyltransferase (UGTs) membrane bound enzymes through a nucleophilic substitution from the glucuronic acid from uridine diphosphate glucuronic acid to the functional group of the steroid molecule[81]. Instead, the sulfonation consists in the transfer of a sulphate moiety by sulfotransferases (SULT), which use the 3'–phosphoadenosine-5'-phosphosulfate (PAPS) as co-substrate, to the acceptor compound[82–84]. 3α-O-β-Glucuronides, glucuronides and sulfates of the secondary and tertiary 17β-hydroxy steroids are the major AAS metabolites. Glucuronidation is the preferential metabolic pathway, unless for 3β-hydroxy steroids (such as DHEA, dehydroepiandrosterone[85]) which are predominantly excreted as sulfate[86]. Genetic polymorphism of gene coding metabolizing enzymes may significantly affect urinary androgens levels and cause relevant inter-individual variations. Among the others (CYP17, UGT2B15, UGT2B7, SULT2A1), polymorphism in the UGT2B17 gene has been widely studied, because its deletion drastically reduces the excretion of testosterone and some other related steroids, making more difficult their misuse detection[87–89].

1.2.1.5 AAS in clinical practice: therapeutic use and adverse effects

Several evidences about the benefits and the risks associated with the use of AAS in clinical practices are widely reported in literature[68,90–95]. In the early nineties, they were usually prescribed by psychiatrists to men suffering depressive disorders, epilepsy and paranoia, also in combination with electroconvulsive therapy. Then, AAS, especially testosterone 17β-esters, 17α-derivatives and nandrolone, have been considered useful in the treatment of cachexia and catabolic conditions in patients with AIDS, chronic obstructive pulmonary disease (COPD), renal and burns failure or malnutrition and growth retardation. AAS (stanozolol) may exert beneficial effects in the treatment of aplastic anemia, even if they have been recently replaced by recombinant human erythropoietin and analogues. The main indication for the administration of androgens remains the male hypogonadism[96]:

testosterone depot preparations (transdermal gel, mucoadhesive buccal tablets, long acting intramuscular injection) show an attractive pharmacokinetic profile to ensure a constant testosterone serum levels in androgens replacement therapies. From the 1990s, however, the selective androgen receptor modulators (SARMs) have become the preferential candidate to treat androgen-deficiency-related diseases: they can be administered orally in low milligram doses and possess high tissue selectivity, consistently reducing the androgenic side effects on prostate, hair and skin[97,98].

AAS can cause a wide range of undesirable effects after a prolonged use, an acute overdose or a use for non-medical purposes[71,74,90,99–101]. They were usually prescribed in cycles of 6-12 weeks followed by a variable suspension period to avoid remarkable disturbance on the hypothalamic-pituitary-gonadal axis (HPG-axis). The suppression of the release of LH and FSH, that regulate the steroidogenesis and spermatogenesis processes respectively, induces a drastic reduction of fertility, azoospermia and atrophy of testes in men, and amenorrhea in women. As a result of the endogenous aromatization process to estrogens, AAS can lead to gynecomastia and higher voice pitches in men. Androgenic steroids enhance the occurrence of thrombotic events and cardiac damage (left ventricular hypertrophy and heart failure), especially if administered in combination with growth hormone and insulin, also abused for anabolic intent[102]. The psychological implications are mostly unpredictable and range from irritability, violent behavior towards himself and the others, to uncontrolled libido. The administration of AAS, particularly the 17α-testosterone derivatives, is also related to a high incidence of hepatotoxicity and increased risk of liver tumor. During puberty, the use of AAS can induce growth stunting, due to a premature closure of the epiphysis. Hirsutism in women and acceleration of baldness in men are adverse effects equally frequent.

1.2.1.6 AAS in doping analysis

The anabolic androgenic steroids represent one of the most group of drugs abused by athletes to improve the sport performances, despite the extent of their adverse effects. In the late 90's, the administration of androgens for not therapeutic purposes have been significantly increased, especially in the German Democratic Republic and other socialist systems, where many cases of young women athletes submitted to virilization/androgenization programs were reported[103]. AAS have been prohibited from 1974; since then, the main analytical challenge has been to differentiate the endogenous from the exogenous steroids, given that they are

both excreted in urine as structural identical phase I and phase II metabolites[104]. The general instructions for measuring and reporting of endogenous anabolic androgenic steroids (EAAS), also called pseudo-endogenous steroids, have been provided by WADA in the Technical Document TD2018EAAS[105]. The current strategy includes an initial testing procedure to estimate the steroid profile of the athlete and a confirmation procedure of any abnormal urinary steroid profiles by GC-C-IRMS analysis. The key parameters of the steroid profile are six steroidal markers (testosterone (**T**), epitestosterone (**E**), androsterone (**A**), etiocholanolone (**Etio**), 5α-androstan-3α,17β-diol (**5αAdiol**), 5β-androstan-3α,17β-diol (**5βAdiol**)) as sum of free and glucuronide fractions) and their relative ratios (T/E, A/T, A/Etio, 5αAdiol/5βAdiol and 5αAdiol/E). More specifically, epitestosterone is the C-17 epimer of testosterone, produced in parallel with T via a not completely known biosynthetic pathway[106]. Even though the daily production of E is only 3 % of that of T, the excretion rates of T and E are similar (T/E approximately 1) due to the poor metabolism of E in man. The exogenous administration of testosterone and its precursors in healthy subjects leads to abnormal increase of the urinary concentration of T glucuronide, but does not distinctly affect the E. Therefore, the T/E ratio is considered the most representative first level index of the intake of AAS. The initial cut-off of 6 has been lowered to 4 to avoid false positive results in case of athletes showing a natural elevated T/E ratio[107,108]. The T/E ratio displays high population variability, mainly caused by the genetic polymorphism of UGT2B17, and intra-individual fluctuations, especially occurring in female athletes during the different phases of the menstrual cycle[109]; the 5α- and 5β-metabolites ratio (5αAdiol/5βAdiol and A/Etio) are more stable steroid profile parameters. They are particularly sensitive to detect application of transdermal formulations of T or DHT administration: DHT is their common precursor, produced from T by the 5α-reductase highly expressed in the skin[110]. A/T ratio was earlier T/A ratio; the numerator has been switched with the denominator to reduce the decimals needed. The 5αAdiol/E ratio is the latest parameter added, following the evidence of its usefulness to detect transdermal T-gel[111].

These steroid concentrations (adjusted to a urine specific gravity (SG) of 1.020, if SG > 1.018) and ratios are compared to population-based reference range.

A sample is suspicious if any of the following parameters are met:

- T/E ratio (calculated from the corrected chromatographic peak areas or height) greater than 4.0;
- A/T ratio less than 20;

- 5αAdiol/5βAdiol ratio greater than 2.4;
- Concentrations of T or E (adjusted for specific gravity (SG)) greater than 200 ng/mL in males or greater than 50 ng/mL in females;
- Concentrations of A or Etio (adjusted for SG) greater than 10000 ng/mL;
- Concentration of 5αAdiol (adjusted for SG) greater than 250 ng/mL in males or greater than 150 ng/mL in females, combined with a 5αAdiol/E greater than 10 in either sex.

The traditional testing of steroid profile is currently supported by an individual reference range evaluation, the Athlete Biological Passport (ABP), firstly proposed by Donike et al. in 1994 and then developed by Sottas et al.[112–114].

The steroidal module of the ABP is a Bayesian screening test for the detection of abnormal values in longitudinal markers. It compares sequential measurements of a steroidal marker with previous readings performed on the same individual: if more measurements are executed, narrower individual reference range can be defined (Figure 1.5)[115–117]. The responsibility for managing and assessing each Passport belongs to the Athlete Passport Management Unit (APMU)[118].

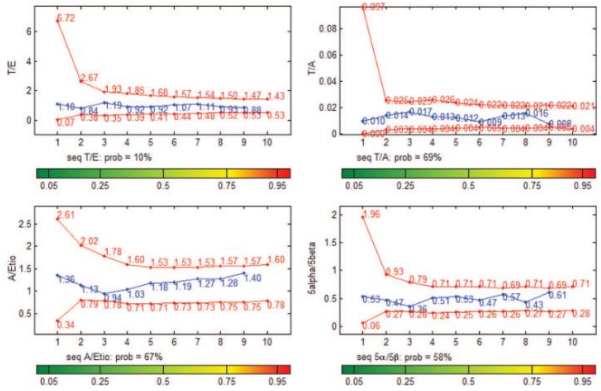

Figure 1.5 Example of the Steroidal Module of the Athlete Biological Passport
Blu lines represent actual test results; red lines indicate individual range[116]

The subsequent confirmation procedure is performed when the estimated steroid profile constitutes an Atypical Passport Finding (ATPF) or represents a "suspicious steroid profile" (SSP) finding, or upon direct request from the APMU, the Testing Authority or WADA. It consists of a GC-MS(/MS) full quantitative determination and

a GC-C-IRMS analysis of the steroid profile markers to assess their exogenous or endogenous origin[105,119].

1.2.2 Glucocorticoids

Glucocorticoids (GCs) are corticosteroids with 21 carbon atoms; cortisol is the main representative one. They have key role in the regulation of carbohydrate, protein, lipid and nucleic acid metabolism[53]. They promote the gluconeogenesis by liver from amino acids and glycerol and reduce the utilization of glucose in the peripherical tissues, resulting in the increase of the blood glucose levels. Glucocorticoids stimulate the catabolism of proteins and activate the lipolysis. They induce the mobilization of fatty acids from adipose tissue and their oxidation in cells; excess of cortisol can causes dramatic redistribution of fat in the body, mainly in the back of the neck ("buffalo hump") and face ("moon facies"). Glucocorticoids are involved in the suppression of inflammatory processes and immune response, since they reduce the release of vasoactive factors, the extravasation of leukocytes and the secretion of lipolytic, proteolytic enzymes and cytokines[120]. Remarkable effects are also induced on bone and cardiovascular system, on which high levels of circulating GCs are responsible for osteopenia, osteoporosis and hypertension. Psychiatric manifestations (anxiety, sleep disturbance, mood disorders, psychotic depression and cognitive disfunctions) in patients with GCs excess or deficiency reveal that the central nervous system is another target of this class of hormones. They seem to regulate the neuronal excitability via mechanisms not yet completely understood[54].

The molecular pathway by which GCs exert their action at intracellular level involves genomic steps similarly to those previously described for AAS. Briefly, glucocorticoids enter the cell and bind the specific GR receptors inducing its nuclear translocation and the activation or repression of target genes (GRE, glucocorticoids response elements). A non-genomic mechanism including signaling through membrane associated receptors are also reported[121–123].

1.2.2.1. Regulation of glucocorticoids secretion

The physiological levels of GCs are regulated by the hypothalamic-pituitary-adrenal (HPA) axis[49] in a complex interactions system among the hypothalamic

corticotropin-releasing hormone (CRH), arginine vasopressin (VPA) and the pituitary adrenocorticotropic hormone (ACTH), through three different mode:

1. negative feedback mechanism: GCs act at several levels of the HPA axis for decreasing the release of CRH from CRH neurons and ACTH from corticotropes;
2. circadian rhythm: a pulsatile secretion dependent on day-night and sleep-wake patterns. The circulating GCs levels peak at 8 A.M. (140-180 ng/mL, while in the evening 20-40 ng/mL);
3. stress response: stress signals, as injury, hemorrhage, surgery, exercise, pain, anxiety, apprehension, nausea, fever and hypoglycemia overcome the negative feedback regulation and diurnal variation. In stress conditions, cortisol rises consistently.

ACTH (39 amino acids peptide) is released from a larger precursor, the pro-opiomelanocortin (POMC), through sequential proteolytic cleavage steps. The interaction of ACTH with its melanocortin receptor (MCR2, a G protein-coupled receptor) on the adrenal cortex triggers the secretion of GCs, mineralocorticoids and the androgens precursor dehydroepiandrosterone (DHEA, peripherally converted to more potent androgens). More specifically, mineralocorticoids (mostly aldosterone) are synthesized in the glomerulosa outer layer, glucocorticoids (such as cortisone) are produced in the middle fasciculata zona, and the reproductive steroids are secreted by the inner reticularis layer.
See Figure 1.2 for the biosynthetic pathway illustration.

1.2.2.2. Synthetic glucocorticoids: chemical structure and clinical use

Since cortisone (1948) and cortisol (1951) became the two drugs of choice in the treatment of rheumatoid arthritis, several studies have been implemented to produce derivatives having fewer side-effects, more separated glucocorticoid and mineralocorticoid activities and longer duration of action[124]. The double bond at C-4, C-5 and the 3-oxo-group are essential for both the glucocorticoid and mineralocorticoid activities, while the 11β and 17α-hydroxyl groups on ring C and D respectively are responsible for an appreciable glucocorticoid activity and high potency. The introduction of an additional C-1, C-2 double bond (prednisone and prednisolone) and a hydroxyl or methyl group at C-16, selectively increases the

glucocorticoid activity, especially if combined with a fluorine atom at C-9 (triamcinolone, dexamethasone, betamethasone). Esterification of the hydroxyl groups at C-17 and C-21 with valerate or propionate enhances the molecular lipophilicity with improved topical/systemic potency ratios[125]. The most common GCs are represented in Figure 1.6. Compounds differing in potency, activity and half-life have been synthetized by providing the opportune chemical modifications. Depending on their half-life, GCs can be divided in short- (≤ 12 h; hydrocortisone and cortisone), intermediate- (12-36 h; prednisone, methylprednisolone), and long- (36-72 h; dexamethasone and betamethasone) acting agents.

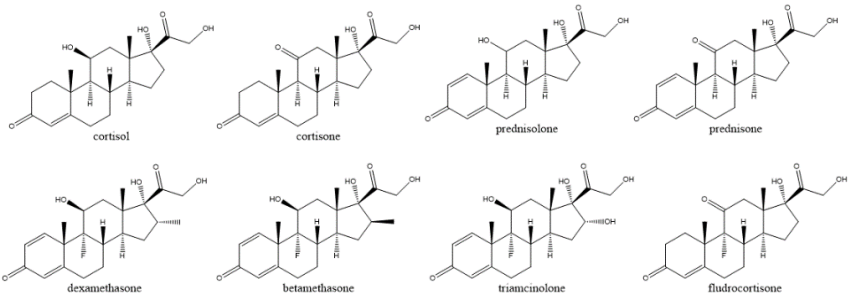

Figure 1.6 The most common glucocorticoids: chemical structures

Due to their prominent anti-inflammatory and immunosuppressive effects, GCs are mainly used for treatment of several inflammatory (psoriasis, eczema, allergies, asthma) and rheumatic (rheumatoid arthritis) diseases[126,127]. They may be also administered in replacement therapy in cases of hypothalamic-pituitary-adrenal axis failure or adrenal insufficiency, and in clinical oncology to reduce the side effects of chemotherapy and treat lymphoproliferative disorders[124]. The benefit-to-risk ratio of glucocorticoids, especially after chronic therapies, has been debated for years. Treatment with systemic GCs is associated with a number of adverse reactions and toxicities of different extents: suppression of the HPA axis, bone fragility, growth arrest, behavioral and psychiatric problems, hypertension, hyperglycemia and increased risk of infections are the most significant ones[123,126,128–131].

1.2.2.3. Metabolism

As already described for AAS, the phase I metabolism of GCs mainly involves the reduction of both the C-4, C-5 double bond and the C-3 carbonyl group. Most of these A-ring reduced compounds are excreted as glucuro- or sulfo- conjugated metabolites[76]: specifically, all metabolites with a 3α-hydroxyl substituent are excreted as glucuronide conjugates and those with a 3β-hydroxy-5-ene group are excreted as sulfates. Other cysteinyl conjugates and N-acetylglucosamines types of conjugated metabolites have been recorded[132]. Metabolism of cortisol and cortisone is illustrated in Figure 1.7: tetrahydrocortisone (THE), 5α-tetrahydrocortisone (Allo-THE), tetrahydrocortisol (THF), 5α-tetrahydrocortisol (Allo-THF) and 11-desoxy-tetrahydrocortisol (THS) are the main metabolites[133]. Corticosteroids with the 11-keto group, as cortisone and prednisone, undergo a pre-receptor metabolism consisting of an enzymatic reduction to the corresponding more active 11β-OH derivatives, cortisol and prednisolone[123]. The enzymatic conversion is catalyzed by 11β-HSD type 1 (*11β-hydroxysteroids dehydrogenase*) in a nicotinamide adenine dinucleotide phosphate (NADPH)-dependent reaction. 11β-HSD1 is a reductase widely expressed in liver, adipose tissue, muscle, pancreatic islets, adult brain and inflammatory cells. Its activity is supported by the microsomal hexose-6-phosphate dehydrogenase (H6PDH), able to regenerate NADPH and supply the reducing sources. Accordingly, in patients suffering from cortisone reductase deficiency, drugs that do not require the enzymatic activation should be preferentially administered[134–136]. The conversion of the 11β-oxo precursors in their 11β-hydroxy metabolites is inhibited by the 11β-HSD type 2, since it catalyzes the opposite reaction. 11β-HSD type 2 is mainly located in the mineralocorticoids target tissues (kidneys) where the GCs inactivation promotes the effects mediated by the aldosterone-mineralocorticoid receptor (MR) binding.

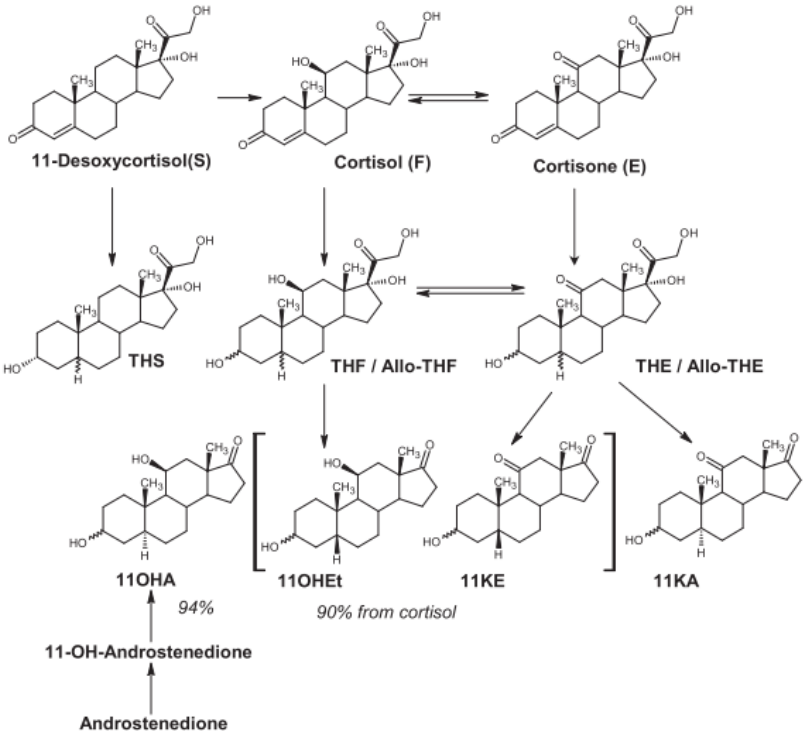

Figure 1.7 Diagram of cortisol and cortisone metabolism[133]

1.2.2.4. GCs as doping agents

Glucocorticoids exert a beneficial impact on the muscle responsiveness and recovery during sport performances, by increasing the availability of metabolic substrates and reducing the feeling of fatigue and the pain of efforts[137]. GCs are widely abused by athletes, despite the not negligible side effects associated with their administration[138,139]. They are included in the section S9 of the WADA Prohibited List and banned "in competition" when administered by systemic (oral, intravenous, intramuscular or rectal) routes[29]. A reporting level of 30 ng/mL has been established by WADA to discriminate the allowed (topical) from the prohibited (systemic) administration: a concentration higher than 30 ng/mL shall be observed in urine samples to report an adverse analytical finding[37]. Synthetic glucocorticoids are routinely screened by LC-MS/(MS)[132,140–142]; the use of GC-MS

based methods is limited by the difficulty in obtaining unique derivatization products[143]. GCs are also included in multitargeting procedures for the simultaneous detection of other banned compounds (anabolic agents, β2-agonists, hormone antagonists and modulators, diuretics, stimulants, narcotics and β-blockers)[144,145]. Moreover, since hydrocortisone and cortisone are naturally produced in the body, the isotope ratio mass spectrometry analysis can be utilized to determine the abuse of endogenous glucocorticoids by measuring the carbon isotope ratio of their resulting metabolites in human urine samples[133,146].

1.3 *Ex-vivo* degradation of endogenous compounds

The usual non-sterile collection or transportation conditions and the presence of normal or pathogenic microbial flora contamination are all favorable circumstances able to change the composition of the collected urine (increase or depletion of endogenous steroids or even the hydrolysis of conjugated metabolites) and alter specific steroid parameters. The free/glucuronide testosterone and 5α-androstanedione/A and 5β-androstanedione/Etio ratios are all criteria monitoring in doping analyses as markers of the bacterial metabolic activity[147–150]. Among the *ex vivo* bacterial degradation of endogenous steroids, 19-demethylation and Δ1-steroid-dehydrogenation have been extensively reported in literature and considered in this PhD thesis. They induce the formation of banned substances (19-norandrosterone, boldenone, prednisolone and prednisone) even if no drugs intake has occurred, affecting the correct interpretation of data.

<u>19-Norsteroids</u>

19-Norsteroids, nandrolone and its precursors, are anabolic androgenic compounds synthetized since the 1950s to enhance the myotrophic action and reduce the androgenic side effects of testosterone, from which they structurally derive[151–153]. They are primarily metabolized into two glucuronic acid conjugated products, 19-norandrosterone (**19-NA**), about 72 %, and 19-noretiocholanolone (**19-NE**), about 28 %. Unfortunately, the presence of 19-NA and 19-NE in urine could also occur in case of not intentional intake of nandrolone and its precursors[154]: after the consumption of non-castrated boar edible tissues[155–157], during pregnancy or norethisterone based contraceptive therapy[158–162] or as the result of an *in situ* androsterone 19-demethylation[163]. The detection of 19-

norsteroids is based on the quali-quantitative determination of 19-NA by GC-MS/MS. In addition, the GC-C-IRMS analysis shall be executed on samples not originated from pregnant female athletes or female athletes taking 19-norethisterone, showing 19-NA concentration between 2.5 and 15 ng/mL[164].

Boldenone

Boldenone (androsta-1,4-diene-17β-ol-3-one, **β-Bold**) is a synthetic derivative of testosterone in which an unsaturation between C1 and C2 was included. Initially used for veterinary use, boldenone is one of the most detected AAS in doping control analyses. It is frequently administered as its prohormone boldione (17a-boldenone, androsta-1,4-diene-3,17-dione, **ADD**) based formulations. In doping analysis, boldenone misuse is detected by the identification of β-Bold and its main metabolite (5β-androst-1en-17β-ol-3-one, **BoldM2**) by GC-MS/MS, even if more new approaches have been recently published[165–170]. Several evidences of endogenous production of boldenone and metabolites (as results of an *ex vivo Δ1-steroid dehydrogenase* activity) in animals as well in humans[78,171–174] lead the WADA to require the GC-C-IRMS confirmation analysis at concentration (adjusted for SG) between 5 and 30 ng/mL[119,175,176].

Prednisone and prednisolone

Prednisone (**PRED**) and prednisolone (**PLONE**) are two synthetic glucocorticoids widely used both in human and in veterinary medicine. As mentioned before, they are enzymatically converted to each other by the hepatic *11β-hydroxysteroid dehydrogenase* in a reversible process: PLONE is the active molecule, usually administered as prednisone in oral formulations[177]. Their major metabolites in urine have been identified and characterized by LC-MS/MS and the most significant and long-term markers (20β-hydroxy-prednisone and 20β-hydroxy-prednisolone) have been selected[142,178–180]. The uncommon presence of prednisone and prednisolone in human urine, due to the Δ1-dehydrogenation of cortisone and cortisol physiologically excreted[181–184], leads the WADA to recommend the GC-C-IRMS confirmation analysis at concentrations between the reporting level of 30 ng/mL and 60 ng/mL[185].

1.4 The GC-C-IRMS analysis

The stable isotopes analysis concerns the so-called isotopic "fingerprints" of natural materials, namely the combination of the stable isotopes' ratios of several elements ($^{2}H/^{1}H$, $^{15}N/^{14}N$, $^{13}C/^{12}C$, $^{18}O/^{16}O$, $^{34}S/^{33}S$ and $^{37}Cl/^{35}Cl$), determined at the time of the earth's formation and then influenced by chemical, biological and physical fractionation processes. Isotopic variations are, therefore, characteristics of the origin and the history of a substance. By convention, the relative abundance of stable isotopes is referenced to the heavy isotope, codified in the "δ value" notation, firstly described by Urey and reported in units of per mil (‰)[186]:

$$\delta\ ‰ = \left(\frac{R_{sample} - R_{standard}}{R_{standard}}\right) * 1000$$

where R is the isotope ratio (heavier/lighter isotope) of the sample and of the international accepted standard, whose δ value is arbitrarily set to 0 ‰. Negative δ values indicate that the sample is depleted in the heavy isotope relative to the standard, whereas positive δ values represent an enrichment of the heavy isotope. The internationally recognized standards are generally calibrated and provided by the International Atomic Energy Agency (IAEA, Vienna, Austria) and the National Institute of Standards and Technology (NIST, Washington, DC, USA).

International standard	Isotope ratio of reference material
PeeDee Belemnite (PDB)	$^{13}C/^{12}C = 0.0112372$ $^{18}O/^{16}O = 0.0020671$
Atmospheric nitrogen (AIR)	$^{15}N/^{14}N = 0.0036765$
Vienna standard mean ocean water (V-SMOW)	D/H ($^{2}H/^{1}H$) = 0.00015576 $^{18}O/^{16}O = 0.0020052$
Canyon Diablo meteorite troilite (CDT)	$^{34}S/^{32}S = 0.0450045$
Standard mean ocean chloride (SMOC)	$^{37}Cl/^{35}Cl = 0.324$

Figure 1.8 International standards for the most common elements analyzed by IRMS

The standards are limited by the availability of the material in the environment: the PBD, the primary reference material for carbon isotopic measurements, was exhausted long ago. It was a calcium carbonate from a belemnite rostrum of the 'Pee-Dee' formation during the Cretaceous period of the State of South Carolina

(USA). Today, the ^{13}C natural isotopic abundance is expressed, by convention, in relation to V-PDB, whose $\delta^{13}C$ value was calculated to be +1.95 ‰ compared to the primary standard[187,188].

The study of stable isotopes by isotope ratio mass spectrometry (IRMS) has experienced several application fields[189]. It is commonly used in pharmaceutical and food industry to establish the authenticity of products and determine eventual adulterations[190–192]; to reconstruct prehistoric diet and lifestyle from organic residues preserved in archaeological artefacts[193,194], to discover the source of environmental contaminants[195]; for studying the animals migratory behaviour[196,197]; in clinical diagnosis (i.e. breath test), and research[198–200]; in doping analyses[133,176,201–205].

1.4.1 Application of the GC-C-IRMS to doping analysis

The IRMS is the mandatory technique required by WADA for detecting the abuse of pseudo-endogenous steroids[119], showing the same chemical structure and physiological functions of their endogenous counterparts, but different ^{13}C content. The endogenous carbon isotopic profile comes from the individual dietary habits, mainly based on the consumption of C3 or C4 plants derivatives.

Plants discriminate against ^{13}C during the photosynthetic process in a variable extent depending on the way by which they assimilate CO_2. C4 plants discriminate against $^{13}CO_2$ less than C3 plants, producing a range in $\delta^{13}C$ value of about 17 ‰ to 10 ‰, whereas C3 plants range between 24 and 32 ‰[206–209] (Fig. 1.9). About 90 % of terrestrial plant species are C3 plants, that fix carbon from the atmospheric CO_2 by the Calvin-Benson cycle. The primary step is catalyzed by the *ribulose bisphosphate carboxylase/oxygenase* enzyme, also called *Rubisco*, and leads to the formation of a C3-atom molecule (phosphoglyceric acid). C4 plants (sugarcane and maize) have been adapted to high light, arid and warm environments and have achieved higher photosynthetic capacity and higher water- and nitrogen-use efficiencies compared with C3 plants, by performing alternative photosynthetic reactions (Hatch-Slack cycle). Since the first stable product formed is an organic acid at four atoms of carbon (oxaloacetate), they are called C4 plants. CAM plants (Crassulacean acid metabolism plants, such as stonecrops and cactus) adapt to extreme arid conditions by using alternatively C3 or C4 metabolism, but their photosynthetic capacity is very low.

Endogenous reference $\delta^{13}C$ values are between -16 to -26 ‰ in the worldwide population depending on the C3 or C4 plants enriched diet: the Americans typically show the least negative $\delta^{13}C$ values (from -16 to -18 ‰), while Scandinavian population displays the most negative ones (from -24 to -26 ‰)[210–212] (Fig. 1.9).
$\delta^{13}C$ values of synthetic drugs, instead, is affected by the natural precursors selected in their manufacturing process. They are mainly produced by a combination of microbial and chemical processes on phytosterols and sapogenins, C3-plant derived natural precursors[213]. Phytosterols are collected as residual products during the soybean-oil production: stigmasterol, β-sitosterol, campesterol and brassicasterol are the most typical ones[214,215]. Sapogenins, such as hecogenin, tiogenin, and diosgenin, are primarily extracted from roots of various Dioscorea species of Mexico yams[216]. The preferential use of C3 plants in the pharmaceutical industries results in $^{13}C/^{12}C$ isotopic ratios more depleted compared to the endogenous ones[217–219].

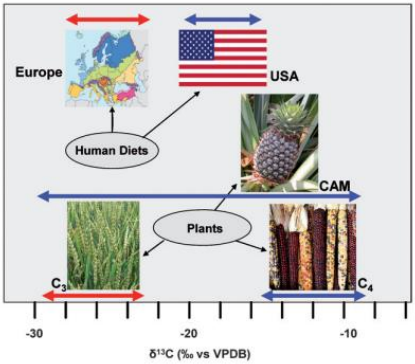

Figure 1.9 Carbon isotopic composition of plants and human diets

The GC-C-IRMS doping analysis is an additional tool performed as confirmation step after obtaining suspicious findings in the previous screening procedure or if directly requested by Testing Authorities. The GC-C-IRMS method relies on the determination of the $\Delta\delta^{13}C$ values between two classes of compounds:

- Target compounds (TCs),

diagnostic markers whose $\delta^{13}C$ values are affected by the exogenous administration of illicit drugs. Currently, they are:

i. Andro, Etio, 5αAdiol, 5βAdiol, T and E (in case of the detection of EAAS);
ii. 19-NA (in case of the detection of nandrolone and precursors). In this PhD thesis, 19-NE was also selected as additional TC;
iii. β-Bold and BoldM2 (in case of the detection of boldenone and pro-hormones);
iv. Pred and Plone (in case of the detection of prednisone and prednisolone).

- Endogenous reference compounds (ERCs),
compounds involved in a different or upper stage of the metabolic pathway of interest. Their ^{13}C composition does not vary after the intake of drugs. They are:

i. products of the cortisol/cortisone metabolic pathway in case of the detection of EAAS: pregnanediol (**PD**), 5α-pregnane-3α,17α,20α-triol (**PT**), 11β-hydroxyandrosterone (**11-OH-A**), 11-ketoetiocholanolone (**11-keto-Etio**);
ii. A, PD and PT (in case of the detection of 19-norsteroids);
iii. 11-ketoEtio, PD and PT (in case of the detection of boldenone and its pro-hormones);
iv. PD, PT and tetrahydro11-deoxycortisol (**THS**) (in case of the detection of prednisone and prednisolone).

The $\Delta\delta^{13}C_{(ERC\text{-}TC)}$ values allow to normalize the GC-C-IRMS results in relation to the individual diet and provide effective criteria to identify doping cases.
The results of the GC-C-IRMS analyses shall be interpreted:
Positive, if one of the following sets of criteria is fulfilled:

i. $\Delta\delta^{13}C$ value of ERC-T > 3‰ and either ERC-5αAdiol or ERC-5βAdiol > 3 ‰;
ii. $\Delta\delta^{13}C$ values of ERC-5αAdiol and ERC-5βAdiol pairs are both > 3 ‰;
iii. E > 50 ng/mL in females or > 200 ng/mL in males (SG-adjusted) and $\Delta\delta^{13}C$ value of ERC-E > 4 ‰;
iv. $\Delta\delta^{13}C$ value of ERC-A > 3 ‰ or ERC-Etio > 4 ‰;
v. $\Delta\delta^{13}C$ value of ERC-A is between 2-3 ‰ or ERC-Etio is between 3-4 ‰, and one of $\Delta\delta^{13}C$ value of ERC-5αAdiol or ERC-5βAdiol > 3 ‰;
vi. $\Delta\delta^{13}C$ value of ERC-5αAdiol > 4 ‰ and $\delta^{13}C$ 5αAdiol ≤ -27 ‰;
vii. The $\Delta\delta^{13}C$ value of either the ERC-formestane, ERC-β-Bold or ERC- BoldM2 pairs is greater than 4 ‰.

Negative when $\Delta\delta^{13}C$ values do not confirm the exogenous origin of TC(s), i.e. when the $\Delta\delta^{13}C$ values of the ERC-TC pairs do not meet any of the criteria specified above. Inconclusive if, in case of combined positive criteria, only one is met; when technical limitations (i.e. interfering peaks) affect the interpretation of the results; when none of the positive criteria is fulfilled and the $\delta^{13}C$ values of TC are not consistent with an endogenous origin[119].

Concerning the reporting procedure of 19-norsteroids testing results, instead, WADA has established threshold values to exclude that the presence of 19-NA in urine (after adjustment for the urine specific gravity, if > 1.01858) is not due to an intentional administration of nandrolone or precursors: 15 ng/mL for pregnant female athletes, 10 ng/mL for female athletes using norethisterone and 2.5 ng/mL for all other cases. As mentioned before, the GC-C-IRMS analysis is executed on samples showing 19-NA concentration between 2.5 and 15 ng/mL and not collecting from pregnant athletes or in therapy with norethisterone, and on samples from pregnant female athletes in which 19-NA is higher than 15 ng/mL. Adverse analytical findings (AAF) are reported when the $\delta^{13}C$ value of 19-NA is consistent with its exogenous origin. In case of IRMS results inconclusive or consistent with endogenous values, the 19-NA/19-NE ratio is determined to confirm or not the atypical results to the anti-doping test[164].

Findings for prednisone and prednisolone are reported according to the recommendation given by WADA in the Technical Letter TL19[185]. An analytical outcome shall be considered AAF when the estimated concentration of prednisone and/or prednisolone (SG-adjusted) is > 30 ng/mL and < 60 ng/mL and the GC/C/IRMS analysis demonstrates an exogenous origin of the substance(s). On the contrary, negative findings shall be reported if the overall metabolic pattern indicates a microbial *in situ* activity and the $\delta^{13}C$ values of both Pred and Plone are consistent with an endogenous origin.

1.4.2 GC-C-IRMS instrumentation: principles of operation for carbon isotopes ratios measurements

The basic components of a GC-C-IRMS system are a gas chromatograph, a mass spectrometer for isotopic ratio and a connection between them (combustion furnace)[189,191,202,220–222].

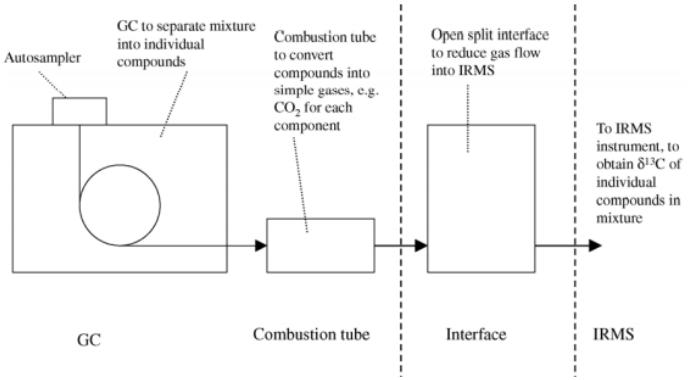

Figure 1.10 Schematic outline of a GC-C-IRMS system configured for C-isotopic analysis[189]

The first relevant constraint for the isotope ratio detection is the analyte quantitative vaporization and transfer to the GC-column to avoid any possible fractionation process during the injection phase. The *splitless* (the split line is closed so that both the solvent and the compounds of interest enter the column) mode injection is the most widely used for analyzing compounds found at low concentrations (nanograms) in the biological matrices. The *large volume* injection (by a *programmed temperature vaporizer,* PTV, injector in which the solvent is totally evaporated before the transfer of target analytes to the analytical column) is an alternative injection mode, normally used in trace or ultra-trace analyses. Regardless of the type of injector, the sample is entered through a silicone rubber septum in the rapid vaporization chamber (the liner, in which the sample in liquid phase passes into gaseous phase and is mixed with the carrier gas, He) arranged at the column head, with the help of a micro-syringe.

At the exit of the chromatographic column there is a glass or metal splitter named "Y" for its shape, through which the sample is transferred into the oxidation reactor, the heart of the GC-IRMS interface. It is a high-temperature (under certain conditions, it reaches 1100 °C) chemical reactor that continuously and quantitatively transforms complex organic molecules into a single gaseous species. The isotopic ratio of $^{13}C/^{12}C$ is then determined on the CO_2 in which the organic compounds are converted into. (Same principle is at the base also of the determination of $^{2}H/^{1}H$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, $^{34}S/^{33}S$ and $^{37}Cl/^{35}Cl$, converted to H_2, N_2, CO, SO_2 and CH_3Cl respectively). The reactor is a non-porous alumina tube (Al_2O_3

99.7%, 0.5 mm internal diameter, 1.55 mm outer diameter, 320 mm length) packed with metal oxide. It contains three cables of the same length of 240 mm and 0.125 mm in diameter twisted together and operating at 900-950 °C (940 °C in our setting conditions): one of copper (Cu), one of nickel (Ni) and one of platinum (Pt). CuO is the better oxidant at temperature below 800 °C, whereas NiO is favorable at high temperature; Pt acts as catalyst. Ni wire is the sole oxidant material for analyzing extremely thermally stable molecules for which temperature up to 1150 °C must be used. O_2 consumed in the combustion of the analytes must be periodically regenerated: typically, in our practice, the reactor undergoes a re-oxidation cycles at the beginning and at the end of each analytical session (for a maximum of 40 samples). The water resultant from the combustion process is removed through a selectively permeable membrane of the sulfonated fluoropolymer Nafion™: H_2O can participate in proton transfer reactions leading to the formation of some isobaric species interfering with the analysis of $^{13}CO_2$ (i.e. $^{12}C^{16}O_2H^+$ against $^{13}C^{16}O_2^+$ at *m/z* 45).

The gaseous samples coming from the GC column reach the isotope ratio-MS consisting of an electron-impact ionization source, a single magnetic-sector analyzer and multiple Faraday detector[223]. In the ionization chamber the electron beam, which is produced by a tungsten filament or rhenium to which is applied a potential difference, orthogonally meets the sample gas flow into the ionization region. The ion beams in the analyzer output, are focused and collected on a collector electrode or Faraday cup. For carbon, there are 3 Faraday cups that monitors the various isotopologues of CO_2:

- *m/z* 44: $^{12}C^{16}O^{16}O^{+\bullet}$
- *m/z* 45: $^{13}C^{16}O^{16}O^{+\bullet}$ (93.5 %) and $^{12}C^{17}O^{16}O^{+\bullet}$ (6.5 %)
- *m/z* 46: $^{12}C^{18}O^{16}O^{+\bullet}$ primarily, but minor contributions also of $^{13}C^{17}O^{16}O^{+\bullet}$ (0.20 %) and of $^{12}C^{17}O^{17}O^{+\bullet}$ (about 0.0036 %).

Measurements of $\delta^{13}C$ values must be corrected for the amount of ^{17}O in the CO_2: ^{17}O abundance is defined by measurements of $\delta^{18}O$ of the sample, obtained through the determination of the ratio of the *m/z* 46 and 44 ion currents. This correction for ^{17}O (Craig correction) relies on an accurate estimation of the ^{17}O-^{18}O abundance relationship in the major terrestrial oxygen pools, without physically measuring the ^{17}O abundance of CO_2[202,224].

In this PhD work, the GC-C-IRMS analyses were performed on a Thermo Delta Plus or Thermo DELTA V™ Advantage isotope ratio mass spectrometer (both from

ThermoElectron, Bremen, Germany), coupled to a Thermo TRACE™ 1310 GC (ThermoElectron, Bremen, Germany) or a HP7890 gas chromatograph (Agilent Technologies, Milan, Italy) through a Thermo Isolink-Conflo IV Interface (ThermoElectron, Bremen, Germany). The next generation system (Thermo DELTA V™ Advantage) is provided of a dual injection mode (split/splitless and PTV) and a Thermo ISQ single quadrupole mass spectrometer (ThermoElectron, Bremen, Germany) connected with a 4-Port Silflow MCD (0.25) to the single TRACE™ 1310 GC.

1.4.3 Sample-pretreatment for the GC-C-IRMS analysis

Before the IRMS analysis, the urinary concentrations of TCs and ERCs was estimated by GC-MS/MS to select the adequate volume of urine (5 – 25 mL, divided into more than one aliquot of max 7 mL) to process. Then, 0.75 mL of phosphate buffer (0.8 M, pH 7.4) and 100 µL of β-glucuronidase from *E. coli* were added to urine samples to perform the enzymatic hydrolysis (55 °C, 60 min). After cooling, pH was adjusted to 9-10 with carbonate buffer (0.50 mL; 20%) and a liquid/liquid extraction was achieved on a mechanical shaker for at least 20 min with 10 mL of organic solvent (tert-butyl methyl ether, TBME, or n-pentane according to the method used). Once separated, the solvent of the different aliquots was combined and taken to dryness (75 °C, under nitrogen stream). The final residue was reconstituted in 50 µL of a methyltestosterone water:methanol 50:50 mixture (ISTDLC, 100 µg/mL), the common internal standard selected for the next HPLC purification step. The sample treatment via HPLC (one or two sequential step(s) depending on the method considered) is crucial in the IRMS procedure to get final urinary extracts of adequate purity able to guarantee reliable $\delta^{13}C$ values. The combustion of organic substances in the oxidation reactor completely disrupts the original molecules: the IRMS data do not give any information about the chemical structure. The purification procedure before the carbon isotopic determination ensures that the CO_2 measured is certainly produced by the compounds of interest.

Derivatization of organic functional groups in the pre-treatment procedure is common in GC-IRMS, just as in GC-MS, but the correction for atoms added by derivative could be problematic, even if conceptually simple. Indeed, all the GC-C-IRMS methods developed in our Laboratory and reported in this work do not involve the steroid molecules derivatization.

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Chapter 2

Objectives and outline of the study

The isotope ratio mass spectrometry coupled to gas chromatography (GC-C-IRMS) is a mandatory procedure in anti-doping analyses performed on urinary samples in which the concentration and/or the ratio concentrations of specific steroid profile markers overcome the limits defined by the World Anti-Doping Agency (WADA) or deviate from the population and individual reference range[1]. It is a confirmatory investigation to establish whether these alterations, if not caused by physiological reasons, can be attributed to a synthetic steroids' intake. Indeed, the determination of their ^{13}C composition, which is typically more depleted for pharmaceutical preparations compared to their physiologically produced counterparts[23], allows to disclose the exogenous or endogenous origin of the steroid compounds.

Applicability and efficacy of the IRMS technique in the routine doping controls shall comply with several requirements and overcome some criticalities.

A. High sensitivity must to be guaranteed to detect the abuse of compounds (as 19-nortestosterone and boldenone and metabolites) found in urine at very low levels (ca. 2 ng/mL or lower)[14].
B. Urinary samples to be analyzed in the GC-C-IRMS procedure must be adequately purified to obtain reliable $\delta^{13}C$ values not affected by potential interferences, requiring a laborious pre-treatment process.
C. Prohibited steroids, such as 19-norsteroids, boldenone and its metabolite and prednisone and prednisolone[5–8], could be produced *ex-vivo* by gut microbial flora from endogenous steroids and then naturally excreted in urine. *In-vitro* formation in urinary specimens by urinary microbial flora cannot be excluded. Specific IRMS methods need to be developed to identify a drug misuse or an enzymatic activity.
D. In order to reduce the risk of inconclusive or false negative results, the selection of new target compound should be also considered and the current IRMS protocols optimized accordingly.
E. The synthesis of products with $\delta^{13}C$ values close to the endogenous reference range have been already shown[9]. The ^{13}C carbon isotopic composition of other pharmaceutical substances prohibited in sport should be determined to assess eventual limitations of the IRMS analysis.

F. Even though $\delta^{13}C$ values are high stable parameters[10] essentially depending on the individual dietary habits, further investigation on possible exogenous factors affecting the individual isotopic profile shall be performed, as has already done for the urinary steroid profile[11].

The research described in this work addresses some these issues, proposing new or alternative GC-C-IRMS approaches.
It is divided into two main parts:
PART I, in which:

i. the advantages of injecting large sample volume in a programmed temperature vaporizing (PTV) inlet to enhance the sensitivity of the methods and reduce the volume of urinary matrices to be processed, are presented;
ii. a method to discriminate the *ex-vivo* or exogenous origin of prednisolone and prednisone is proposed;
iii. the inclusion of 19-norethiocolanolone as additional target compound is proposed to prevent some limitations of the current protocol for the detection of the abuse of 19-norsteroids.

PART II, where:

i. new commercially available prednisolone and prednisone-based formulations are analyzed to verify the applicability of the method previously developed;
ii. the effect of 5α-reductase inhibitors as potential confounding factors on the IRMS findings is investigated.

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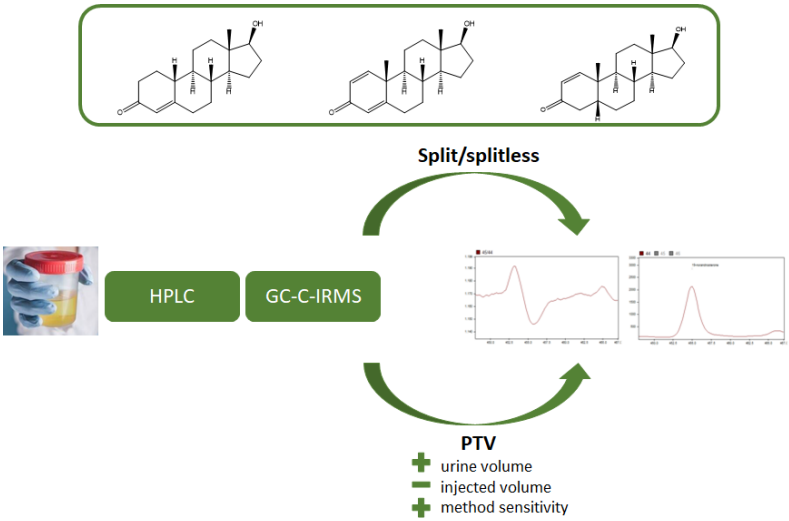
Part I

Improved confirmation methods for compounds *ex-vivo* produced

Chapter 3

Advantages of using the large volume injection technique in GC-C-IRMS doping control analyses



3.1 Abstract

The $^{13}C/^{12}C$ ratio, expressed as $\delta^{13}C$ (‰) value, is determined through a GC-C-IRMS (gas chromatography combustion isotope ratio mass spectrometry) analysis to discriminate the exogenous or endogenous origin of pseudo-endogenous steroids, included in the List of the Prohibited Substances and Methods published yearly by the World Anti-Doping Agency (WADA). The GC-C-IRMS method is a confirmation procedure performed in all accredited doping laboratories when urinary concentration of specific steroid profile markers or metabolites exceeds the limits defined in reference Technical Documents or after specific request of the Testing Authorities. The criteria to establish the origin of boldenone and 19-norandrosterone (19-NA, the main metabolite of nandrolone) require high analysis sensitivity: the GC-C-IRMS method must be able to detect very low urinary levels (2 ng/mL). According to the standard operating protocols, a large urine sample volume (up to 25 mL) is usually processed, and 2 or 3 µL of the purified samples are injected in splitless mode in the IRMS equipment to obtain adequate signals within the linearity range (210 – 6000 mV). Data collected after injecting large sample volume (up to 9 µL) are discussed in order to propose alternative instrumental settings to improve sensitivity and, at the same time, to reduce the needed urine volume.

3.2 Introduction

The isotope ratio mass spectrometry coupled to gas chromatography (GC-C-IRMS) is the technique used in anti-doping controls to detect the abuse of pseudo-endogenous steroids, compounds showing the same chemical structure but different ^{13}C content compared to their respective endogenous analogues[1,2]. The IRMS was firstly introduced in a testing program of a worldwide sportive event in 1998, during the Japanese Olympic Winter Games[3]. Since then, even if it involves a laborious and time-consuming samples pre-treatment process, it was applied to an increasing number of suspicious or atypical samples.

The IRMS procedure must ensure high sensitivity for confirming the origin of testosterone metabolites (excreted in urine in relatively high amount: 10 to 10000 ng/mL)[2], but also of steroids (as 19-norsteroids, boldenone and metabolite) possibly produced *ex-vivo* in low concentrations by the gut or urinary microbial flora. In details, WADA requires the GC-C-IRMS analysis when the urinary levels of 19-norandrosterone (19-NA) range from 2.5 to 15 ng/mL[4] and those of boldenone and/or its metabolite are between 5 and 30 ng/mL[2].

The GC-C-IRMS ordinary protocols provide for the use of variable volume of urine, 21 - 25 mL, divided into more than one aliquot, and for a final injection (in splitless mode) of 2 – 3 µL in a split/splitless inlet[5–7]. The injection in splitless mode, in which the split line is closed so that both the solvent and the target compounds enter the column, is an essential condition in trace analyses. In the measurement of carbon isotope ratio (CIR), the complete vaporization of the sample before entering the column is a must because evaporation and partial adherence of the analyte to the column head can produce significant changes in the resulting delta values. However, the need of high volume of urine to process could be a critical issue, considering that part of the disposable volume (less than 70 mL) is commonly consumed in the initial screening tests and that additional volumes should be reserved to other requested analyses or confirmation procedures. In a previous work, it has already showed that the selection of a new type of injection, by using a solvent vent injection instead of splitless one, may increase the sensitivity of the IRMS method, drastically reducing the amount of urine required. It has been applied to the analysis of the key markers of the steroid profile, also selected as target compounds (TC) to detect the abuse of pseudo-endogenous steroids:

testosterone (T), epitestosterone (E), androsterone (A), etiocholanolone (Etio), 5α-androstan-3α,17β-diol (5αAdiol), 5β-androstan-3α,17β-diol (5βAdiol)[8].

In this chapter, we evaluated the advantages of the large volume injection (LVI) in the GC-C-IRMS methods for the analysis of 19-norsteroids, and boldenone and its metabolite.

3.3 Materials and methods

3.3.1 Chemicals and reagents

Certified reference material of 19-norandrosterone (3α-hydroxy-5α-estrane-17-one, **19-NA**) was from NMIA (Lindfield, Australia). Boldenone (17β-hydroxy-1,4-androstadien-3-one, **β-Bold**) and 5b-androst-1-ene-17b-ol-3-one (**BM2**) were supplied by Steraloids (Newport, RI, USA), as well as the 5α-androstan-3β-ol (**ISTDRI**), the internal standard used to dissolve the purified and dried extracts before the instrumental analysis. 17α-methyltestosterone (**MT**), the selected internal standard for the HPLC steps, was from Sigma-Aldrich (Milan, Italy).
All solvents and reagents were of analytical or HPLC grade and purchased from Carlo Erba (Milano, Italy). β-Glucuronidase from *Escherichia coli K12* was provided by Roche Diagnostic (Mannheim, Germany). Water was from a Milli Q water purification system (Millipore S.p.A., Milan, Italy).
The calibration of the CO_2 reference gas (Solgas, Monza, Italy) for the isotope ratio mass spectrometer was performed against underivatized steroids (CU/PCC 34-3) with certified delta values traceable to VPDB, obtained from Prof. Brenna (Cornell University Certified Reference Material)[9].

3.3.2 Sample preparation

Blank urine samples (BUR) were spiked with 19-NA or β-Bold and BM2 reference materials to obtain positive urine samples (USP) at the limit of quantification (LOQ) of their corresponding methods (2 and 4 ng/mL respectively). After the enzymatic hydrolysis with β-glucuronidase (pH 7.4) and the liquid/liquid extraction at pH 9 with n-pentane or *tert*-butyl methyl ether (TBME) respectively, samples have been adequately pre-treated under the conditions elsewhere described[7,9]. A suitable HPLC step has been performed before the determination of $\delta^{13}C$ values to obtain pure compounds free of interferences that could be affect the reliability of the IRMS findings.

3.3.3 GC-C-IRMS analysis in a large volume injection mode

The GC-C-IRMS analyses were carried out on a Thermo DELTA V™ Advantage isotope ratio mass spectrometer connected to a combustion reactor (at 940 °C) coupled to a Thermo TRACE™ 1310 GC through a Thermo Isolink-Conflo IV Interface (all from ThermoElectron, Bremen, Germany). The instrument is equipped with a programmed temperature vaporizing (PTV) injector, that allows to perform a large volume injection, besides the most frequently used split and splitless ones.

The large volume injection is an injection mode normally selected in case of trace and ultra-trace analyses[10–12]. Its operating principle can be divided into different steps, as represented in Figure 3.1:

a) cold injection. The sample is injected at temperature (65°C) below the boiling point of the solvent and the gas flow is increased. Less thermal stress and better recovery of thermolabile compounds are guaranteed.

b) Solvent elimination. The injector temperature is increased to 280°C very rapidly, allowing the evaporation of the solvent and a subsequent pre-concentration of the compounds. In this phase, the split valve is open, and the analytes are retained in the liner.

c) Transfer of analytes to the GC column in splitless mode.

d) Cleaning process. The temperature and the gas flow are increased; the split line is re-opened. High boiling compounds left in the liner are removed.

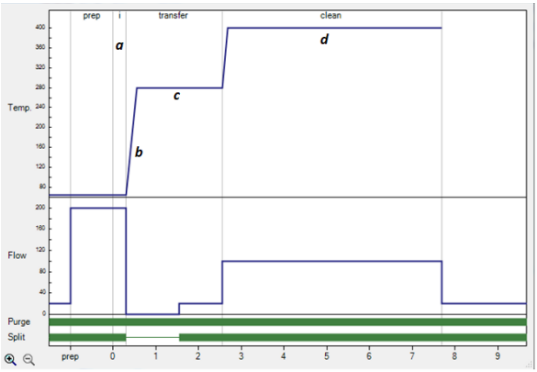


Figure 3.1 Schematic representation of the large volume injection phases in a programmed temperature vaporizer inlet

In our experimental conditions, the instrument was equipped with a PTV liner (2 mm ID, L=120 mm) and a HP5MS (J&W Scientific) 5% phenylmethyl fused-silica

capillary column (30 m x 0.25 mm i.d., 0.25 µm film thickness). As solvent, a mixture of cyclohexane/isopropanol showing boiling points of 80.7 and 82.6 °C (> 65 °C, the injection temperature in the cold vaporizer), respectively, was used. In Figure 3.2a-b the instrumental setting conditions of splitless and LVI injection mode are compared.

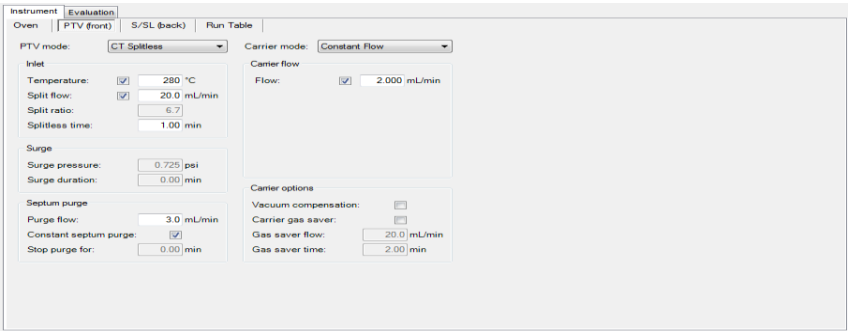


Figure 3.2a Instrumental setting in splitless injection mode

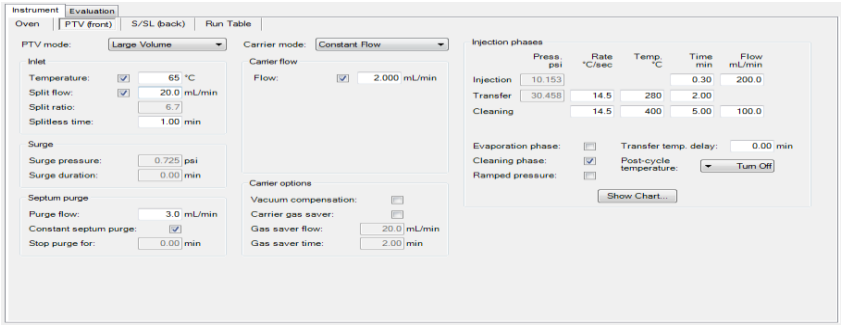


Figure 3.2b Instrumental setting in large volume injection mode

GC-C-IRMS analysis of boldenone and its metabolite

Blank urine samples spiked with BM2 at 4 ng/mL (LOQ of the method already described[7]) were processed starting from 21 mL of urine (3 x 7 mL) in accordance with the routine confirmation method, or from a single aliquot of 7 mL. Positive urine sample at 2 ng/mL of β-Bold and BM2 have been injected in PTV mode, selecting increasing injection volumes (from 1 to 9 µL) and assessing the acceptability of the delta values and the signal and background (BGD) amplitudes.

Each test was repeated three times. The sensitivity of the method was re-evaluated in the new injection conditions.

GC-C-IRMS analysis of 19-norandrosterone

Three positive urine samples (21 mL, divided into 3 aliquots of 7 mL) were obtained by the same urinary matrix fortified with 19-NA reference material at 2 ng/mL (LOQ). They were injected in splitless (3 µL) or PTV (3 or 9 µL) mode.

3.4 Results and Discussion

3.4.1 GC-C-IRMS analysis of boldenone and its metabolite

Twenty-one µL of blank urine sample fortified with adequate amount of BM2 to obtain positive urine sample at LOQ of 4 ng/mL were injected according to the standard operating protocol (splitless injection; 2 µL) (see Figure 3.3a). The LOQ of the method was verified by processing lower volume of urine (7 ml instead of 21 mL) and injecting in the PTV injector larger volume (6 µL rather than 2 µL) of the purified sample (see Figure 3.3c). The LOQ of 4 ng/mL and the signal amplitude within the linearity range of the instrument (210 to 6000 mV) have been guaranteed in both the circumstances. This preliminary outcome has led us to repeat the test on 21 mL of a positive urine sample at 2 ng/mL: as shown in Figure 3.3b, the signal was acceptable (inside the instrumental linearity) and with optimal peak shape.

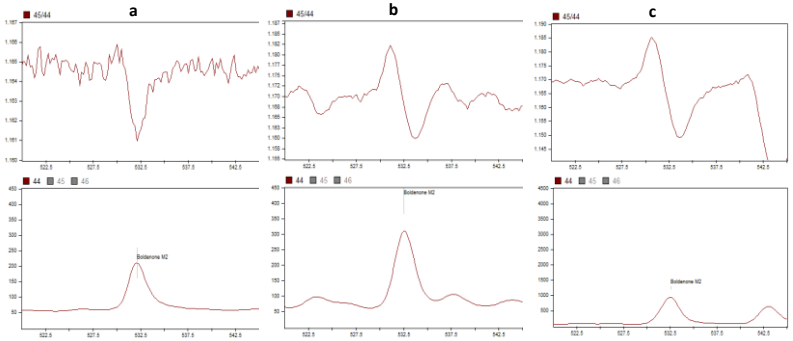


Figure 3.3 Chromatographic GC-C-IRMS profile analysis of BM2 (m/z 44 lower and 45/44 upper) after different volumes and injection conditions

A lower LOQ (2 ng/mL) has been defined, accordingly. The suitability of the LVI in the analysis of β-bold and BM2 was verified after 1, 2, 4, 6 and 9 µL of injection (in triplicate) of positive urine samples (21 mL) at 2 ng/mL. Each test was repeated three times to verify the method linearity and reproducibility. The $\delta^{13}C$ values obtained were within the acceptability ranges (-30.0 to -28.0 ‰ for β-boldenone; -31.0 to -29.0 ‰ for boldenone M2), showing good repeatability among the triplicates. A standard deviation < 0.5 ‰, the maximum inter-assay variation accepted by the instrument manufacturer, was measured along the different signals. The response amplitude was higher than 210 mV for all samples injected, except for the injection of 1 µL of β-bold; the background amplitude was not remarkable influenced by the increasing injection volumes. (See Figure 3.4a-b)

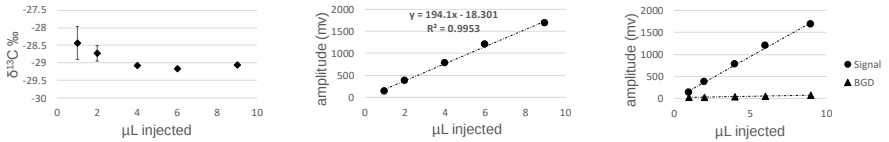


Figure 3.4a β-Bold: comparison of $\delta^{13}C$ values, signal and BGD amplitudes using PTV injection at different volumes (1, 2, 4, 6, 9 µL)

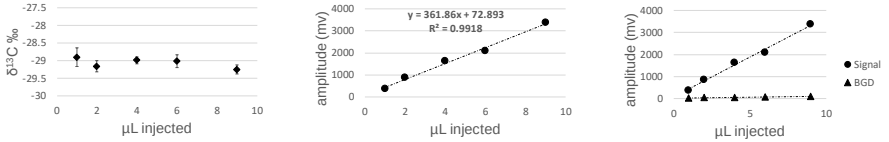


Figure 3.4b BM2: comparison of $\delta^{13}C$ values, signal and BGD amplitudes using PTV injection at different volumes (1, 2, 4, 6, 9 µL)

3.4.2 GC-C-IRMS analysis of 19-norandrosterone

Twenty-one mL of urine samples fortified with 19-NA reference material at 2 ng/mL were processed and injected in splitless and PTV injection mode. The GC-C-IRMS chromatographic profile are shown in Figure 3.5. For equal injected volume (3 µL), the PTV injection mode, allowed to improve the chromatographic peak shape, by removing peak tailing effect and reducing the background noise. The large volume injection (9 µL) ensured the better chromatographic response in terms of Gaussian peak and signal amplitude. The larger amount of urinary purified matrix transferred to the GC column did not negatively affect the background signal.

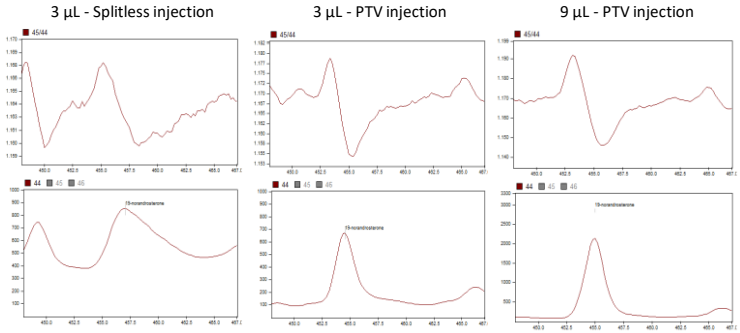


Figure 3.5 Chromatographic GC-C-IRMS profile analysis of 19-norandrosterone (m/z 44 lower and 45/44 upper) after different volumes and injection conditions

3.5 Conclusions

The large volume injection by a programmed temperature vaporizing injector is an alternative injection mode useful to ensure a pre-concentration of trace compounds and a better recovery of thermolabile substances, minimizing the thermal degradation in the vaporization chamber. It has proved to be an efficient tool for the evaluation of fatty acids, essential oils, pesticides in food analysis[13,14], and of androgenic steroids in doping control procedures[8].

In this work we showed that the adoption of a large volume injection allows to improve the sensitivity of the current methods for detecting the abuse of 19-norsteroids and boldenone and its metabolite, guaranteeing reliable $\delta^{13}C$ values and signal amplitude within the instrumental linearity range (210 – 6000 mV).

The results proved that the ability to adjust the injection volumes, choosing volumes larger than those traditionally used in the splitless mode, enables to reduce and standardize the initial urine sample volumes, making the preliminary sample preparation steps faster and less laborious. The reduced urine volume also lowers the risk of column overloading and the matrix effect in the instrumental analysis.

The benefits of using a PTV injector was further investigated in the next chapters, also in the determination of the exogenous or *ex-vivo* origin of prednisolone and prednisone, glucocorticoids for which the IRMS confirmation test is required at urinary concentrations between 30 and 60 ng/mL.

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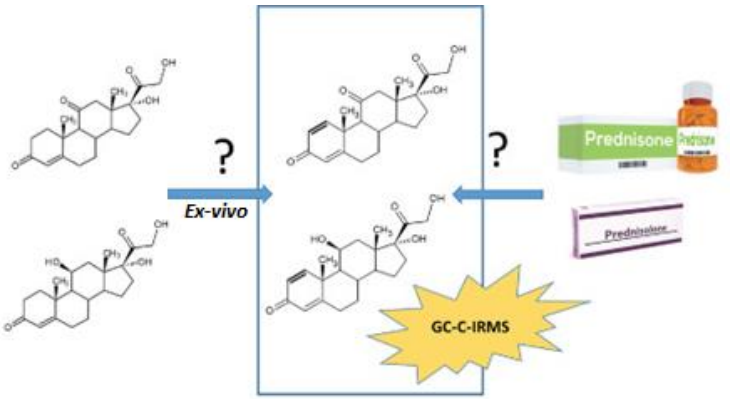
Chapter 4

Development and validation of a method to confirm the exogenous origin of prednisone and prednisolone by GC-C-IRMS



Adapted from

L. Iannella, C. Colamonici, D. Curcio, F. Botrè, X. de la Torre (2019)

Development and validation of a method to confirm the exogenous origin of prednisone and prednisolone by GC-C-IRMS

Drug Testing and Analysis, 11: 1615-1628

4.1 Abstract

Prednisone and prednisolone are two anti-inflammatory steroidal drugs listed by the World Anti-Doping Agency (WADA) within the class of glucocorticoids, which are prohibited "in competition" and when administered systemically. Their presence in collected urine samples may be attributed, if no exogenous administration occurred, to an *in situ* microbial formation from endogenous steroids. In this chapter, a gas chromatography coupled to carbon isotope ratio mass spectrometry (GC-C-IRMS) method was developed and validated to distinguish their exogenous origin from the endogenous one. Eight prednisone/prednisolone pharmaceutical preparations commercially available in Italy were analysed to establish an exogenous $\delta^{13}C$ values reference range (-28.96 ± 0.39 ‰). No more than 25 mL of urine were processed, and no derivatization nor intentional steroids structure modifications were performed before the GC-C-IRMS analysis. A first HPLC purification step was set up to isolate the three endogenous reference compounds (ERCs) selected (tetrahydro-11-deoxycortisol (THS), pregnanediol (PD) and pregnanetriol (PT)), while a second LC purification was necessary to separate prednisone from prednisolone. In the GC-C-IRMS analysis, two different GC run methods were set up to guarantee the better sensitivity and selectivity for each compound. Both prednisone and prednisolone showed signals (m/z 44) with amplitudes within the method linearity range until a lower urinary concentration of 20 ng/mL (< WADA reporting level, 30 ng/mL). The method was fully validated according to the WADA requirements. As a proof of concept, urine samples collected from two excretion studies in healthy male volunteers after a prednisone or prednisolone administration were analysed by the proposed method, demonstrating its applicability for the analysis of real samples.

4.2 Introduction

Prednisolone and prednisone are two synthetic glucocorticoids widely used in clinical practice to treat inflammatory and autoimmune diseases both in human and in veterinary medicine. Prednisolone is the active substance usually administered as prednisone in oral formulations. The biotransformation of prednisone into prednisolone is catalysed by the hepatic 11β-hydroxysteroid dehydrogenase (11β-HSD1). The enzymatic conversion between the two compounds is a reversible process, which makes prednisone not only a prodrug, but also a prednisolone metabolite[1]. Prednisolone and prednisone show higher anti-inflammatory potency and longer pharmacological activity than their structurally related natural glucocorticoids, cortisol and cortisone[2,3]. Due to their pharmacological properties, they are misused by athletes to enhance the physical endurance and tolerance for pain and, therefore, they are listed by WADA as prohibited substances[4]. More specifically, they are included in the section S9 of the WADA Prohibited List and banned "in competition" when administered by oral, intravenous, intramuscular or rectal routes[5]. A reporting level of 30 ng/mL for parent compounds and/or their metabolites has been established by WADA to discriminate the permitted administration routes from the forbidden ones[6]. This has led the antidoping laboratories to investigate the excretion profile of prednisone and prednisolone administered by permitted and prohibited routes. Their major metabolites in urine have been identified and characterized by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) and the most significant and long-term markers (20β-hydroxy-prednisone and 20β-hydroxy-prednisolone) have been selected[4,7–10].

WADA has recently underlined the possible enzymatic formation of prednisone/prednisolone in collected human urine. The usual non-sterile collection or transportation conditions and the presence of normal or pathogenic microbial flora contamination are all favourable circumstances able to change urine specimens' composition. The bacterial activity may lead to an increase or depletion of endogenous steroids or even to the hydrolysis of conjugated metabolites, altering the specific steroid profile parameters[11–14]. Among the *in situ* bacterial reactions on endogenous steroids, 19-demethylation and Δ1-steroid-dehydrogenation, which lead to the formation of 19-norandrosterone (19-NA) and

boldenone, respectively, have been extensively reported in the literature[15,16]. The uncommon presence of prednisone and prednisolone in human urine may be attributed to the activity of Δ^1-SDH on steroids (respectively cortisone and cortisol) physiologically excreted into urine[17–20]. To discriminate the endogenous or exogenous origin of prednisone and prednisolone, WADA has established the GC-C-IRMS analysis as a recommended confirmation at concentrations between the reporting level of 30 ng/mL and 60 ng/mL.

The aim of this work is to disclose the origin of prednisone and prednisolone by GC-C-IRMS, developing and validating a specific method in accordance with the WADA Technical Document TD2019IRMS[21] and the ISO17025 requirements.

The procedural conditions were set up starting from the experience of the previous already validated for the confirmation analysis of pseudo-endogenous glucocorticoids[22,23]. The method was validated selecting tetrahydro-11-deoxycortisol (THS) as endogenous reference compound (ERC) since 11-desoxycortisol (S) is in an upper stage of the glucocorticoids metabolism and there is no conversion from cortisol to S. Urine samples are processed without any derivatization or steroids structure intentional modification before the instrumental analysis, but after an adequate purification step necessary to remove potential interferences in the determination of $\delta^{13}C$ values. For the method here developed, pregnanediol (PD) and pregnanetriol (PT) were also included, since they are not affected by the exogenous administration of prednisone or prednisolone. The operating procedure was performed using the largest urine volume (25 mL) available for the GC-C-IRMS confirmation analysis. The opportunity to reduce the initial urine volume needed by injecting larger volume in a programmed temperature vaporizing (PTV) inlet[24] was also evaluated.

4.3 Materials and methods

4.3.1 Standards and reagents

The standards of prednisolone (11β,17α, 21-trihydroxypregna-1,4-diene-3,20-dione), prednisone (17α,21-dihydroxypregna-1,4-diene-3,11,20-trione), tetrahydro-11-deoxycortisol (**THS**, 3α,17α,21-trihydroxy-5β-pregnan-20-one), pregnanetriol (**PT**, 5β-pregnane-3α,17α,20α-triol), 17α-methyltestosterone (**MT**), dexamethasone (**DESA**), (11β,16α)-9-fluoro-11,17,21-trihydroxy-16-methylpregna-

1,4-diene-3,20-dione), adrenosterone (androst-4-ene-3,11,17-trione), cortisol (11β,17α, 21-trihydroxypregna-4-ene-3,20-dione), cortisone (17,21-dihydroxypregna-4-ene-3,11,20-trione), tetrahydrocortisol (**THF**, 3α,11β,17α,21-tetrahydroxy-5β-pregnane-20-one), allo-tetrahydrocortisol (**allo-THF**, 3α,11β,17α,21-tetrahydroxy-5α-pregnane-20-one), tetrahydrocortisone (**THE**, 3α,17α-21-trihydroxy-5β-pregnane-11,20-dione) and allo-tetrahydrocortisone (**allo-THE**, 3α,17α-21-trihydroxy-5α-pregnane-11,20-dione) were supplied by Sigma-Aldrich (Milan, Italy). Pregnanediol (**PD**, 5β-pregnane-3α, 20α-diol) and 5α-androstan-3β-ol were from Steraloids (Newport, RI, USA). Δ1-adrenosterone (andros-1,4-diene-3,11,17-trione) was purchased from LGC (Milan, Italy).

Solvents (*tert*-butyl methyl ether, acetonitrile, methanol, cyclohexane and isopropanol) and reagents (sodium bicarbonate, potassium carbonate, sodium phosphate, sodium hydrogen phosphate) were of analytical or HPLC grade and provided by Carlo Erba (Milano, Italy). β-Glucuronidase from *Escherichia coli* K12 was obtained from Roche Diagnostic (Mannheim, Germany). Water was from a Milli Q water purification system (Millipore S.p.A., Milan, Italy).

CO_2 reference gas (Solgas, Monza, Italy) for isotope ratio mass spectrometer calibration was calibrated against underivatized steroids (CU/PCC 34-3) with certified delta values traceable to VPDB, obtained from Prof. Brenna (Cornell University Certified Reference Material)[25].

4.3.2 Pharmaceutical preparations containing prednisone or prednisolone

Eight different commercially available formulations were analysed: six were prednisone oral pharmaceutical preparations and two were prednisolone preparations clinically administered by oral and ocular route (see Table 1). They were all produced by Italian pharmaceutical companies and purchased from Italian pharmacies. The tablets containing prednisone or prednisolone that were not already in solution were crushed in a mortar and dissolved in adequate volume of methanol to obtain a 1 mg/mL solution. The mixture was sonicated for 5 min, centrifuged and the solution transferred to a new vial. For the pharmaceutical preparations already in solution, a direct dilution in methanol was prepared to get the same final 1 mg/mL solution. An adequate dilution using a mixture of

cyclohexane/isopropanol (4:1) containing 5α-androstan-3β-ol as internal standard was prepared before their analysis by GC-C-IRMS.

4.3.3 Urine samples

Urine samples were collected from two male volunteers (27 and 36 years old) before and after the oral administration of a single dose of prednisone (Deltacortene® 5 mg, Bruno Farmaceutici, Italy) or prednisolone (Sintredius® 1 mg/mL, Dompè, Italy) to prove the method applicability to real cases. Prednisone, prednisolone, THS, PD and PT were extracted from urine samples, purified and injected in the GC-C-IRMS system. The endogenous glucocorticoids metabolites selected as ERCs for the detection of cortisone misuse (tetrahydrocortisone, THE; 5α-tetrahydrocortisone, allo-THE; tetrahydrocortisol, THF; and 5α-tetrahydrocortisol, allo-THF) were also analysed.

4.3.4 Sample preparation

Samples were prepared as described previously[23,26]. Briefly, phosphate buffer (0.8 M, pH 7.4) 1.5 mL and 100 µL of β-glucuronidase from *E. coli* were added to 25 (4 x 6.25) or 10 (2 x 5) mL of urine to perform the hydrolysis (55 °C, 60 min). After cooling, pH was adjusted to 9-10 with carbonate buffer (20%) and extraction performed with 10 mL of *tert*-butyl methyl ether (at least 20 min on a mechanical shaker). Once separated, the solvent of the different aliquots was combined and take to dryness (75 °C, under nitrogen stream). The final residue was reconstituted in 50 µL of a methyltestosterone solution (100 µg/mL in a water:methanol 50:50 mixture) for the next HPLC purification steps.

4.3.5 HPLC sample purification

The analytes of interest were prednisone and prednisolone as target compounds (TCs), and THS, PD and PT as ERCs. Two sequential HPLC steps were necessary to obtain final extracts from the urine samples of adequate purity to get reliable results during the IRMS analyses.

The first purification was executed injecting 50 µL of the pre-treated samples in an Agilent 1100 Series liquid chromatograph (Agilent Technologies S.p.A., Cernusco sul Naviglio, Milan, Italy) equipped with an ACE® C18 column (25 cm, 4.6 mm, 5 µm) and an ACE® C18 precolumn (2 cm, 4.6 mm, 5 µm) from CPS Analitica (Milan, Italy).

The mobile phase consisted of water (solvent A) and acetonitrile (solvent B) with a flow rate of 1 mL/min at 38 °C. An isocratic program was set up at 38% of B until 32.5 min; a first increment to 55% of B in 0.01 min was followed by a further increase to 65% of B in 1 min for 4.5 min to finally reach 100% of B in 0.01 min for 3.99 min for a total run of 42 min. MT was selected as internal standard to check the repeatability of the elution conditions, monitoring the compounds signals at a UV lamp (192 nm, Agilent 1100 UV DAD detector). The THS, PD and PT collected fractions were taken to dryness (75 °C, under nitrogen stream) and dissolved with a mixture of cyclohexane:isopropanol (4:1) containing 5α-androstan-3β-ol (10 µg/mL) as the GC-C-IRMS internal standard[23,26]. Variable volumes of the dissolution mixture were used depending on the THS, PD and PT estimated concentration in the original samples to obtain adequate signal during the IRMS analysis. Prednisolone and prednisone eluted during the isocratic part of the program, respectively at the retention time of 5.5 and 5.7 min. They were collected in a single fraction that was dried and reconstituted in 50 µL of dexamethasone solution (100 µg/mL in a water:methanol 2:1 mixture) the second HPLC purification internal standard.

The second purification was performed using an ACE® EXCEL 5 C18 AMIDE column (25 cm, 4.6 mm, 5 µm) from CPS Analitica (Milan, Italy) at 20 °C. Water (solvent A) and methanol (solvent B) at flow rate of 1 mL/min were selected as a mobile phase. The chromatographic run started with 50% of B until 24.5 min, then increasing B to 100% in 0.01 min for 8.49 min for a total run of 33 min. Prednisone was eluted at 10.8 min, whereas prednisolone at 15.6 min: the two fractions separately collected were taken to dryness (75 °C, under nitrogen stream) and dissolved with 16 µL of a mixture cyclohexane:isopropanol (4:1) containing 5α-androstan-3β-ol (10 µg/mL). The absorbance signals of the analytes were detected at 254 nm by the Agilent 1100 UV DAD lamp.

4.3.6 GC-C-IRMS instrumental analysis

The purified extracts from 25 mL (4 x 6.25 mL) of initial urine volume were analysed in a HP7890 gas chromatograph (Agilent Technologies, Milan, Italy) connected to a combustion furnace (at 940 °C) linked to a Thermo Delta Plus isotope ratio mass spectrometer (ThermoElectron, Bremen, Germany) through a Thermo Isolink-Conflo IV Interface. The chromatographic run was conducted on a 5%-Phenyl-

methylpolysiloxane (30m x 0.25 mm i.d. x 0.25 µm film thickness) column from J&W Scientific. To guarantee an adequate sensitivity and sensibility for each compound, two different GC methods were implemented. For prednisolone, THS, PD and PT, helium as carrier gas was set at the opportune flow rate able to provide an internal standard retention time between 400 and 411 sec. The temperature ramp was programmed as follows: 150 °C (1 min hold), 25 °C/min to 260 °C for 3.6 min, 25 °C to 270 °C for 0.9 min, 40 °C/min to 290 °C (hold 1.20 min), 40 °C/min to 310 °C for 1.6 min. The purge time was set at 1 min, while the purge flow at 100 mL/min. A helium flow rate at 1.5 mL/min and an initial oven temperature of 130 °C (for 0.5 min) were established in the GC method specifically developed for prednisone. The temperature was increased 100 °C/min to 265 °C (held for 8.5 min) to finally reach (100 °C/min) 310 °C for 4 min. Two µL of each sample was injected in splitless mode at 280 °C.

The purified extracts from 10 mL of initial urine samples were analysed in a TRACE 1310 gas chromatograph (ThermoElectron, Bremen, Germany) connected to a combustion furnace (at 940 °C) linked to a Thermo Delta Advantage isotope ratio mass spectrometer (ThermoElectron, Bremen, Germany). The GC column features, the instrumental interface (Thermo Isolink-Conflo IV) and the chromatographic methods applied, were the same of just previously described. The PTV injection mode (Figure 4.1) supported by this GC system as well as the split/splitless one, allowed to obtain signals of an amplitude inside the linearity method range by processing lower urine volume and injecting larger extracts volume: 2 µL for ERCs, 3 µL for prednisolone and 8 µL for prednisone.

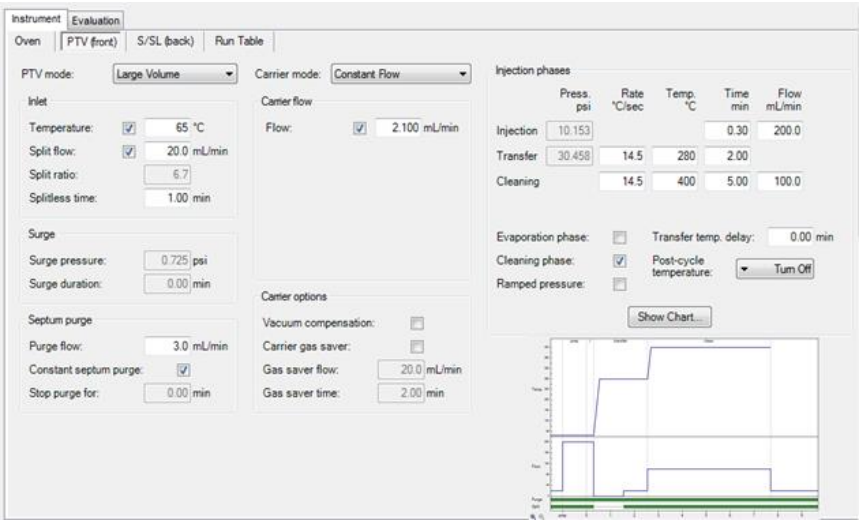


Figure 4.1 Instrumental conditions and phases of the PTV injection

4.3.7 GC-MS instrumental conditions

The purity of the extracted samples was verified before the $\delta^{13}C$ (‰) values determination through a GC-MS full scan analysis to ensure that no interferences could affect the carbon compounds isotopic composition. The HP6890 GC coupled to HP5973 mass spectrometer (MS) (Agilent Technologies SpA, Cernusco sul Naviglio, MI, Italy) was equipped with the same type of column and under the same chromatographic conditions used for the IRMS analysis. During the validation process, fractions just before and after the fractions of interest were also analyzed to verify the completeness of the peaks of interest collection.

Validation parameters

Blank urine samples were collected from healthy donors of different gender, age and covering usual pH (5 to 8) and specific gravities (SG from 1.003 to 1.030) ranges found in routine doping control samples. These selected urines were fortified with a mixture of prednisone and prednisolone standards solution (100 µg/mL) to obtain positive controls at specific concentrations (both at 10, 20 and 30 ng/mL).

The method validation was performed in compliance with the requirements of the WADA TD2019IRMS. Linearity, selectivity, limit of quantification (LOQ), recovery, repeatability and measurement uncertainties were evaluated. The new developed

procedure was applied to the prednisone and prednisolone analysis, whereas the HPLC and GC-C-IRMS already validated conditions were used for THS, PD, PT, THE, allo-THE, THF and allo-THF[23].

The linearity of the method was assessed by injecting six serially diluted standard prednisone and prednisolone solutions in splitless mode to define the response ranges producing consistent $\delta^{13}C$ (‰) values within the instrumental linearity range (from 0.2 to 7 V). All standard solutions were analysed in triplicate through the GC method specifically developed for each compound. A maximum standard deviation (SD) < 0.5 ‰ for each triplicate determination was considered acceptable (as mentioned in the TD2019IRMS). It was verified that the $\delta^{13}C$ (‰) mean values of each replicate did not deviate by more than 0.5 ‰ from the $\delta^{13}C$ (‰) mean value resulting from the overall 18 measurements. The mean value was assigned to the reference material. The selectivity was ensured by checking the presence of any interfering peak at the prednisone and prednisolone retention times in ten blank urine samples of ten different healthy donors. The chromatograms obtained with blank samples were compared to those of ten spiked positive urine samples at the reporting level of 30 ng/mL. The limit of quantification (LOQ) was calculated through the analysis of three sets of blank urine samples spiked with prednisone and prednisolone standard solutions to obtain positive samples at the reporting level of 30 ng/mL and below (20 ng/mL and 10 ng/mL). Three positive samples for each selected concentration were processed. The LOQ was defined as the lowest analyte concentration giving a delta values mean and SD < 1 ‰ (n = 3) showing a response in the linear range. The recovery percentage was studied by comparing the signals resulting from the analytes submitted to the overall operating procedure with that extrapolated from the linearity calibration curve. The repeatability of delta values was evaluated by checking that their SD in ten spiked positive samples injected in different days were below 0.5 ‰ (intermediate precision). The measurement uncertainty (u_c) was estimated by analysing ten different spiked positive urine samples and combining the intermediate precision and the bias from the delta values assigned to the reference materials during the linearity study (28.97 ± 0.20 ‰ for prednisone and 28.85 ± 0.27 ‰ for prednisolone). A maximum value of 1 ‰ was accepted.

4.4 Results and discussion

The aim of the newly developed method was to disclose whether the presence of prednisone and prednisolone in urine could be attributed to an exogenous administration or to an *in situ* microbial formation from endogenous cortisone and cortisol respectively.

4.4.1 $\delta^{13}C$ (‰) values determination of synthetic prednisone and prednisolone

In order to reach the proposed scope, the first step was to preliminarily confirm that the pharmaceutical preparations containing prednisone or prednisolone present a carbon isotopic composition (ratio of ^{13}C to ^{12}C) distinguishable from that of the endogenously produced compounds. To do so, 7 different oral pharmaceutical preparations and one eye drops formulation commercially available in Italy were prepared as described previously and analyzed directly by GC-C-IRMS. The $\delta^{13}C$ (‰) measurements were performed in triplicate. The mean value and the relative standard deviation (SD), obtained for each drug, is shown in Table 4.1. The exogenous average $\delta^{13}C$ (‰) value of the 8 pharmaceutical products was -28.96 ± 0.39 ‰, which is more depleted than that of urinary endogenous steroids in agreement with the data previously published on TCs and ERCs from European Caucasian population reference ranges (from -20 to -25 ‰)[27,28].

Brand name	Active principle	Manufacturer	Formulation	Batch	$\delta^{13}C$ (‰) Mean ± SD (n=3)
Lodotra®	Prednisone	Mundipharma Pharmaceuticals, Italy	Tablets	18W03503	-29.23 ± 0.13
Deltacortene®	Prednisone	Bruno Farmaceutici, Italy	Tablets	850560	-29.19 ± 0.12
Prednisone Teva®	Prednisone	Teva, Italy	Tablets	180833	-28.39 ± 0.07
Prednisone Mylan®	Prednisone	Mylan Pharma, Italy	Tablets	163066	-28.86 ± 0.11
Prednisone EG®	Prednisone	BF Research, Italy	Tablets	B155140	-28.45 ± 0.05
Prednisone DOC Generici®	Prednisone	DOC Generici, Italy	Tablets	173224	-29.43 ± 0.20
Sintredius®	Prednisolone	Dompé, Italy	Oral Solution	1090818	-29.28 ± 0.25
Solprene®	Prednisolone	Farmigea, Italy	Eye Drops	290318	-28.85 ± 0.27

Table 4.1 $\delta^{13}C$ values of prednisone and prednisolone pharmaceutical preparations available in Italy

4.4.2 Method development

A preliminary adequate liquid chromatography step was performed to guarantee reliable delta values: the initial operating conditions adopted to purify the extracted urine samples were the same already validated for the pseudo-endogenous anabolic androgenic steroids analysis[26]. The single HPLC step set up through an ACE® C18 column was sufficient to allow the adequate purification of THS, PD and PT, but not suitable for prednisone and prednisolone. Among the several tests performed to configure the convenient phase columns and chromatographic conditions combination, a second purification in an ACE® EXCEL 5 C18 AMIDE column was selected. The purified fractions were collected, dried and dissolved in a fixed volume of 16 µL of a cyclohexane:isopropanol solution containing 5α-androstan-3β-ol as internal standard and injected in the isotope ratio mass spectrometer. The GC-C-IRMS analysis of prednisolone, THS, PD and PT was executed through the GC method already validated for the pseudo-endogenous steroids and able to provide adequate peak shape and compounds separation. A specific chromatographic ramp was implemented for prednisone to avoid losing analytical selectivity. The injection volume was established depending on the urine sample volume and on the presence of a split/splitless or a PTV instrumental injection mode. The chromatograms of prednisone and prednisolone from pharmaceutical preparations and those of their standards subjected to the entire preparation procedure showed that the defined HPLC collection windows and the GC-C-IRMS conditions were adequate.

As already known, the corticosteroids undergo an intramolecular elimination of the side chain during the vaporization process in a gas chromatographic analysis[29,30,31]. To completely convert prednisone and prednisolone to Δ1-adrenosterone, and cortisone and cortisol to adrenosterone before the oxidation at the injection port, other recently proposed methods for the prednisone and prednisolone detection in GC-C-IRMS have added an oxidation step in the pre-instrumental procedure. The production of the corresponding 17-ketosteroids compounds has been described as profitable action to reduce the potential isotopic fractionation caused by the spontaneous chemical modification occurring during the vaporization process, independently from the injection mode selected[32]. Instead, in our operative conditions, no structural modification was intentionally conducted on the examined analytes during the preparation protocol. The two possible 17-ketosteroids oxidation products (Δ1-adrenosterone and adrenosterone) were

injected as standards in the same GC conditions set up for prednisolone, prednisone, cortisol and cortisone. The spectra of the compounds resulting from the injection of prednisolone and prednisone were those of 11beta-hydroxyandrosta-1,4-diene-3,17-dione and andros-1,4-diene-3,11,17-trione (Δ1-adrenosterone). The same C-17 side chain elimination was observed for cortisone and cortisol, thermally degraded to adrenosterone and 11beta-hydroxyandrostenedione (see Figure 4.2a-c). All the spectra below are based on pure reference materials.

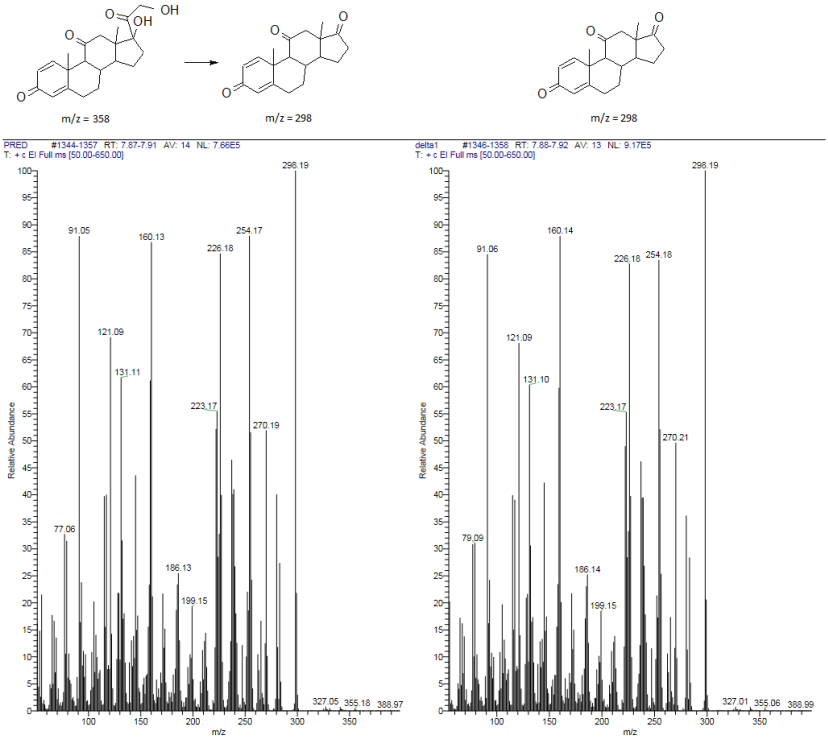


Figure 4.2a Mass spectra comparison: the prednisone native form and its oxidation product (on left) and Δ1-adrenosterone (on right)

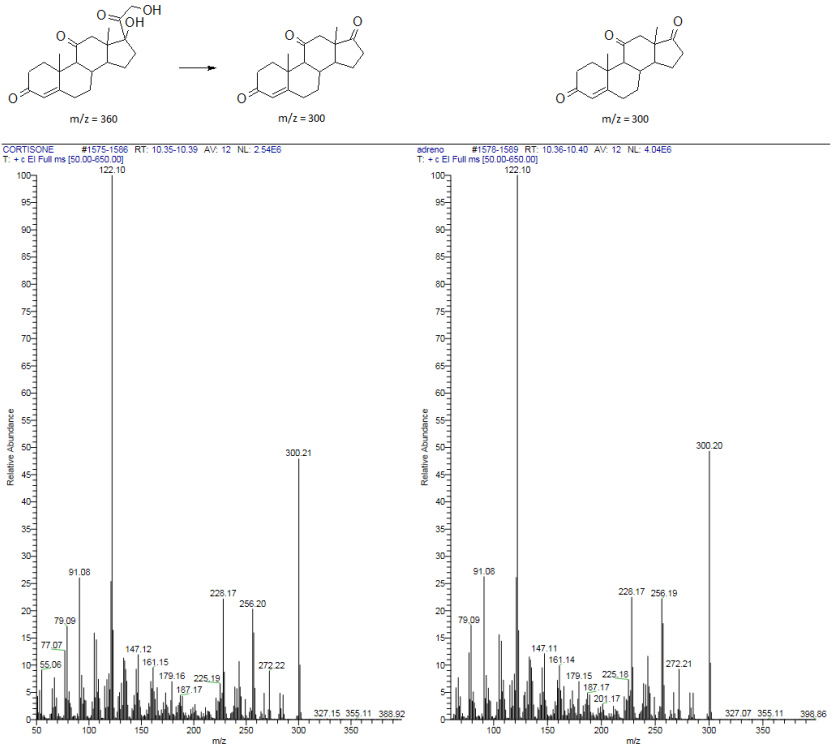


Figure 4.2b Mass spectra comparison: the cortisone native form and its oxidation product (on left) and adrenosterone (on right)

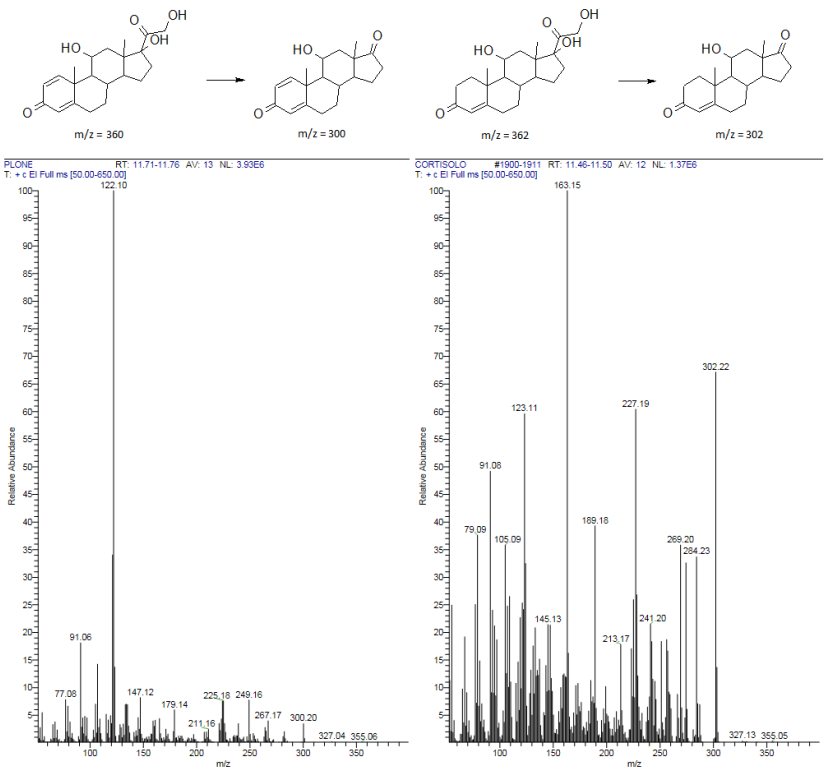


Figure 4.2c Mass spectra of the prednisolone (on left) and cortisol (on right) oxidation products

No evidences have been found to support not only other side chain cleavage (hydroxyl to keto group at C-11) on the steroidal structure, but also any possible isotopic fractionation during the spontaneous oxidation process. Indeed, the prednisone and prednisolone delta values obtained during the validation procedure and the excretion studies were robust, repeatable and in agreement with those of pharmaceutical preparation administered: whatever fractionation occurred, this was irrelevant for the final and correct interpretation of the data. Δ1-adrenosterone and adrenosterone standards were also injected in the liquid chromatograph under the instrumental conditions described above: both compounds were eluted during the isocratic part of the program, five to ten minutes away from prednisone, prednisolone, cortisol and cortisone (Figure 4.3).

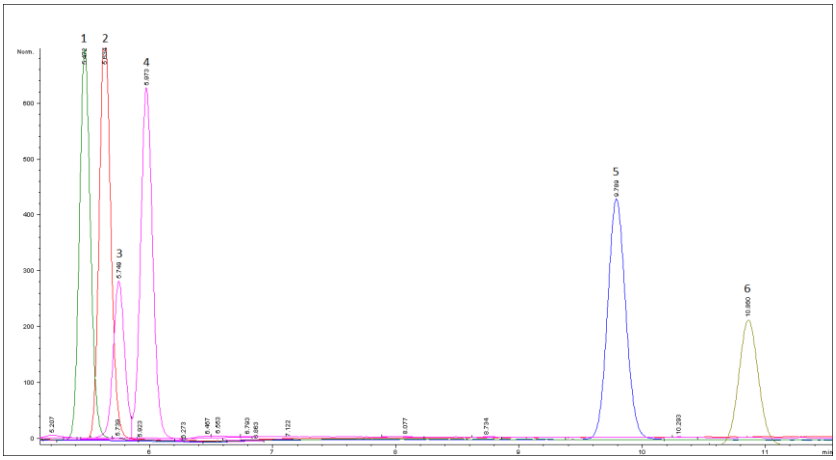


Figure 4.3 HPLC chromatograms of a mixture of standards analysed during the first purification step. (1) prednisolone; (2) prednisone; (3) cortisol; (4) cortisone; (5) Δ1-adrenosterone; (6) adrenosterone

Therefore, neither Δ1-adrenosterone nor adrenosterone may be collected in the prednisone and prednisolone LC fractions: they necessarily originated from the spontaneous elimination reaction.

4.4.3 Method validation

The method was validated following the requirements established by WADA in the TD2019IRMS [21].

- *Linearity*

The GC-C-IRMS linearity response of prednisone and prednisolone was evaluated by injecting (2 µL splitless mode) three replicates of six serially diluted standard solutions. Prednisone showed a linear response (r^2 = 0.999) within 300 to 3200 mV, corresponding to 10-240 ng injected on column. The $\delta^{13}C$ (‰) average value was of -28.94 ± 0.33 ‰. Prednisolone exhibited linear response (r^2 = 0.997) between 250 and 2860 mV, by injecting from 17 to 250 ng on column. The mean $\delta^{13}C$ (‰) value observed was of -28.74 ± 0.27 ‰ and the SD < 0.5 ‰ for both compounds. Being the observed SD < 0.5‰ for both compounds this defines the instrumental linear range (Figure 4.4). The linearity study was repeated by injecting 8 µL of prednisone (-28.97 ± 0.20 ‰) and 3 µL of prednisolone (-28.85 ± 0.27 ‰) standard solutions in a PTV injection provided system, confirming previous findings.

- *Selectivity, LOQ and recovery*

Ten different blank urine samples and the same urine sample spiked with prednisone and prednisolone standards (30 ng/mL) were processed to prove the absence of interferences at the expected retention times of the two target steroids in the GC-C-IRMS analysis. HPLC extracts from 25 or 10 mL of initial urine volume were respectively injected in splitless (2 µL) and in a PTV (3-8 µL) mode. The chromatography was illustrated in Figure 4.5a-b. In both methods the assessment of interfering peaks was verified through a GC-MS analysis. The background was not affected either by the initial matrix volume or by the type/injection volume.

The lowest prednisone and prednisolone detectable concentrations with acceptable amplitude signals and $\delta^{13}C$ (‰) values in the linear range, were estimated through the analysis of three sets of spiked urine at decreasing levels (30, 20 and 10 ng/mL). The LOQ was established for both compounds at 20 ng/mL (< 30 ng/mL, the reporting level) since at 10 ng/mL either the SD was too high, and/or the mean value deviated too much from the reference value. The overall $\delta^{13}C$ (‰) values and the relative recovery percentages are summarized in Table 4.2a-b. An additional test was performed to confirm the delta values reliability and repeatability at the higher level of the concentration range for which WADA has established the IRMS as a recommended confirmation analysis. Two set of three different male volunteer urine samples were spiked with prednisone and prednisolone standards to obtain a final concentration of 60 ng/mL. The samples were analysed in split/splitless (2 µL injected for both TCs) or PTV mode (8 µL injected for prednisone and 3 µL for prednisolone) by processing respectively 10 or 25 mL of urine. The related delta values were presented in Table 4.3a-b. As it can be seen, both at the LOQ and the upper limit, the SD was < 1‰.

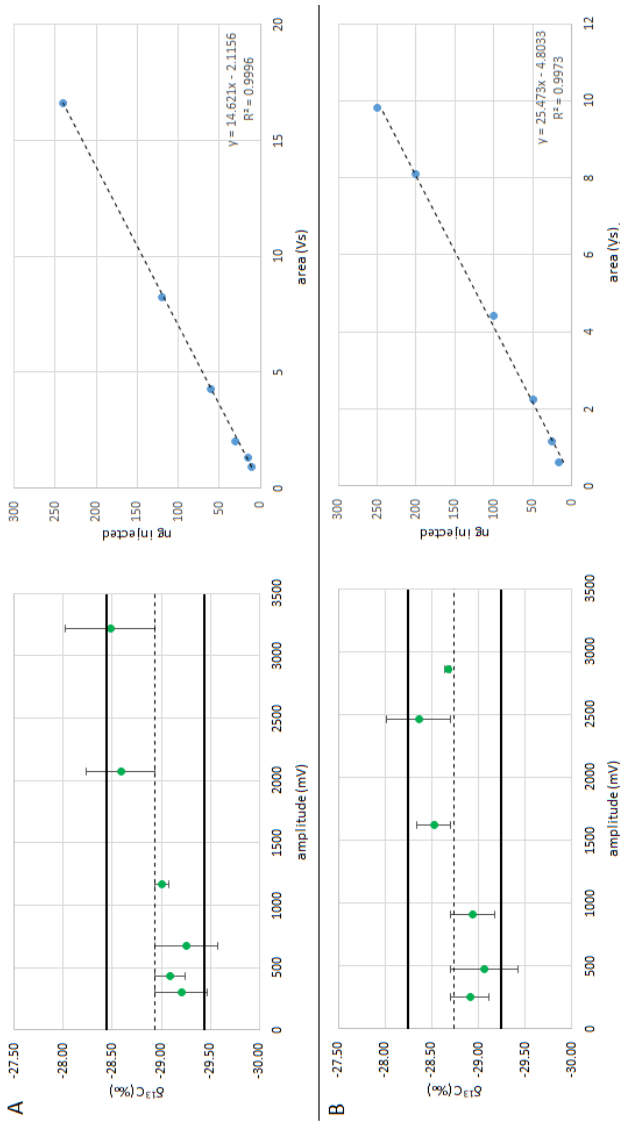


Figure 4.4 Linearity

(A) The graphs represent the mean measurements of 3 injections of prednisone, the lower and upper acceptance limits (mean ± 0.5 ‰) and the line of best fit

(B) The graphs represent the mean measurements of 3 injections of prednisolone, the lower and upper acceptance limits (mean ± 0.5 ‰) and the line of best fit

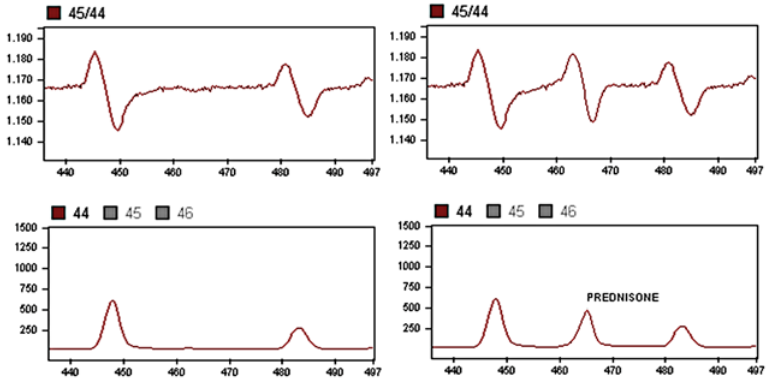


Figure 4.5a Selectivity

Prednisone: the GC-C-IRMS analysis comparison between the blank and a positive sample

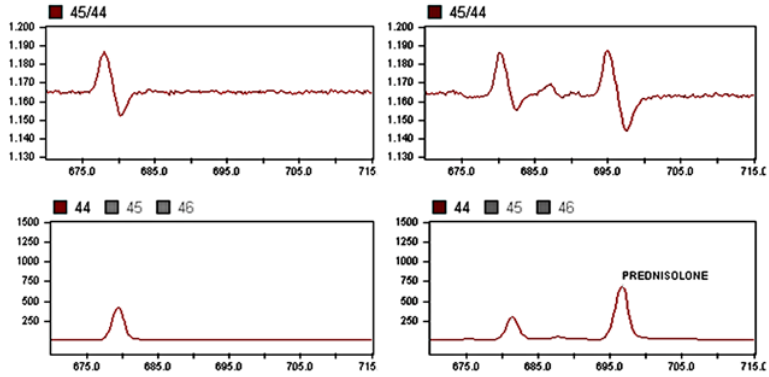


Figure 4.5b Selectivity

Prednisolone: the GC-C-IRMS analysis comparison between the blank and a positive sample

	$\delta^{13}C$ (‰)	Mean (‰) (n=3)	SD (‰) (n=3)	Recovery (%)
USP 30 (ng/mL)	-28.83	-28.89	0.14	34
	-29.05			
	-28.78			
USP 20 (ng/mL)	-28.68	-28.69	0.18	28
	-28.88			
	-28.52			
USP 10 (ng/mL)	-30.56	-30.19	0.52	25
	-29.60			
	-30.42			

Table 4.2a Prednisone: LOQ and recovery. Mean, SD and recovery of three replicates for each considered spiked positive control urine (USP) at 30, 20 and 10 ng/mL

	$\delta^{13}C$ (‰)	Mean (‰) (n=3)	SD (‰) (n=3)	Recovery (%)
USP 30 (ng/mL)	-28.44	-28.54	0.24	46
	-28.37			
	-28.81			
USP 20 (ng/mL)	-28.89	-28.64	0.23	50
	-28.56			
	-28.46			
USP 10 (ng/mL)	-29.45	-29.28	0.41	55
	-28.81			
	-29.58			

Table 4.2b Prednisolone: LOQ and recovery. Mean, SD and recovery of three replicates for each considered spiked positive control urine (USP) at 30, 20 and 10 ng/mL

	$\delta^{13}C$ (‰)	Mean (‰) (n=3)	SD (‰) (n=3)
A	-29.44	-29.53	0.09
	-29.61		
	-29.54		
B	-28.92	-29.52	0.50
	-29.72		
	-29.86		

Table 4.3a Prednisone injected in a splitless (A) or solvent venting mode (B)

	$\delta^{13}C$ (‰)	Mean (‰) (n=3)	SD (‰) (n=3)
A	-28.78	-29.07	0.25
	-29.19		
	-29.23		
B	-28.94	-28.88	0.15
	-28.71		
	-28.99		

Table 4.3b Prednisolone injected in a splitless (A) or solvent venting mode (B)

- *Repeatability and uncertainty*

The repeatability of the method was verified through the analysis in different days of 10 mL of urine collected from four males (36-50 years) and four females (25-50 years). The urine samples were spiked with prednisone and prednisolone standard solutions used as reference materials (RM) to obtain samples at 30 ng/mL. The $\delta^{13}C$ (‰) values of samples deviated less than 0.50 ‰ from the RM delta values (-28.97 ± 0.20 ‰ for prednisone; -28.85 ± 0.27 ‰ for prednisolone) also demonstrating that no fractionation occurred during sample processing, independently of the urinary matrix (Table 4.4).

	prednisone			prednisolone		
	$\delta^{13}C$ (‰)	Ampl. (mV)	Δδ (‰)	$\delta^{13}C$ (‰)	Ampl. (mV)	Δδ (‰)
M1	-28.99	347	0.02	-28.67	420	-0.18
M2	-28.87	447	-0.11	-28.78	664	-0.07
M3	-28.96	513	-0.01	-28.60	409	-0.25
M4	-28.43	465	-0.54	-29.26	421	0.41
F1	-28.81	735	-0.16	-28.67	661	-0.18
F2	-28.85	606	-0.12	-28.78	667	-0.07
F3	-28.97	773	0.00	-28.70	386	-0.15
F4	-28.70	452	-0.27	-28.91	305	0.06
MEAN	-28.82	542		-28.80	492	
SD	0.18			0.21		

Table 4.4 Repeatability. Analysis of samples from four male (M) and four female (F)

For the estimation of the uncertainty, ten replicates of the same urine spiked at 30 ng/mL were analysed through the whole procedure. The intermediate precision was estimated by the SD calculated during the analyses of the replicates obtained in different batches, prepared by different analysts and analysed in different days. The bias was estimated as RMSbias considering the difference of each determination from the reference value assigned to the RM during the linearity experiments. (Table 4.5). The combined uncertainty of the method was estimated at 0.3 ‰ for both compounds.

	prednisone			prednisolone		
	$\delta^{13}C$ (‰)	Ampl. (mV)	Δδ (‰)	$\delta^{13}C$ (‰)	Ampl. (mV)	Δδ (‰)
USP1	-29.07	449	-0.10	-28.69	903	0.16
USP2	-28.64	466	0.33	-28.85	996	0.00
USP3	-28.77	423	0.20	-28.87	542	-0.02
USP4	-28.71	378	0.27	-28.57	1353	0.28
USP5	-29.08	602	-0.11	-29.11	1516	-0.26
USP6	-28.98	383	-0.01	-28.77	1402	0.08
USP7	-29.19	629	-0.22	-28.76	1526	0.10
USP8	-28.97	425	0.00	-28.67	889	0.19
USP9	-28.93	344	0.04	-29.14	1452	-0.29
USP10	-28.89	483	0.08	-28.81	1878	0.04
MEAN	-28.92	458	0.05	-28.82	1246	0.03
SD	0.17			0.18		

Table 4.5 Repeatability. Analysis of 10 replicated of the same urine spiked at 30 ng/mL

The method once validated was then applied to real urine samples collected in two excretion studies on two male healthy volunteers.

4.4.4 Excretion studies

The fit for purpose of the method was assessed by analysing urine samples collected from two healthy male volunteers administered with prednisone (Deltacortene® 5 mg, one tablet) and prednisolone (Sintredius® 1 mg/mL, one vial). Prednisone and prednisolone concentrations were extrapolated from the instrumental linearity curve: urinary levels decreased below 30 ng/mL within the 24 hours after oral administration. The GC-C-IRMS analysis was performed on samples in which prednisone and prednisolone were at concentrations just below, between and above the range of 30 – 60 ng/mL. Delta values of the two target steroids and the three selected ERCs were determined. The $\delta^{13}C$ (‰) values obtained and presented in Table 4.6a-b were in agreement with those previously determined of the two pharmaceutical preparations administered.

		PREDNISONE		PREDNISOLONE		PD	PT	THS
	TIME (h)	Conc. (ng/mL)	$\delta^{13}C$ (‰)	Conc. (ng/mL)	$\delta^{13}C$ (‰)	$\delta^{13}C$ (‰)	$\delta^{13}C$ (‰)	$\delta^{13}C$ (‰)
	0	-	-	-	-	-22.45	-22.44	-22.20
	3	62	-29.15	348	-29.00	-22.31	-22.43	-22.61
	20	29	-29.26	28	-29.16	-21.93	-22.21	-22.31
MEAN			-29.21		-29.08	-22.23	-22.36	-22.37
SD			0.08		0.11	0.27	0.13	0.21

Table 4.6a Volunteer 1 before and after a Sintredius® 1 mg/mL one vial administration

		PREDNISONE		PREDNISOLONE		PD	PT	THS
	TIME (h)	Conc. (ng/mL)	$\delta^{13}C$ (‰)	Conc. (ng/mL)	$\delta^{13}C$ (‰)	$\delta^{13}C$ (‰)	$\delta^{13}C$ (‰)	$\delta^{13}C$ (‰)
	0	-	-	-	-	-22.77	-23.45	-22.84
	3	90	-28.99	534	-28.81	-22.33	-23.18	-22.47
	8	52	-29.84	153	-28.73	-22.43	-23.58	-
	12	182	-29.12	89	-29.06	-22.50	-22.91	-22.79
MEAN			-28.98		-28.87	-22.51	-23.28	-22.70
SD			0.14		0.17	0.19	0.29	0.20

Table 4.6b Volunteer 2 before and after a Deltacortene® 5 mg one tablet administration

The $\Delta\delta^{13}C$ (‰) values were calculated for each ERC-TC pair and summarised in Table 4.7a-b. By the $\Delta\delta^{13}C$ (‰) values determined and the other IRMS interpretation criteria applied in analogues cases in which no population data are available, a $\Delta\delta^{13}C$ ‰ greater than 4 ‰ has been proposed as adequate limit to confirm a positive result.

	prednisone			prednisolone		
TIME (h)	**PD**	**PT**	**THS**	**PD**	**PT**	**THS**
0						
3	6.85	6.72	6.54	6.70	6.57	6.39
20	7.34	7.06	6.96	7.24	6.95	6.85

Table 4.7a Volunteer 1: $\Delta\delta^{13}C$ values (‰) for each ERC-TC pair

	prednisone			prednisolone		
TIME (h)	PD	PT	THS	PD	PT	THS
0						
3	6.66	5.81	6.52	6.48	5.63	6.35
8	6.41	5.27	-	6.30	5.16	-
12	6.62	6.20	6.33	6.56	6.15	6.28

Table 4.7b Volunteer 2: $\Delta\delta^{13}C$ values (‰) for each ERC-TC pair

The study was extended to include the THE, allo-THE, THF and allo-THF delta values determination. These are endogenous glucocorticoids metabolites that, as demonstrated in a previous study[23], can be used as target compounds to detect the cortisone misuse (prohibited by WADA but also from endogenous origin). As shown in Table 4.8a-b, their delta values were not affected by the prednisone and prednisolone pharmaceutical preparations intake. Their extraction, purification and IRMS analysis were performed according to the method mentioned before. The SD in each set of measurements was below 0.50 ‰, except for allo-THE. The allo-THE signals were not considered acceptable: the amplitude was outside the instrumental linearity range (< 0.20 V) and the delta values SD were higher than 0.5 ‰.

TIME (h)	THE	allo-THE	THF	allo-THF
0	-21.88	-21.73	-23.37	-23.21
3	-21.91	-22.51	-23.47	-23.54
20	-21.81	-23.33	-23.14	-22.76
MEAN	-21.87	-22.52	-23.33	-23.17
SD	0.06	0.80	0.17	0.39

Table 4.8a Volunteer 1: endogenous glucocorticoids metabolites $\delta^{13}C$ ‰ before and after a Sintredius® 1 mg/mL one vial administration

TIME (h)	THE	allo-THE	THF	allo-THF
0	-22.50	-22.58	-23.35	-23.23
3	-21.92	-23.10	-23.36	-23.14
8	-22.80	-22.12	-22.69	-22.89
12	-22.28	-21.38	-22.43	-22.61
MEAN	-22.38	-22.29	-22.96	-22.97
SD	0.37	0.73	0.47	0.28

Table 4.8b Volunteer 2: endogenous glucocorticoids metabolites $\delta^{13}C$ ‰ before and after a Deltacortene® 5 mg, one tablet administration

The THE, THF and allo-THF $\delta^{13}C$ (‰) study could allow to identify the type of corticosteroids abused (prednisone/prednisolone or cortisone/cortisol). In case of prednisone/prednisolone administration, none THE, THF and allo-THF $\delta^{13}C$ (‰) deviation from the characteristic endogenous range would be obtained. Instead, typically exogenous values not only of prednisone and prednisolone, but also of THE, THF and allo-THF, were presumably explained with the cortisone/cortisol intake also resulting in their degradation into exogenous prednisone/prednisolone.

4.5 Conclusions

The pharmaceutical preparations sold so far in Italy allowed developing a method able to distinguish the origin of prednisolone detected in urine. For the future, it will be necessary to extend the study to a broader variety of preparations available worldwide. A method for the detection of synthetic prednisone and prednisolone by GC-C-IRMS has been developed and validated in compliance with the ISO17025, the WADA International Standard for Laboratories (ISL) and the Technical Documents requirements. The analysis was performed using 25 or less mL of urine, purifying the extracts in two sequential HPLC steps and selecting a different GC method for each of the two compounds in order to achieve adequate selectivity and sensitivity. No derivatization nor additional oxidative step before the instrumental analysis was necessary.

The set up operating conditions allowed to detect prednisone and prednisolone, producing reproducible and reliable delta values for acceptable amplitude signals

(> 250-300 mV), compatible with urinary concentrations (20 ng/mL) below the WADA reporting level (30 ng/mL). The method can be applied to discriminate the exogenous origin from the *in situ* bacterial production of prednisone and prednisolone, as required by WADA, in case of administration of drugs with $\delta^{13}C$ (‰) values that could be distinguishable from the values of the steroids produced endogenously.

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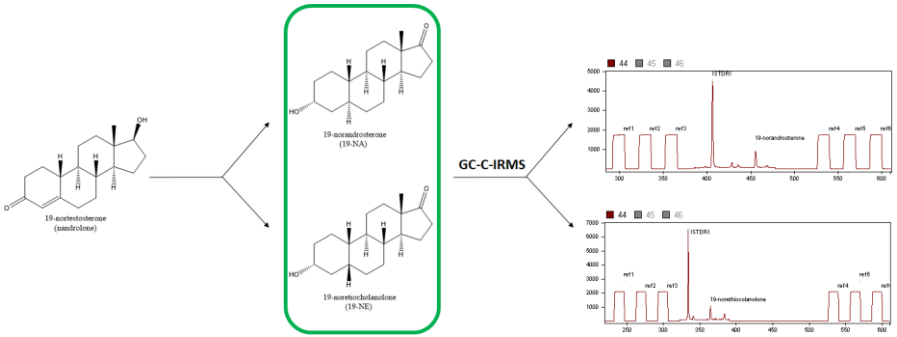
Chapter 5

Detecting the abuse of 19-norsteroids in doping controls: a new gas chromatography coupled to isotope ratio mass spectrometry method for the analysis of 19-norandrosterone and 19-noretiocholanolone



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5.1 Abstract

The detection of 19-norsteroids abuse in doping controls currently relies on the determination of 19-norandrosterone (19-NA) by gas chromatography tandem mass spectrometry (GC-MS/MS). An additional confirmatory analysis by gas chromatography coupled to isotope ratio mass spectrometry (GC-C-IRMS) is performed on samples showing 19-NA concentrations between 2.5 and 15 ng/mL and not originated from pregnant female athletes or female treated with 19-norethisterone. 19-Noretiocholanolone (19-NE) is typically produced to a lesser extent as a secondary metabolite. The aim of this work was to improve the GC-C-IRMS confirmation procedure for the detection of 19-norsteroids misuse. Both 19-NA and 19-NE were analyzed as target compounds (TC), while androsterone (A), pregnanediol (PD) and pregnanetriol (PT) were selected as endogenous reference compounds (ERC). The method was validated and applied to urine samples collected by three male volunteers after the administration of nandrolone based formulations. Before the instrumental analysis, urine samples (< 25 mL) were hydrolyzed with β-glucuronidase from *E. coli* and extracted with n-pentane. Compounds of interest were purified through a single (for PT) or double (for 19-NE, 19-NA, A, and PD) liquid chromatographic step/s, to reduce the background noise and eliminate interferences that could have affect the accuracy of $\delta^{13}C$ values. The limit of quantification (LOQ) of 2 ng/mL was ensured for both 19-NA and 19-NE. The 19-NE determination could be helpful in case of "unstable" urine samples, in late excretion phases or when co-administration with 5α-reductase inhibitors occur.

5.2 Introduction

Nandrolone and its precursors are anabolic androgenic 19-norsteroids synthetized since the 1950s to enhance the myotropic action and reduce the androgenic side effects of testosterone, from which they structurally derive[1–3]. Nandrolone, 19-nortestosterone, is a model 19-norsteroid widely studied in clinical situations to treat various catabolic disorders[4–13]. It is usually administered by intramuscular injection as decanoate or phenyl propionate ester, even if several oral nandrolone prohormones (19-norandrostenedione and 19-norandrostenediol) formulations have been easily accessible for years as dietary supplements and are still freely available on Internet market[14–19]. 19-Norsteroids undergo a phase I metabolism similar to that of testosterone, through the oxidation of hydroxyl group on C17 (catalyzed by 17β-hydroxysteroid-dehydrogenase), reduction of double bound C4-C5 (by 5α and 5β-reductase) and reduction of 3 keto-function (by 3α and 3β -hydroxysteroid-dehydrogenase) enzymatic steps[20,21]. Unlike dihydrotestosterone, DHT, the 5α-dihydro 19-norderivatives possess a weak binding affinity to the androgen receptors in prostate and hair follicles, reducing the incidence of prostatic growth and alopecia and justifying the beneficial effect of nandrolone in male hypogonadism therapy[22–25]. 19-Norandrosterone (the 5α-reduced isomer) and 19-noretiocholanolone (5β-isomer) are the main 19-norsteroids metabolites, mostly excreted as glucurono- and sulfo-conjugates[26,27]. The 3β-hydroxy isomers (norepiandrosterone and norepietiocholanolone) have been also identified as minor enzymatic products in the sulphate fraction[28].

19-Norsteroids have been extensively abused by athletes to increase the muscle mass and improve the overall sports performance, especially in sports where the muscle strength is relevant. They are included as anabolic androgenic steroids (AAS) in the section S1.1 of the Prohibited List written and regularly updated by the World Anti-doping Agency (WADA)[29]. In doping analysis, the detection of 19-norsteroids is currently performed through a GC-MS/MS semi-quantitative determination of their major metabolite 19-norandrosterone (19-NA), typically produced about three times more than 19-noretiocholanolone (19-NE)[30,31], even if different excretion ratios have been also determined.

19-NA and 19-NE could be found at low concentrations in urine even if no exogenous and intentional administration of nandrolone or its precursors

occurred[32–34]. Nandrolone is naturally produced and accumulated in edible tissues of some animal species such as wild or non-castrated boars, horses and sheep and it is one of the most frequently growth promoter in livestock. The consumption of meat from such animals could lead to an excretion of nandrolone metabolites[35–37]. Endogenous 19-NA traces have been identified in female during pregnancy or after norethisterone based contraceptive therapy as intermediate product in the aromatization process of androgens to oestrogens[38,39] or in the de-ethynylation reaction of norethisterone[40–42] respectively. Findings of low amount of 19-NA and 19-NE in stored urine samples also confirmed the hypothesis of an *in situ* 19-demethylation of the physiologically excreted androsterone and etiocholanolone[43]. Similar evidences have been also reported about the *in situ* bacterial production of boldenone[37,44,45] and prednisolone/prednisone[46–48] from endogenous steroids; in the case of 19-NA a methyl group is removed from the steroid skeleton.

WADA has established threshold values to exclude that the presence of 19-NA in urine (after adjustment for the urine specific gravity, if > 1.018[49]) is due to one of the above-mentioned conditions: 15 ng/mL for pregnant female athletes, 10 ng/mL for female athletes using norethisterone and 2.5 ng/mL for all other cases. An additional confirmatory analysis by GC-C-IRMS is executed on samples showing 19-NA concentration between 2.5 and 15 ng/mL and not collecting from pregnant athletes or in therapy with norethisterone[50]. The IRMS analysis allows the discrimination between the exogenous or endogenous origin of steroid compounds, by exploiting their different ^{13}C content (expressed as $\delta^{13}C$ ‰). Adequate strategies has been adopted in some WADA Accredited Antidoping Laboratories to determine the origin of 19-norsteroids metabolites[31,51,52]. A fast and simplified GC-C-IRMS procedure on underivatized 19-NA has been effectively set up and routinely applied in our Laboratory[53]. In case of IRMS results inconclusive or consistent with endogenous values, the 19-NA/19-NE ratio is determined to confirm or not the atypical results to the anti-doping test[50].

The aim of this study was to improve the current GC-C-IRMS confirmation procedure of 19-norsteroids and align it to the approach already established for the detection of boldenone and prednisolone/prednisone, in which two target compounds are examined. For this purpose, both main metabolites of nandrolone (19-NA and 19-NE) were selected as TC. The complementary evaluation of 19-NE could be critical to prevent the risk of inconclusive or false negative outcomes in

case of analytical issues, like interferences signals or high background noises, on the 19-NA determination. In addition, even if it is typically a minor metabolite, 19-NE may no longer be negligible when certain conditions occur. Indeed, the co-administration of 19-norsteroids with drugs, as 5α-reductase inhibitors, affecting the steroids metabolic pathway, results in a consistent drop of 5α-reduced metabolites urinary excretion and a corresponding increase of the formation of 5β-products, as 19-NE[54–56]. Moreover, the preferential production of 19-NE in the so-called "unstable" urine samples has been already demonstrated[43].

In this work, a specific GC-C-IRMS method for the detection of 19-NE was developed accordingly and applied to the analysis of positive urine samples collected from three male subjects administered with 19-norandrostenedione. The trend of urinary levels and $\delta^{13}C$ values of 19-NA and 19-NE was examined. $\delta^{13}C$ values of A, PD and PT, selected as ERC, were also determined in every sample.

5.3 Materials and methods

5.3.1 Materials and chemicals

Certified reference standards of 19-noretiocholanolone (3α-hydroxy-5β-estrane-17-one, **19-NE**), 19-norandrosterone (3α-hydroxy-5α-estrane-17-one, **19-NA**) and androsterone (3α-hydroxy-5α-androstane-17-one, **A**) were from NMIA (Lindfield, Australia). Pregnanetriol (5β-pregnane-3α,17α,20α-triol, **PT**) and 17α-methyltestosterone (**MT**) were supplied by Sigma-Aldrich (Milan, Italy). Pregnanediol (5β-pregnane-3α, 20α-diol, **PD**), 5β-estran-17α-ethynyl-3α,17β-diol (**NET**) and 5α-androstan-3β-ol (**ISTDRI**) were from Steraloids (Newport, RI, USA).

All solvents (n-pentane, acetonitrile, methanol, cyclohexane and isopropanol) and reagents (sodium bicarbonate, potassium carbonate, sodium phosphate, sodium hydrogen phosphate) were of analytical or HPLC grade and purchased from Carlo Erba (Milano, Italy). β-Glucuronidase from *Escherichia coli* K12 was provided by Roche Diagnostic (Mannheim, Germany). Water was from a Milli Q water purification system (Millipore S.p.A., Milan, Italy).

The calibration of the CO_2 reference gas (Solgas, Monza, Italy) for the isotope ratio mass spectrometer was performed against underivatized steroids (CU/PCC 34-3) with certified delta values traceable to VPDB, obtained from Prof. Brenna (Cornell University Certified Reference Material)[57].

5.3.2 Urine samples

Urine samples fortified with 19-NA and 19-NE reference standards (USP, spiked positive control urine) were tested during the development of the procedure and for its validation. Positive urine samples from excretion studies after the oral administration of the nandrolone precursor 19-norandrostenedione, were analyzed to confirm the reliability of the method: 3 male volunteers (29, 38 and 57 years old) collected their urine at regular intervals for at least two days, after the intake of 5 or 10 mg of 19-norandrostenedione (Genetic Evolutionary Nutrition, Los Angeles), whose $\delta^{13}C$ value was previously determined (-29.70 ± 0.30 ‰)[53]. Urine were stored at -20 °C until analysis. Each volunteer, opportunely informed about the aim of the project, signed a written consent allowing the use of urine samples for research purpose.

The study was conducted fulfilling the recommendations for research involving human subjects described in Declaration of Helsinki[58].

5.3.3 Sample pre-treatment

The enzymatic hydrolysis (55 °C, 60 min) was carried out on each urine aliquot by the addition of 1.5 mL of phosphate buffer (0.8 M, pH 7.4) and 100 µL of β-glucuronidase from *E. coli*. After incubation, the pH was adjusted to 9 - 10 with 0.5 mL of carbonate buffer 20 % and sample extracted with 10 mL of n-pentane on a mechanical shaker for at least 20 min. Samples were then centrifuged for 2 minutes at 3000 rpm, and organic solvent separated. The organic layers from different aliquots of the same sample were combined and taken to dryness under nitrogen stream (75 °C). Fifty µL of a MT solution (internal standard) at 100 µg/mL in water:methanol (50:50) was used to reconstitute the dried residues for the next HPLC purification step.

A GC-MS/MS analysis was previously performed to estimate the concentrations of TC and ERC and select the adequate volume of urine (7 – 25 mL, divided into more than one aliquot of max 7 mL) to be processed[59–61]. Sample pre-treatment for the GC-MS/MS procedure accredited according to ISO 17025 and currently in use in our Laboratory involves a solid phase extraction (SPE) before the enzymatic hydrolysis, even it is not yet traceable in the mentioned literature.

5.3.4 First HPLC purification step

The purification of urine samples was necessary to eliminate any interferences which may impact on the accuracy and reliability of the $\delta^{13}C$ values of the compounds of interest. Two sequential HPLC purification steps were developed in this work to guarantee adequate purity of all analytes (19-NE, 19-NA, A, PD and PT). Fifty µL of the pre-treated samples were injected on an Agilent 1200 Series liquid chromatograph (Agilent Technologies S.p.A., Cernusco sul Naviglio, Milan, Italy) provided of an Ascentis® phenyl column (15 cm, 4.6 mm, 5 µm) and an Ascentis® phenyl Supelguard™ guard cartridge (2 cm, 4.0 mm, 5 µm), both from Sigma-Adrich (Milan, Italy). The column temperature was set at 60 °C and the UV detector at 192 nm (TC and ERC absorbance wavelength) and 254 nm (MT absorbance wavelength). Water (solvent A) and acetonitrile (solvent B) were used as mobile phase at constant flow rate of 1 mL/min. An isocratic program was executed at 50 % of B until 8.50 min; an increment to 100 % of B was reached in 0.01 min and held until the end of the ramp, for a total run of 15 min. The compounds were collected in the following order: PT, 19-NE, 19-NA, A and PD (in the same window). Their corresponding collection time windows were indicated in Table 5.1.

Analytes	1st HPLC* ISTD MT (RT 5.60 ± 0.20)	2nd HPLC# ISTD NET (RT 6.80 ± 0.20)
PT	4.80 ÷ 5.29	(not required)
19-NE	5.69 ÷ 6.14	4.80-5.45
19-NA	6.17 ÷ 6.73	5.20-5.70
A	6.95 ÷8.15	5.35-5.95
PD		11.75-12.70

Table 5.1 Collection intervals for the two HPLC purification steps
*: Ascentis® phenyl (15 cm, 4.6 mm, 5 µm)
#: ACE® Excel 5 C18 Amide (25 cm, 4.6 mm, 5 µm)

The collected fractions were taken to dryness under nitrogen stream (75-90 °C). Only PT was reconstituted in a volume of the GC-C-IRMS internal standard solution (ISTDRI solution at 10 µg/mL, in cyclohexane:isopropanol 4:1) depending on its previously estimated urinary concentration[59–61]. 19-NE, 19-NA, A and PD were dissolved in 50 µL of the internal standard selected for the second HPLC clean-up (solution of NET at 200 µg/mL in a mixture of methanol:acetonitrile 1:300. With the

2nd LC column used, greater methanol percentage has proved to be responsible for a high solvent front and interfering signals next to the peaks of interest).

5.3.5 Second HPLC purification step

The purity of the collected fractions was evaluated by performing a GC-MS full scan analysis in the same chromatographic conditions previously described[53,62]. An additional LC procedure was set up in order to eliminate the presence of co-eluting endogenous interferences on 19-NE, reduce the background noise on 19-NA and ISTDRI signals and conveniently separate A from PD. It was carried out on an Agilent 1200 Series liquid chromatograph (Agilent Technologies S.p.A., Cernusco sul Naviglio, Milan, Italy) equipped with an ACE® EXCEL 5 C18 AMIDE column (25 cm, 4.6 mm, 5 µm) from CPS Analitica (Milan, Italy) at 25 °C. Acetonitrile 100 % was selected as the mobile phase at constant flow of 1 mL/min in a fast isocratic program (total runtime of 15 min). The Agilent 1200 UV DAD lamp was set at 192 nm (TC and ERC absorbance wavelength). The collection time windows were reported in detail in Table 5.1. The purified fractions were evaporated to dryness under nitrogen stream (75-90 °C) and dissolved in a volume of the ISTDRI solution suitably selected on their urinary levels estimated by GC-MS/MS[59–61].

The repeatability of the specific internal standards (MT and NET) retention times was monitored in both the LC sessions to assess the stability of the elution conditions: a maximum variation of ± 0.15 min was accepted along the sequence of injections.

During the first stages of the method development, the collected fractions and the fractions just before and after those expected for the selected TC and ERC, were analyzed by GC-MS to check the absence of co-eluting interferences and exclude any possible isotopic fractionation caused by a non-complete collection of the analytes. The injection was performed on a HP6890 GC coupled to HP5973 mass spectrometer (Agilent Technologies SpA, Cernusco sul Naviglio, MI, Italy) provided with the same column and chromatographic parameters set for the subsequent IRMS analysis.

5.3.6 GC-C-IRMS instrumental analysis

Two different GC methods were developed to guarantee an adequate sensitivity and selectivity to all the compounds of interest.

GC-C-IRMS analyses were carried out on a Thermo DELTA V™ Advantage isotope ratio mass spectrometer connected to a combustion reactor (at 940 °C) coupled to a Thermo TRACE™ 1310 GC through a Thermo Isolink-Conflo IV Interface (all from ThermoElectron, Bremen, Germany). The instrument is equipped with a programmed temperature vaporization (PTV) injector (injector initial temperature at 65°C), that allowed to inject variable volumes (2 – 10 µL) of purified samples starting from initial volume lower than 25 mL (7 – 21 mL). Efficacy of the method was also verified on an HP7890 gas chromatograph (Agilent Technologies, Milan, Italy) isotope ratio mass spectrometer (Thermo DELTA V™ Plus from ThermoElectron, Bremen, Germany) system, in which a maximum volume of 2 - 3 µL may be injected in splitless through the split/splitless injector (2 units of this configuration are available in the laboratory).

The specific oven temperature ramp for 19-NE was set as follows: 150 °C (1 min held), 35 °C/min to 290 °C for 5 min, 40 °C/min to 310 °C for 4.5 min, for a total run of 15 min. The GC parameters selected for the chromatographic runs of 19-NA and ERC were the same as those previously defined[53,62]. Briefly: 150 °C for 1 min, 25 °C/min to 260 °C for 3.6 min, 25 °C/min to 270 °C for 0.9 min, 40°C/min to 290 °C for 1.2 min, 40 °C/min to 310 (1.6 min held) for a total run of 14.10 min.

Chromatographic separations were executed on a 5 % Phenyl-methylpolysiloxane (30 m x 0.25 mm i.d. x 0.25 µm film thickness) column from J&W Scientific (CPS Analitica, Milan, Italy). Helium was used as carrier gas at constant flow rate of about 2.0 mL/min to ensure an ISTDRI retention time between 330 and 350 s (19-NE method) or 399 and 414 s (19-NA, A, PD and PT method). The identity of compounds was determined for comparison with the retention times of steroid standard solutions injected at the beginning and the end of each sequence and confirmed by GC-MS analysis. After the GC separation, the flow is split by a 4-Port Silflow MCD (0.25) between the Thermo ISQ single quadrupole mass spectrometer (ThermoElectron, Bremen, Germany) and the IRMS interface (main part).

Validation parameters

The new developed method for the determination of 19-NE carbon isotopic composition was fully validated in compliance with the criteria required by WADA

and ISO17025. Linearity, selectivity, limit of quantification (LOQ), repeatability, uncertainty and recovery were evaluated. The reliability of delta values was verified on various disposable GC-C-IRMS equipment. Real samples after 19-norandrostenedione intake have been analyzed to show that the method was fit for purpose.

Linearity of the GC-C-IRMS response

The instrumental linearity was estimated by injecting in triplicate serially diluted solutions of 19-NE reference standard. We identified the range within which the difference between the mean $\delta^{13}C$ value of each triplicate and the overall mean $\delta^{13}C$ value was not more than 0.50 ‰ and ensured that the signals amplitude (in mV) was linearly correlated to the nanograms injected ($R^2 \geq 0.99$). Only triplicates showing SD < 0.5 ‰ were accepted. The overall $\delta^{13}C$ value mean was assigned to the 19-NE reference standard as its actual $\delta^{13}C$ value.

Selectivity

The selectivity of the method has been proven by the non-occurrence of any interfering peak at the 19-NE retention time in a set (n = 10) of blank urine samples (BUR) in comparison with the same aliquots fortified with 19-NE reference standard at 2.0 ng/mL. Five different aliquots of 19-NE standard solutions at concentrations within the linearity instrumental range were examined in five different days to monitor the repeatability of the retention time. For each working session, the 19-NE and ISTDRI retention time relative ratio, RRT, was calculated: a maximum deviation of 1 % was considered acceptable.

Limit of quantification (LOQ) and linearity of the method

Positive urine samples were prepared at scalar concentrations of 19-NE and analyzed in triplicate to find the lowest level (LOQ) of 19-NE producing reliable delta values within the instrumental linearity range (SD < 0.50 ‰). The linearity of the method was assessed on different urine matrices, at constant volume of 21 mL, fortified with appropriate amount of 19-NE standard solution to obtain positive urine samples at LOQ and 15 ng/mL. We verified that the standard deviation (SD) among $\delta^{13}C$ values of each triplicate was < 0.50 ‰ and that the bias of the mean $\delta^{13}C$ value of each triplicate from the overall mean calculated during the instrumental linearity was < 0.50 ‰.

Variability of delta values

The repeatability of delta values (intermediate precision) was tested in different days on ten positive urine samples prepared at 19-NE concentration within the instrumental linearity range. It was checked that the overall SD was below 0.50 ‰.

Uncertainty and recovery

The measurement uncertainty was estimated by combining the standard deviation related to the repeatability of delta values with the bias from the $\delta^{13}C$ reference value of 19-NE standard. The percentage recovery was calculated through the analysis of three different spiked urine samples (5 ng/mL) undergoing the entire operative procedure (extraction, HPLC purification and GC-C-IRMS analysis) and three 19-NE standard solutions, equivalent to a urine sample at 5 ng/mL, directly injecting in the isotope ratio mass spectrometer system without any pre-treatment steps.

Optimization of the GC-C-IRMS analysis of 19-NA, A and PD

Additional tests were performed on 19-NA to ascertain whether the validation parameters already evaluated in its former protocol[53] were guaranteed also after the addition of the second LC purification step.

19-NA, A and PD fractions from different control urine samples were pre-treated and injected in different days following the new developed procedure and the previously described methods[53,62] to examine the accuracy of their $\delta^{13}C$ values.

PT was analyzed under the conditions previously validated[53].

Reliability of delta values on different GC-C-IRMS instruments

The same positive control (positive urine sample at 2 ng/mL) was analyzed in all the three GC-C-IRMS instruments available in our Laboratory to ensure that the delta values were not affected by different variables (urinary matrices volume, injection volume and instrumental injector).

Excretion studies

As a proof of concept, the method was tested on samples collected from male subjects (n = 3) after the administration of one single oral dose of nandrolone precursor (19-norandrostenedione), whose $\delta^{13}C$ value was previously defined[53]. The $\delta^{13}C$ values and urinary levels of all TC and ERC were determined.

5.4 Results and discussion

Linearity of the GC-C-IRMS response

Three replicates of six serially diluted solutions of 19-NE reference standard corresponding to 5-100 ng on column (2 µL injected in splitless in a split/splitless injector) were analyzed in the same analytical session. The standard deviation of $\delta^{13}C$ values of each triplicate was below 0.50 ‰. The overall $\delta^{13}C$ average value (-29.70 ± 0.13 ‰) was assigned to the reference standard as its $\delta^{13}C$ actual value; the $\delta^{13}C$ mean value of none of the six points of the serial dilution diverged from it for more than 0.11 ‰. A linear response ($R^2 > 0.99$) within the range 240 to 5100 mV was obtained (see Figure 5.1a-b).

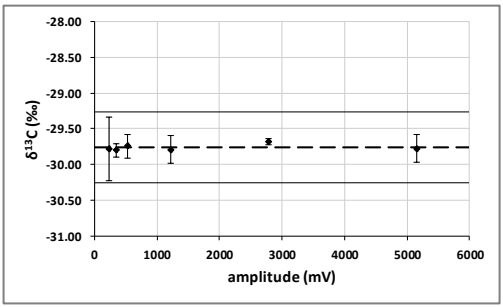


Figure 5.1a Linearity of $\delta^{13}C$ values: amplitude *vs* $\delta^{13}C$ values
The mean $\delta^{13}C$ value (dotted line), the upper and the lower limit (solid lines; µ ± 0.5 ‰) are represented in the graph

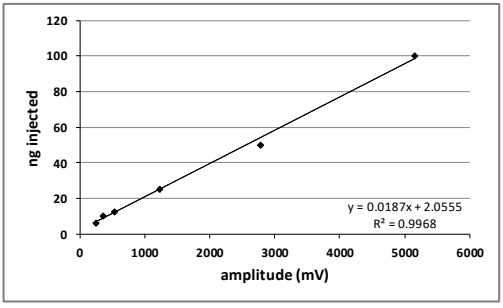


Figure 5.1b Instrumental linearity: amplitude *vs* ng injected

Selectivity

No interfering peaks were detected at the same retention time of 19-NE (366 s, against an internal standard retention time of 335 s) in a series of ten blank urine samples (21 mL) processed and injected in the same conditions (PTV injection; 9 µL injected) used for the corresponding matrices fortified with 19-NE reference standard at 2 ng/mL. The identity of 19-NE was confirmed through the injection in a Thermo ISQ single quadrupole mass spectrometer, according to the TD2015IDCR criteria[63]. The following 3 diagnostic ions (m/z) were monitored: 276, 232, 258.

Limit of quantification (LOQ) and linearity of the method

Since no threshold values are currently reported on the TD2019NA Technical Document about the determination of 19-NE by GC-C-IRMS, we verified if the new developed method fulfilled the same LOQ already defined for 19-NA. Twenty-one mL of blank urine matrices were spiked with 19-NE to obtain positive urine sample at 2 ng/mL. Once purified by HPLC and reconstituted with 16 µL of ISTDRI solution, they were injected in triplicate in PTV mode (4 µL), giving signals with amplitude within the instrumental linearity range. As shown in Table 5.2A, their $\delta^{13}C$ values exhibited a standard deviation of 0.09 ‰ and a drift from the 19-NE reference value (-29.70 ‰) < 0.30 ‰. A sequence of three spiked urine samples at 15 ng/mL, the upper limit of the range within which WADA requires the GC-C-IRMS confirmatory analysis, was also conducted in the same operating conditions (21 mL of urine processed and 4 µL injected) to confirm the linearity of the method. A standard deviation of 0.20 ‰ and a $\Delta\delta^{13}C$ value against the reference value of 0.12 ‰ were obtained (see Table 5.2B). An additional assay was performed in a split/splitless injector by processing higher urine volume (25 mL) to compensate the lower injection volume (2 - 3 µL). At 2.0 ng/mL, the $\delta^{13}C$ SD of the three replicates was 0.12 ‰ and the $\Delta\delta^{13}C$ value between the mean value of the triplicate and the 19-NE reference value was below 0.20 ‰ (Table 5.2C).

		mV	$\delta^{13}C$ (‰)	Mean (n=3)	SD (n=3)	$\Delta\delta^{13}C$ (%)
A	USP 2 ng/mL PTV injector	296	-29.99	-29.91	0.09	-0.21
		260	-29.82			
		300	-29.92			
B	USP 15 ng/mL PTV injector	3491	-29.73	-29.83	0.20	-0.12
		3402	-30.06			
		2986	-29.69			
C	USP 2 ng/mL splitless injector	299	-29.78	-29.89	0.12	-0.19
		290	-30.02			
		290	-29.86			

Table 5.2 LOQ and method linearity in splitless and solvent vent injection mode
The $\Delta\delta^{13}C$ is against the $\delta^{13}C$ value of 19-NE reference standard (-29.70 ± 0.13 ‰)

Variability of delta values

Five standard mixture of 19-NE and ISTDRI were injected in five different analytical sessions. The retention time of 19-NE was measured relative to the reference compound, noting its consistent repeatability (SD < 0.01).

Ten blank urine samples were fortified with appropriate volume of 19-NE reference standard to obtain 10 different positive samples at concentrations within the instrumental linearity range (signals amplitude between 240 to 5100 mV). They were processed and analyzed in 10 different days, showing a $\delta^{13}C$ average value of -29.77 ± 0.22 ‰ (Table 5.3).

	$\delta^{13}C$ (‰)	Amplitude (mV)
Day 1	-29.53	4208
Day 2	-30.04	4940
Day 3	-29.85	2087
Day 4	-29.85	1703
Day 5	-29.90	2573
Day 6	-29.62	1931
Day 7	-30.11	3040
Day 8	-29.65	2803
Day 9	-29.73	2057
Day 10	-29.42	3493
Mean	-29.77	
SD	0.22	

Table 5.3 $\delta^{13}C$ values repeatability

Uncertainty and recovery

The standard deviation related to the delta values repeatability (0.22 ‰) was combined to the bias from the 19-NE reference value (0.22 ‰), resulting in a measurement uncertainty of 0.31 ‰.

The signals amplitude and the $\delta^{13}C$ values of a set of three USP at 5 ng/mL were compared to those obtained from three solution of 19-NE reference standard at the same concentration: a percentage recovery of 60 % have been estimated.

Optimization of the GC-C-IRMS analysis of A and PD

A and PD were previously analyzed under the methods, both based on a single HPLC step, used for the confirmation analysis of 19-norsteroids and pseudo-endogenous steroids respectively[53,62]. In this work, a second step was added in their pre-treatment to collect them individually before the $\delta^{13}C$ value determination. In Table 5.4, $\delta^{13}C$ values obtained from A and PD fractions of five control urine samples processed in various analytical sessions following the former and the new developed method were reported. The comparison shows the very good agreement in terms of accuracy and repeatability of the results, regardless of the procedures applied.

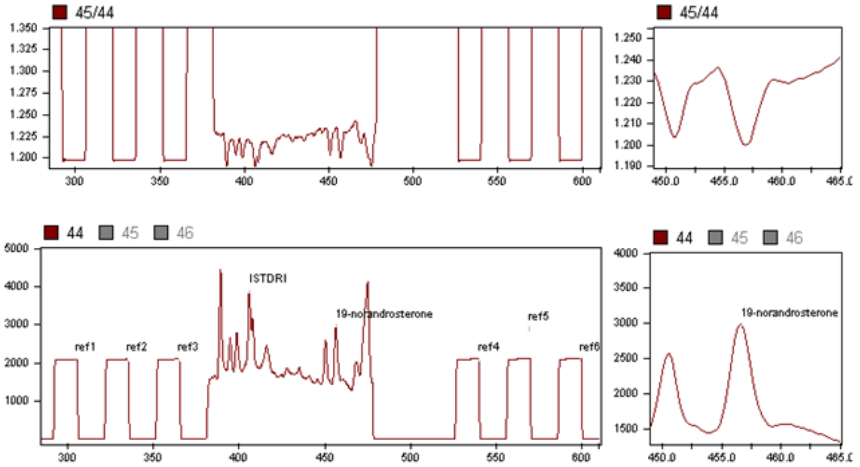
	19-NA			A			PD		
	$\delta^{13}C$ (‰)								
	A	B	$\Delta\delta^{13}C_{(A-B)}$	A	B	$\Delta\delta^{13}C_{(A-B)}$	A	B	$\Delta\delta^{13}C_{(A-B)}$
1	-29.10	-28.76	-0.34	-22.94	-22.84	-0.10	-23.25	-22.90	-0.35
2	-28.94	-28.56	-0.38	-22.77	-22.99	0.22	-23.20	-23.00	-0.20
3	-29.05	-29.19	0.14	-22.41	-22.87	0.46	-23.33	-23.05	-0.28
4	-29.64	-28.53	-1.11	-22.47	-22.70	0.23	-23.07	-23.07	0.00
5	-28.98	-29.36	0.38	-22.60	-22.93	0.33	-23.08	-22.84	-0.24
Mean	-29.05	-29.15	0.10	-22.64	-22.87	0.23	-23.19	-22.97	-0.21
SD	0.28	0.38		0.22	0.11		0.11	0.10	

Table 5.4 IRMS analysis of 19-NA, A and PD: comparison between the new developed procedure (A) and the method previously in use (B)

Optimization of the GC-C-IRMS analysis of 19-NA

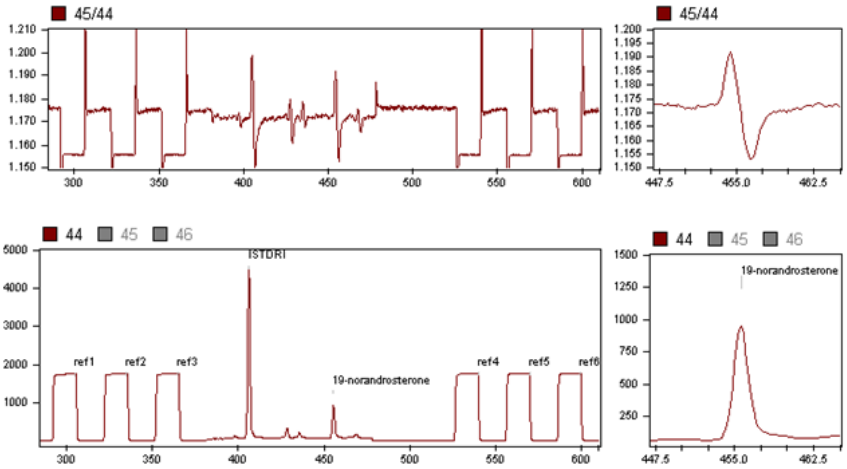
The previous protocol used in our Laboratory for the confirmatory analysis of 19-NA included a fast sample pre-treatment procedure: an enzymatic hydrolysis, a liquid/liquid extraction and a single HPLC purification step. It has allowed to adequately purify the 19-NA extracted fractions, by removing interferences potentially affecting the $\delta^{13}C$ values in the subsequent IRMS analysis[53]. Some of the samples our laboratory receives from other geographical areas and that present signs of microbial activity, frequently show an unusual background (see Figure 5.2a-b) not observed in fresh urine samples. Indeed, the occurrence of similar condition in the daily anti-doping procedures would be reported as inconclusive findings. In Figure 5.2, two GC-C-IRMS plots from the same sample (4 ng/mL, 21 mL processed, 5 µL injected) after one (5.2a) or two sequential LC purification stages (5.2b) are reported, showing the improvement obtained by the new approach. The background noise shifted from 1456 to 67 mV and the 19-NA peak shape improved significantly. No interfering nearby signals were detected. Similar positive effects were observed on the ISTDRI signal. The $\Delta\delta^{13}C$ value of 19-NA between the two measurements (0.36 ‰) remained below 0.50 ‰, the maximum inter-assay variation accepted by the instrument manufacturer. In both cases, the signals exhibited amplitude within the instrumental linearity range: the loss of recovery (of about 40 %) did not negatively affect the reliability of the method. The 19-NE fraction was also collected and subjected to the instrumental analysis (5 µL

injected): its $\delta^{13}C$ value was consistent with the exogenous origin of nandrolone metabolites and in agreement with what previously obtained on 19-NA (Figure 5.3). The validation parameters previously described[53] were verified to assess their applicability in the new developed procedure. In Figure 5.4, a GC-C-IRMS plot of a spiked urine sample at 2 ng/mL injected in PTV mode (9 µL) confirmed the LOQ already determined[53]. Additional data have been included in Table 5.4 supporting the reliability of 19-NA data compared to the already published method.



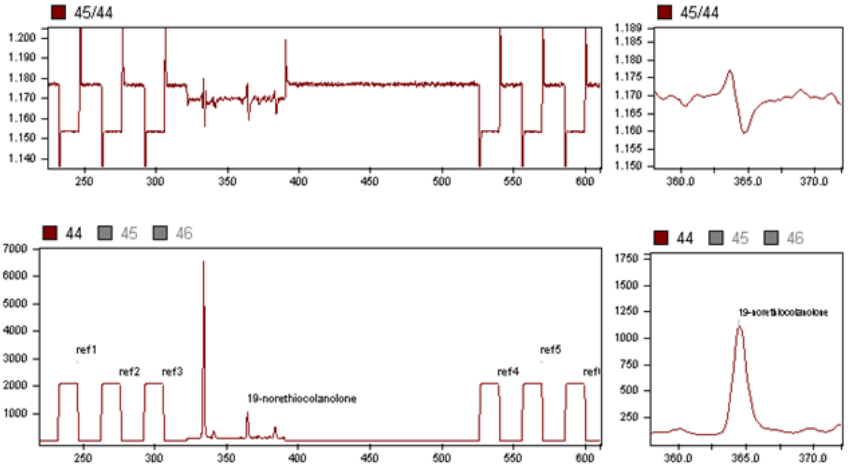
Peak Nr.	Component	Rt [s]	Ampl. 44 [mV]	BGD 44 [mV]	d 13C/12C [per mil] vs. VPDB
1	ref1	305.6	2103	0.9	-39.599
2*	ref2	335.4	2103	1.6	-39.700
3	ref3	365.3	2105	1.7	-39.733
7	ISTDRI	406.1	2112	1773.5	-28.657
11	19-norandrosterone	456.7	1524	1456.1	-30.061
13	ref4	539.6	2108	2.1	-39.822
14	ref5	569.3	2108	2.3	-39.804
15	ref6	599.2	2109	2.3	-39.816

Figure 5.2a 19-NA (4 ng/mL): urine sample showing a high background noise injected after one single HPLC step



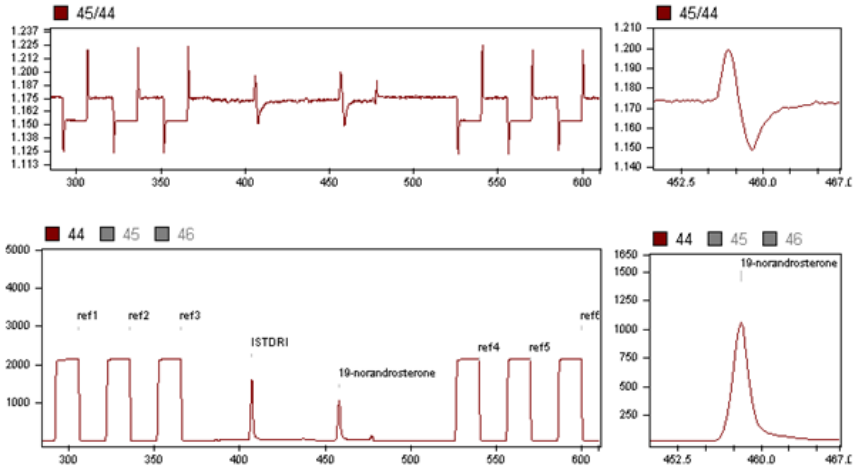
Peak Nr.	Component	Rt [s]	Ampl. 44 [mV]	BGD 44 [mV]	d 13C/12C [per mil] vs. VPDB
1	ref1	305.6	1758	3.2	-39.610
2*	ref2	335.4	1760	6.0	-39.700
3	ref3	365.3	1761	6.7	-39.723
4	ISTDRI	406.5	4489	72.8	-30.569
6	19-norandrosterone	455.6	881	66.9	-29.696
7	ref4	539.6	1768	4.3	-39.718
8	ref5	569.5	1767	6.7	-39.819
9	ref6	599.2	1769	7.2	-39.813

Figure 5.2b 19-NA (4 ng/mL): urine sample showing a high background noise injected after two sequential HPLC steps in PTV mode



Peak Nr.	Component	Rt [s]	Ampl. 44 [mV]	BGD 44 [mV]	d 13C/12C [per mil] vs. VPDB
1	ref1	246.0	2092	0.8	-39.402
2*	ref2	275.9	2089	1.5	-39.500
3	ref3	305.6	2090	1.7	-39.526
4	ISTDRI	334.0	6421	94.8	-29.854
6	19-norethiocolanolone	364.5	1048	85.6	-29.772
8	ref4	539.4	2092	0.9	-39.439
9	ref5	569.3	2094	1.6	-39.520
10	ref6	599.2	2093	1.8	-39.549

Figure 5.3 19-NE (4 ng/mL): injection in PTV mode (5 µL)



Peak Nr.	Component	Rt [s]	Ampl. 44 [mV]	BGD 44 [mV]	d 13C/12C [per mil] vs. VPDB
1	ref1	305.8	2149	1.2	-39.741
2*	ref2	335.7	2149	2.6	-39.900
3	ref3	365.5	2148	2.9	-40.000
4	ISTDRI	407.1	1582	28.8	-30.653
5	19-norandrosterone	458.1	1030	24.7	-29.684
6	ref4	539.6	2153	1.6	-39.879
7	ref5	569.5	2155	2.8	-39.978
8	ref6	599.4	2155	3.1	-39.993

Figure 5.4 19-NA (2 ng/mL): injected after two purification steps in PTV mode (9 µL)

Reliability of delta values on different GC-C-IRMS instruments

Urine samples (21 or 25 mL, depending on the injector used, PTV or split/splitless respectively) spiked with 19-NE at 2 ng/mL were injected (2 or 4 µL) in the three IRMS instruments available in our Laboratory, displaying a $\Delta\delta^{13}C_{(max\text{-}min)}$ value of 0.43 ‰ (Table 5.5).

	Instrument 1	Instrument 2	Instrument 3
Injector	splitless	splitless	PTV
Urine volume	25	25	21
Volume injected	2 µL	2 µL	4 µL
$\delta^{13}C$ ‰	-28.92	-29.35	-29.11
$\Delta\delta^{13}C_{(max-min)}$ ‰	0.43		

Table 5.5 Spiked urine sample at 2 ng/mL injected in different GC-C-IRMS instruments

A method for the IRMS measurement of 19-NE has been developed. Compared to the already proposed ones[64,65], no derivatization step is included, and two different LC column phases were selected. This allows obtaining extracts of adequate purity for the subsequent GC-C-IRMS analysis. Previous described methods showed some limitations as stated by their authors, and no evidences of their application have been reported. Once validated, the method herein presented has been applied to a set of excretion studies as a proof of concept

Excretion studies

Three male volunteers (volunteer 1: 29 years old, 67 kg; volunteer 2: 38 years old, 86 kg; volunteer 3: 57 years old, 86 kg) collected their urine before and at regular intervals for at least 48 hours after the administration of one single dose of 10 mg (volunteer 1 and 2) and 5 mg (volunteer 3) of 19-norandrostenedione ($\delta^{13}C$ value: -29.70 ± 0.30 ‰[53]) . Before the instrumental analysis, the urinary concentrations of both 19-NE and 19-NA were determined through the GC-MS/MS method commonly used in the screening routine tests for the detection of androgenic anabolic steroids[59–61] and, if necessary, adjusted for their specific gravity (SG >1.018[58]). In order to get $\delta^{13}C$ values within the linearity range of the method, we suitably selected for each sample the volume of urine to process (7 – 21 mL), the volume of ISTDRI for dissolving the HPLC fractions and the final volume with which inject the analytes (2 – 9 µL). $\delta^{13}C$ values of 19-NA, 19-NE as TC and of A, PD and PT as ERC were measured for all samples.

Volunteers 1 and 2: both the target compounds reached the highest urinary level within 3-6 hours after the oral intake of nandrolone precursor. As expected, 19-NA was the main excreted metabolite over the entire observational period: it remained detectable above 2.0 ng/mL for 45 hours (volunteer 1) and for more than 80 hours

(volunteer 2). 19-NE quickly decreased below the LOQ of the GC-C-IRMS method after 45 (volunteer 1) and 57 hours (volunteer 2). For volunteer 1, the 19-NA/19-NE ratio was above 3 throughout the study, while for volunteer 2 it was between 1 and 5 (Figures 5.5 and 5.6). $\delta^{13}C$ values of A ($\delta^{13}C$ mean value of volunteer 1: -22.26 ± 0.36; of volunteer 2: -23.80 ± 0.34), PD ($\delta^{13}C$ mean value of volunteer 1: -22.25 ± 0.25; of volunteer 2: -22.25 ± 0.25) and PT ($\delta^{13}C$ mean value of volunteer 1: -23.09 ± 0.12; of volunteer 2: -23.09 ± 0.12) were highly stable and comparable among them. 19-NA and 19-NE showed $\delta^{13}C$ values consistent with their exogenous origin ($\Delta\delta^{13}C_{(ERC-TC)} > 3$), mostly overlapping with each other and in agreement with the value of the administered norandrostenedione (Figures 5.7 and 5.8)

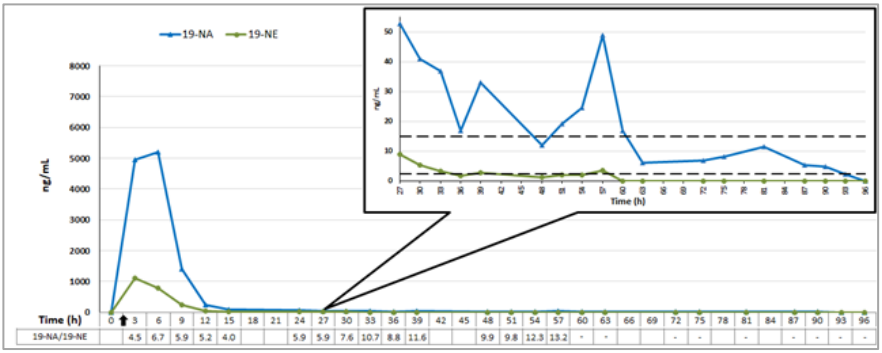


Figure 5.5 Volunteer 1: 19-NA and 19-NE urinary excretion profile and their corresponding ratios
In the box: the last stage of the excretion study; the dotted lines indicate the range within the IRMS procedure should be performed (2.5 – 15 ng/mL)

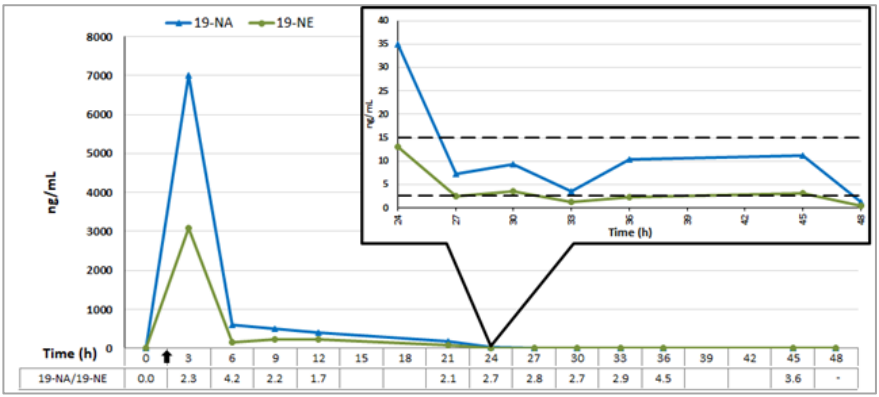


Figure 5.6 Volunteer 2: 19-NA and 19-NE urinary excretion profile and their corresponding ratios In the box: the last stage of the excretion study; the dotted lines indicate the range within the IRMS procedure should be performed (2.5 – 15 ng/mL)

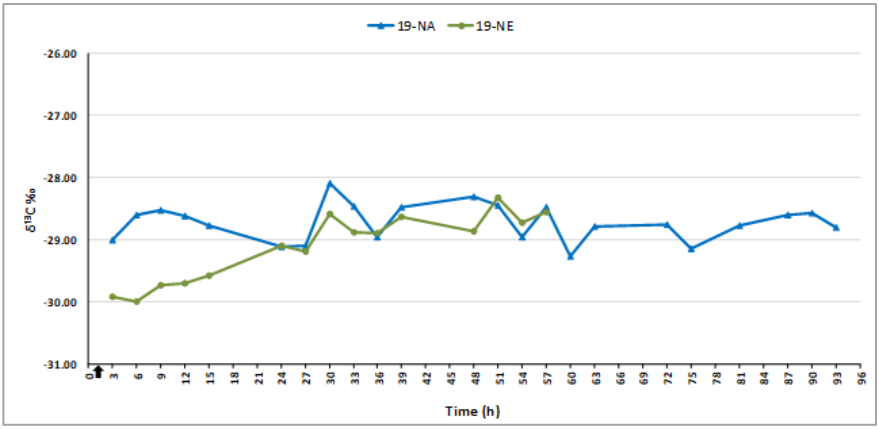


Figure 5.7 Volunteer 1: $\delta^{13}C$ values trend of 19-NA and 19-NE

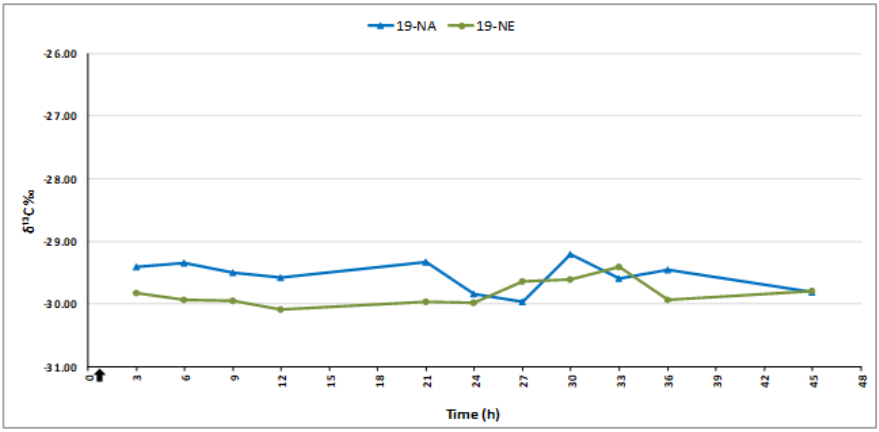


Figure 5.8 Volunteer 2: $\delta^{13}C$ values trend of 19-NA and 19-NE

Volunteer 3: the urinary excretion profile of 19-NA and 19-NE within the 15 hours after the administration of 19-norandrostenedione was similar to what already observed for the previous subjects. Positive urine samples collected from 25 to 45 hours after the beginning of the study exhibited an unexpected concentrations trend for the TC: the 19-NE levels exceeded those of 19-NA, resulting in a 19-NA/19-NE ratio < 1. Instead, only 19-NA was detected in the last samples analyzed (Figure 5.9). As reported in Figure 5.10, the mean $\delta^{13}C$ value for A was of -22.37 ± 0.28, for PD of -22.14 ± 0.32 and for PT of -21.89 ± 0.26. 19-NE $\delta^{13}C$ values were slightly more enriched than 19-NA. Volunteer 3 was periodically subjected to chronic dutasteride (5α-reductase inhibitor) therapy. He stopped its administration 6 months before the administration of nandrolone precursor. Evidences on the influence of 5α-reductase inhibitors on steroid excretion profile have already reported in literature and support our preliminary results[54–56,66,67]. The effect of a co-administration of nandrolone or its precursors with 5α-reductase inhibitors in acute and chronic treatment on the detection of 19-norsteroids misuse by GC-C-IRMS procedure should be better investigated.

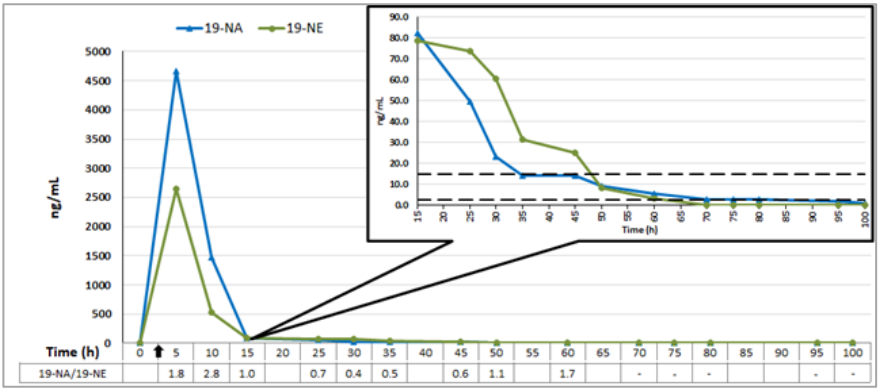


Figure 5.9 Volunteer 3: 19-NA and 19-NE urinary excretion profile and their corresponding ratios In the box: the last stage of the excretion study; the dotted lines indicate the range within the IRMS procedure should be performed (2.5 – 15 ng/mL)

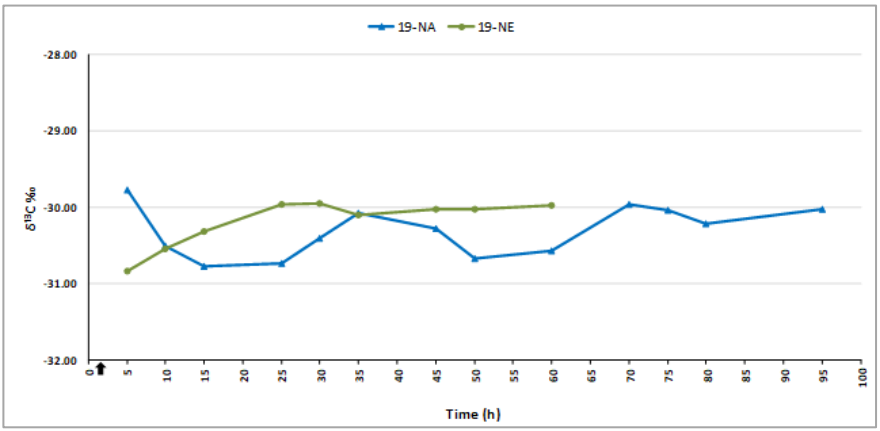


Figure 5.10 Volunteer 3: $\delta^{13}C$ values trend of 19-NA and 19-NE

In all the graphs reported above blanks refer to not collected samples; missing values refer to samples in which 19-NE was not detected, since below the LOD of GC-MS/MS method (2.5 ng/mL). The first point refers to blank urine sample.

5.5 Conclusions

The GC-C-IRMS analyses to confirm the abuse of 19-norsteroids is currently based on the identification of the exogenous or endogenous origin of 19-NA. A fast and simplified procedure on underivatized 19-NA has long been in use in our Laboratory fulfilling the WADA requirements. The detection of 19-NA in case of urine samples affected by degradation processes could sometimes be difficult; inconclusive outcomes could arise from not reliable delta values due to the increased biological background. The introduction of a second HPLC clean-up in the pre-treatment step of 19-NA allowed to reach a consistent decrease of the background noise and guarantee the same sensitivity (LOQ = 2 ng/mL) of the previously described method[53].

Currently, WADA does not require the determination of 19-NE except in case of results of the IRMS analysis inconclusive or consistent with an endogenous origin of 19-NA: urine specimens are then stated as atypical findings if their 19-NA/19-NE ratio is > 3, or negative if they show 19-NA/19-NE ratio ≤ 3. However, the decrease of 19-NA concentration below 2.5 ng/mL and of the 19-NA/19-NE ratio below 3 in urine samples commonly called “unstable” or collected in the late excretion phases or potentially after long-lasting pharmacologic treatment with 5a-reductase inhibitors, may completely mask the prohibited intake of nandrolone, leading to false negative findings.

We herein proposed the alternative use of 19-NE as additional target compound to ensure more confident IRMS findings and improve the doping control procedures for the detection of 19-norsteroids misuse when factors preventing the measurement of 19-NA occur. We proved that, even if typically excreted to a lesser extent than 19-NA, 19-NE remains detectable in urine at concentrations higher than 2.5 ng/mL over 40 hours after the administration of a single dose (10 mg) of nandrolone precursor and could also overcome the urinary levels of 19-NA due to the already known effect of 5α-reductase inhibitors on the 5α-metabolites production[54–56,66,67]. The new protocol was completely validated in accordance with the ISO17025, the WADA International Standard for Laboratories (ISL) and the WADA TD2019NA Technical Document. Suitable and reliable $\delta^{13}C$ values within the linearity range (240 - 5100 mV) were obtained for 19-NE at 2 ng/mL, by processing from 7 to 25 mL of urine depending on the selected injector system (PTV or

split/splitless). The applicability of the method was confirmed on real samples collected after the administration of 19-norandrostenedione.

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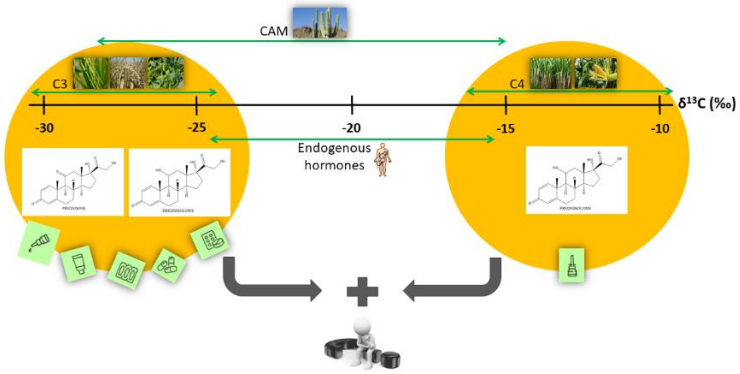
Part II

The IRMS anti-doping analysis: critical issues and current challenges

Chapter 6

Carbon isotopic characterization of prednisolone and prednisone pharmaceutical formulations: implications in antidoping analysis



Adapted from

L. Iannella, F. Botrè, C. Colamonici, D. Curcio, C. Ciccarelli, M. Mazzarino, X. de la Torre (2020)

Carbon isotopic characterization of prednisolone and prednisone pharmaceutical formulations: implications in antidoping analysis

Drug Testing and Analysis, DOI: 10.1002/dta.2876

6.1 Abstract

Twenty-two pharmaceutical formulations containing prednisolone or prednisone commercially available in Italy, Belgium, Spain, Brazil, and India, were analyzed through a specific gas chromatography combustion isotope ratio mass spectrometry, GC-C-IRMS, method. All of them showed typical non-endogenous $\delta^{13}C$ values, except for the Belgian nasal spray, Sofrasolone®, with less depleted ^{13}C content (-17.84 ± 0.18 ‰). Observational studies were performed on two volunteers in therapy with Sofrasolone® to confirm the applicability of the method and suggest adequate interpretation criteria also in case of drugs with less negative $\delta^{13}C$ values. Urine samples were collected before, during, and within the 36 hours after the administration of the spray. Both $\delta^{13}C$ values and urinary concentrations of prednisolone and prednisone were evaluated. All samples were subjected to an adequate pre-treatment (enzymatic hydrolysis, liquid/liquid extraction, and two sequential HPLC steps) before the GC-C-IRMS instrumental injection, according to the method recently developed and validated in our laboratory. Pregnanediol (PD), tetrahydro-11-deoxycortisol (THS), and pregnanetriol (PT) were selected as endogenous reference compounds. The excretion profile was estimated through the liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) method routinely used for the quali-quantitative detection of glucocorticoids. $\delta^{13}C$ values and urinary levels of prednisolone and prednisone were also determined after the intake of one single vial of Sintredius®, a prednisolone oral formulation with conventional more negative $\delta^{13}C$ value (-29.28 ± 0.25 ‰). Finally, the potential masking effect that a combined therapy with Sofrasolone® and Sintredius® could induce on the IRMS findings was investigated.

6.2 Introduction

Prednisolone and prednisone are two synthetic glucocorticoids banned by the World Anti-Doping Agency (WADA) when administered "in competition" by oral, intravenous, intramuscular or rectal routes[1]. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) analysis is routinely performed to estimate the concentrations of prednisolone, prednisone, and their metabolites and discriminate against the forbidden administration routes from the allowed ones[2–5]. At urinary concentrations between the reporting level (30 ng/mL) and 60 ng/mL, WADA has recently recommended an additional GC-C-IRMS confirmatory analysis to determine their exogenous or endogenous origin[6]. Indeed, as widely reported in literature, the non-sterile collection and transport conditions and the presence of normal or pathogen microbial flora in urine samples could lead to the *ex-vivo* degradation of endogenous compounds to banned substances[7–9]. Specifically, cortisol and cortisone physiologically excreted in urine could be converted into prednisolone and prednisone by the Δ1-steroid-dehydrogenase microbial enzyme[10–13]. The discrimination between the exogenous drugs and the endogenously or *ex-vivo* produced compounds is effectively allowed by the study of their ^{13}C composition. Synthetic anabolic-androgenic steroids are mainly produced by a combination of microbial and chemical processes on phytosterols and sapogenins, C3-plant derived natural precursors[14]. Phytosterols are collected as residual products during the soybean-oil production: stigmasterol, β-sitosterol, campesterol and brassicasterol are the most typical ones[15,16]. Sapogenins, such as hecogenin, tiogenin, and diosgenin, are primarily extracted from roots of various Dioscorea species of Mexico yams[17]. The natural discrimination against ^{13}C of the atmospheric CO_2 during the C3 photosynthetic pathway induces a $^{13}C/^{12}C$ isotopic ratios ranging from -24 to -34 ‰, more depleted that the endogenous ones[18–21]. Indeed, the endogenous isotopic profile is affected by the dietary habits: differences between people living in different geographical areas reflect their C3 or C4 plant enrich diet[22]. Endogenous reference $\delta^{13}C$ values range from -16 ‰ to -26 ‰ in the worldwide population: the Americans typically show the least negative $\delta^{13}C$ values (from -16 to -18 ‰), while Scandinavian population display the most negative ones (from -24 to -26 ‰)[23–25].

Recently, a urine sample from an athlete submitted to our routine initial testing procedure showed prednisolone and prednisone urinary concentrations of ca. 20 ng/mL. It was analyzed by the IRMS method already developed and fully validated in our laboratory according to the WADA requirement[26], resulting in a $\delta^{13}C$ value of -18 ‰. Despite still being distinguishable from the European Caucasian endogenous reference values, this unexpected delta value is within the endogenous steroid values range measured in the Americas. It can be reasonably explained by presuming the use of raw materials from C4 or CAM (crassulacean acid metabolism) plants in the synthetic pharmaceutical process. Therefore, an extensive investigation was performed on 22 products commercially available in different countries to verify the existence of pharmaceutical preparations with uncommon exogenous delta values. Data of observational studies on subjects administered with a single or a combination of selected prednisolone formulations with different $\delta^{13}C$ values were also included in this work.

6.3 Materials and methods

6.3.1 Standards and reagents

The standards of prednisolone (**PLONE**, 11β,17α, 21-trihydroxypregna-1,4-diene-3,20-dione), prednisone (**PRED**, 17α,21-dihydroxypregna-1,4-diene-3,11,20-trione), tetrahydro-11-deoxycortisol (**THS**, 3α,17α,21-trihydroxy-5β-pregnan-20-one), pregnanetriol (**PT**, 5β-pregnane-3α,17α,20α-triol), 17α-methyltestosterone (MT), dexamethasone, (11β,16α)-9-fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione), were supplied by Sigma-Aldrich (Milan, Italy). Pregnanediol (**PD**, 5β-pregnane-3α, 20α-diol) and 5α-androstan-3β-ol were from Steraloids (Newport, RI, USA).

Solvents (*tert*-butyl methyl ether, acetonitrile, methanol, cyclohexane and isopropanol) and reagents (sodium bicarbonate, potassium carbonate, sodium phosphate, sodium hydrogen phosphate) were of analytical or HPLC grade and provided by Carlo Erba (Milano, Italy). β-Glucuronidase from *Escherichia coli K12* was obtained from Roche Diagnostic (Mannheim, Germany). Cholesterol esterase from porcine pancreas was purchased from Sigma-Aldrich (Milan, Italy). Water was from a Milli Q water purification system (Millipore S.p.A., Milan, Italy).

Certified $\delta^{13}C$ values traceable to VPDB, obtained from Prof. Brenna (Cornell University Certified Reference Material), was used for the CO_2 reference gas (Solgas, Monza, Italy) for isotope ratio mass spectrometer calibration against underivatized steroids (CU/PCC 34-3)[27].

6.3.2 Prednisolone or prednisone pharmaceutical formulations

Twenty-two prednisolone and prednisone-based preparations produced by pharmaceutical companies from different countries were analyzed. The tablets were crushed in a mortar and dissolved in an adequate volume of methanol to obtain a 1 mg/mL solution. The mixture was sonicated for 5 min and centrifuged; the solution was transferred to a new vial. Pharmaceutical preparations already in solution were directly diluted in methanol to get the same final 1 mg/mL concentration. Esterified forms of the active principle were hydrolyzed by a solution of cholesterol esterase (1 mg/mL), extracted (*tert*-butyl methyl ether) and purified with HPLC before the instrumental analysis. All preparations were diluted with an adequate volume of cyclohexane/isopropanol (4:1) containing 5α-androstan-3β-ol as internal standard and injected in the gas chromatographic-mass spectrometric system.

6.3.3 Excretion studies

Two Caucasian volunteers (volunteer 1: female, 23 years old; volunteer 2: male, 28 years old) in therapy with Sofrasolone® (0.25 mg/mL; 2 puff every three hours for four times a day in a single day, as suggested in the package insert) collected their urine samples before and within the 36 hours after the nasal administration. Additional samples from volunteer 1 after the intake of one single dose of Sintredius® (5 mL; 1.0 mg/mL; -29.28 ± 0.25 ‰) and after a combined administration of Sofrasolone® (0.25 mg/mL; 2 puff every three hours for four times a day) with Sintredius® (one single oral vial) were analyzed.

Each volunteer has signed a written informed consent allowing the use of urine samples for research purposes. Ethical approval of the study was granted by the local Ethical Committee (Lazio 1 Ethical Committee, Rome, Italy), fulfilling the recommendations for research involving human subjects described in Declaration of Helsinki[28].

6.3.4 Urine sample pre-treatment

Urine samples were processed according to the preparative procedure routinely used in our laboratory for the confirmation analysis of pseudo-endogenous steroids[29]. Briefly, ten (2 x 5) or 25 (4 x 6.25) mL of urine were initially hydrolyzed by adding 1.5 mL of phosphate buffer (0.8 M, pH 7.4) and 100 µL of β-glucuronidase from *E. coli* (1 hour at 55°C). After cooling, the pH was adjusted to 9-10 with carbonate buffer (1 M, 2 mL). A liquid/liquid extraction was performed with 10 mL of *tert*-butyl methyl ether by mixing the samples on a mechanical shaker for at least 20 min. Once separated, the solvent of the different aliquots was combined and taken to dryness (75°C, under nitrogen stream). The final residue was dissolved in 50 µL of a methyltestosterone solution (100 µg/mL in a water: methanol 50:50 mixture) and submitted to the next HPLC purification process.

6.3.5 HPLC sample purification

Two LC purification steps were developed to remove potential interferences. All separations were performed on an Agilent 1200 Series liquid chromatograph (Agilent Technologies S.p.A., Cernusco sul Naviglio, Milan, Italy). In the first clean up, the instrument was equipped with an ACE® C18 (25 cm, 4.6 mm, 5 µm) column and an ACE® C18 pre-column (2 cm, 4.6 mm, 5 µm) from CPS Analitica (Milan, Italy); signal at 192 nm was monitored (Agilent 1200 UV DAD detector). Water (solvent A) and acetonitrile (solvent B) at a flow rate of 1 mL/min at 38°C were selected as a mobile phase. The gradient program was as follows: 38% of B until 32.5 min; a first increment of B to 55% in 0.01 min; a further increase of B to 65% in 1 min for 4.5 min to finally reach the concentration of 100% B in 0.01 min for 3.99 min for a total run of 42 min. The analytes eluted in the following order: prednisolone and prednisone (5.15 ÷ 6.35 min), THS (19.65 ÷ 20.70 min) PT (32.95 ÷ 34.85 min) and PD (39.80 ÷ 40.65 min) as against an internal standard (17α-methyltestosterone) retention time of 25.00 ± 0.20 min. The repeatability of the elution conditions was checked by using MT as the internal standard. THS, PD, and PT were separately collected, taken to dryness (75°C, under nitrogen stream) and dissolved with an opportune volume of a mixture of cyclohexane:isopropanol (4:1) containing 5α-androstan-3β-ol (10 µg/mL) as GC-C-IRMS internal standard[29,30]. Prednisolone and prednisone were collected in the same fraction, dried, and dissolved in 50 µL of

dexamethasone solution (100 µg/mL in a water: methanol 2:1 mixture), the internal standard selected for the second HPLC purification.

The second HPLC purification step, necessary to obtain adequate selectivity and sensitivity for both compounds in the following GC-C-IRMS analysis, was performed on an ACE® EXCEL 5 C18 AMIDE column (25 cm, 4.6 mm, 5 µm) from CPS Analitica (Milan, Italy) at 20°C. Water (solvent A) and methanol (solvent B) at a flow rate of 1 mL/min were selected as mobile phase, and the analytes absorbance signal was detected at 254 nm by the Agilent 1200 UV DAD lamp. The chromatographic program was set up starting from 50% of B until 24.5 min, then increasing B to 100% in 0.01 min for 8.49 min for a total run of 33 min. Prednisolone (10.60 ÷ 11.95) and prednisone (15.60 ÷ 17.40) fractions were separately collected, taken to dryness (75°C, under nitrogen stream) and reconstituted with 16 µL of cyclohexane: isopropanol (4:1) containing 5α-androstan-3β-ol (10 µg/mL). The collection windows were defined in relation to a dexamethasone retention time of 25.00 ± 0.20 min.

6.3.6 GC-C-IRMS instrumental analysis

The GC-C-IRMS analysis was performed in splitless mode on an HP7890 gas chromatograph (Agilent Technologies, Milan, Italy) connected to a combustion furnace (at 940°C) linked to a Thermo Delta Plus isotope ratio mass spectrometer through a Thermo Isolink-Conflo IV Interface (ThermoElectron, Bremen, Germany). A TRACE 1310 gas chromatograph (ThermoElectron, Bremen, Germany) connected to a combustion furnace (at 940°C) linked to a Thermo Delta Advantage isotope ratio mass spectrometer (Thermo Electron, Bremen, Germany) through the same interface Thermo Isolink-Conflo IV, was also used. Its distinctive PTV (programmed temperature vaporization) injection mode allowed to reach the same analysis sensitivity by processing lower volume of urine (only 10 mL, instead of 25 mL) and injecting more volume of the pre-treated purified samples (3 µL for prednisolone and 8 µL for prednisone, instead of the routinely 2 µL). The opportune injection volume by splitless or PTV mode was selected according to the initial urine volume disposable for the IRMS confirmation analysis and the urinary concentrations of prednisolone and prednisone previously estimated by LC-MS/MS.

The gas chromatographic conditions have already been described[26,29–31]. Briefly, the separation was achieved on a 5%-phenyl-methylpolysiloxane (HP-5MS, 30 m x

0.25 mm i.d. x 0.25 µm film thickness) column from J&W Scientific. The temperature ramp for prednisolone, THS, PD, and PT was programmed as follows: 150°C (1 min hold), 25°C/min to 260°C for 3.6 min, 25°C to 270°C for 0.9 min, 40°C/min to 290°C (hold 1.20 min), 40°C/min to 310°C for 1.6 min. The helium as the carrier gas was set at the opportune constant flow rate able to provide an internal standard retention time at 400 ± 11 sec. Specific conditions were set up for prednisone: 100°C/min to 265°C (held for 8.5 min) to finally reach (100°C/min) 310°C for 4 min. The helium flow rate was set at the opportune constant value to ensure an internal standard retention time at 260 ± 11 sec.

6.4 Results and discussion

The IRMS analysis is a confirmatory anti-doping procedure currently performed to detect the exogenous or *ex vivo* origin of endogenous anabolic androgenic steroids (EAAS) and distinguish the synthetic forms from their physiological counterparts. Typically, synthetic steroids show an isotopic composition more ^{13}C depleted compared to endogenous ones, due to the characteristic lower ^{13}C content of the natural precursors, mainly phytosterols and sapogenins, selected in the industrial processes[14].

In this work, we focused on the application of GC-C-IRMS analysis for the detection of synthetic forms of prednisolone and prednisone, required by WADA whenever their urinary concentration is comprised between 30 and 60 ng/mL, after adjustment for the specific gravity of the sample according to the WADA rules. No IRMS additional procedure was provided for samples with concentrations above 60 ng/mL, that, when confirmed by LC-MS/MS analysis, are directly reported as adverse analytical findings (AAF).

Prednisolone and prednisone from pharmaceutical formulations commercially available in Italy, Belgium, Spain, Brazil, and India were analyzed in triplicate by GC-C-IRMS to define their $\delta^{13}C$ range. As shown in Table 6.1, all the products displayed typical exogenous $\delta^{13}C$ values, except for the Belgian prednisolone nasal formulation, Sofrasolone® (-17.84 ± 0.18 ‰). Its $\delta^{13}C$ value was confirmed by examining two different batches (18J02 and 18J26) from Belgian market sources. It is less depleted and within the $\delta^{13}C$ endogenous range found in the Americas, suggesting a possible selection of initial starting materials different from C3 plants.

Brand name	Active principle	Manufacturer	Formulation	$\delta^{13}C$ (‰) Mean (n=3)	SD
Lodotra®	Prednisone	Mundipharma Pharmaceuticals, Italy	Tablets	-29.23	0.13
Deltacortene®	Prednisone	Bruno Farmaceutici, Italy	Tablets	-29.19	0.12
Prednisone Teva®	Prednisone	Teva, Italy	Tablets	-28.39	0.07
Prednisone Mylan®	Prednisone	Mylan Pharma, Italy	Tablets	-28.86	0.11
Prednisone EG®	Prednisone	BF Research, Italy	Tablets	-28.45	0.05
Prednisone DOC Generici®	Prednisone	DOC Generici, Italy	Tablets	-29.43	0.20
Sintredius®	Prednisolone	Dompé, Italy	Oral Solution	-29.28	0.25
Solprene®	Prednisolone	Farmigea, Italy	Eye Drops	-28.85	0.27
Scheriproct®	Prednisolone	Bayer, Belgium	Rectal cream	-30.39	0.28
Sofrasolone®	Prednisolone	Melisana, Belgium	Nasal spray	-17.84	0.18
Prednisone Cinfa®	Prednisone	Cinfa, Spain	Tablets	-29.97	0.05
Prednisone Alonga®	Prednisone	Sanofi-Aventis, Spain	Tablets	-29.91	0.24
Prednisone Kernpharma®	Prednisone	Kern Pharma, Spain	Tablets	-30.90	0.23
Prednisone Tarbis®	Prednisone	Tarbis Pharma, Spain	Tablets	-31.25	0.06
Prednisone Pensa®	Prednisone	Pensa Pharma, Spain	Tablets	-30.86	0.24
Estilsona®	Prednisolone	Laboratorios Sonphar, Spain	Oral drops	-29.01	0.08
Prednisona Neo Quimica®	Prednisone	Neo Quimica, Brazil	Tablets	-28.78	0.16
Meticorten®	Prednisone	Super RX Medicamentos Ltda, Brazil	Tablets	-29.38	0.12
PredSim®	Prednisolone	Mantecorp, Brazil	Tablets	-28.80	0.15
Prelone®	Prednisolone	Achè Laboratórios Farmacêuticos, Brazil	Tablets	-28.91	0.10
Wysolone®	Prednisolone	Pfizer, India	Dispersible tablets	-28.58	0.14
Omnacortil®	Prednisolone	Macleods Pharmaceuticals Ltd, India	Dispersible tablets	-29.44	0.16

Table 6.1 $\delta^{13}C$ values of prednisolone and prednisone pharmaceutical preparations available in Italy, Belgium, Spain, Brazil and India

Here, we verified the suitability of the criteria established by WADA to confirm the administration of Sofrasolone®, an exogenous prednisolone formulation showing a not conventional $\delta^{13}C$ value and administered by a not forbidden route. Urine samples of one female and one male Caucasian subjects in therapy with the nasal spray Sofrasolone® were collected each three hours over the 36 hours throughout the study and analyzed by LC-MS/MS and GC-C-IRMS. For a more accurate framework, data obtained from a similarly conducted study in which prednisolone was administered by oral prohibited route were included. Evaluations on possible combined therapy were also reported.

6.4.1 Excretion profile of prednisolone and prednisone when administered by nasal or oral routes

Urinary concentrations of prednisolone and prednisone were determined through the specific LC-MS/MS method routinely adopted in our laboratory for the qualitative and quantitative detection of glucocorticoids[32]. In volunteer 1, the two TC excretion profile rapidly increased within the 12 hours, reaching values ranging from 5 to 61 ng/mL for prednisolone and from 5 to 210 ng/mL for prednisone. Urinary levels of prednisone remained above 60 ng/mL until 24 hours after the first nasal puff (Figure 6.1). Volunteer 2 showed different excretion trend: both compounds were detectable over 24 hours at concentrations between or above the range required by WADA to perform the IRMS confirmation analysis; prednisolone slightly declined below 30 ng/mL after 15 hours (see Figure 6.2).

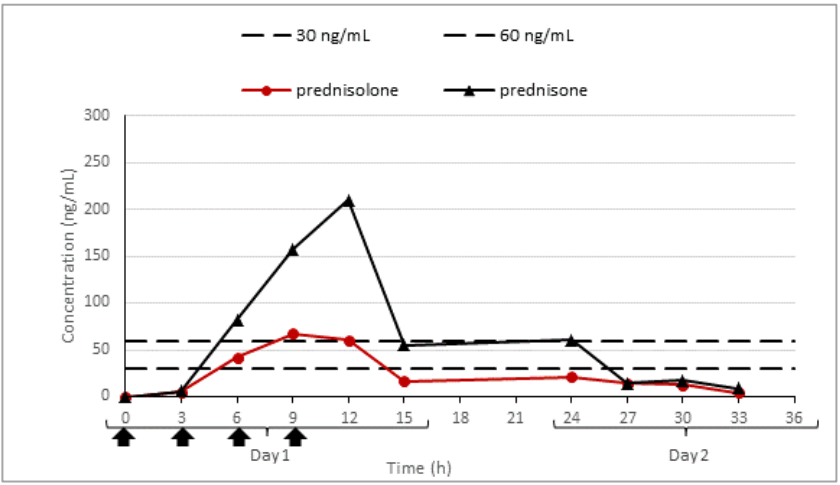


Figure 6.1 Volunteer 1: urinary excretion profile of prednisolone and prednisone after the nasal administration of Sofrasolone®

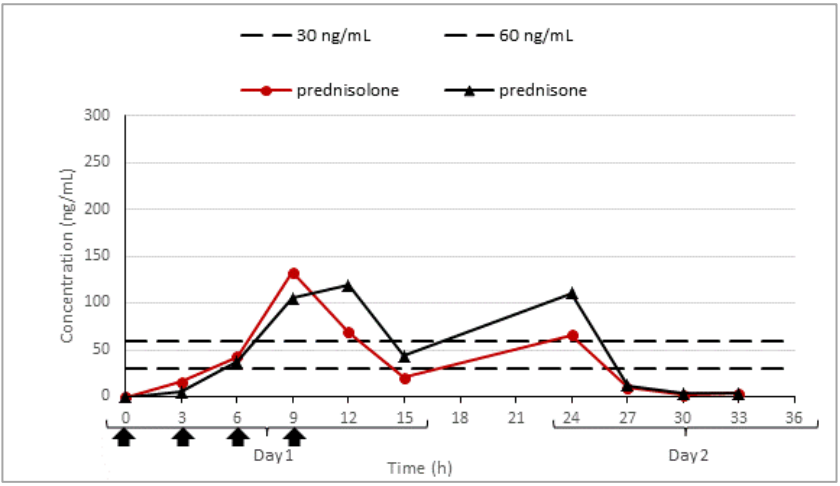


Figure 6.2 Volunteer 2: urinary excretion profile of prednisolone and prednisone after the nasal administration of Sofrasolone®

Results were compared with prednisolone administration by oral route in the same subject 1 involved in the previous study. Urine specimens were collected at regular intervals of three hours after the intake of one single vial of a prednisolone oral formulation, Sintredius®. Concentration of prednisolone reached the highest level (785 ng/mL) 3 hours after dosing and then quickly dropped below 60 ng/mL in the first 15 hours from drug administration. The maximum concentration of prednisone was measured after 6 hours from drug intake; it gradually fell below the reporting level within 24 hours after the oral administration (Figure 6.3).

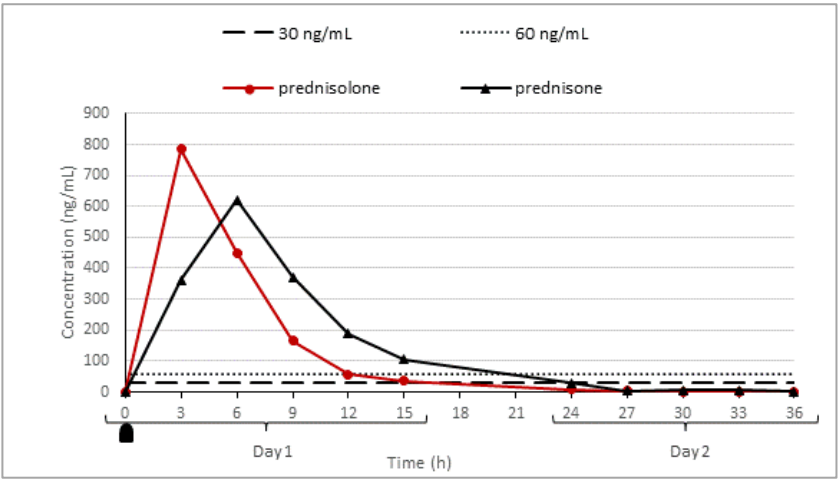


Figure 6.3 Volunteer 1: urinary excretion profile of prednisolone and prednisone after the oral administration of Sintredius®

Even if a significant lower amount of prednisolone and prednisone was measured in urine following nasal administration, the excretion profile produced by Sofrasolone® administration have been shown to be similar to that observed following Sintredius® administration. The specimens collected in the first 3-9 hours contained the higher concentrations of prednisolone and prednisone, while none of the two TC was detectable beyond the 24 hours. All the samples collected within the 6-12 after the first nasal puff would be reported as AAF (concentrations > 60 ng/mL). All the specimens from 12 to 24 hours after the administration (concentrations between 30 and 60 ng/mL) would be analyzed by GC-C-IRMS confirmatory procedure, as in case of oral intake. The nasal administration of glucocorticoids is not currently prohibited. Since the urinary levels reached after a daily nasal administration of prednisolone would impose the GC-C-IRMS confirmation analysis, the criteria used to distinguish the forbidden administration routes from the allowed ones should be reconsidered. This is consistent with previous findings obtained in our laboratory, where the kinetic profiles of prednisolone, prednisone, and metabolites were monitored[32].

6.4.2 GC-C-IRMS analysis after administration of formulations with different $\delta^{13}C$ values

All the samples collected after the nasal administration of Sofrasolone® were processed and suitably pre-treated for the GC-C-IRMS analysis, regardless of the prednisolone and prednisone concentrations previously estimated by LC-MS/MS. The $\delta^{13}C$ values of the two target compounds were reported in Figures 6.4 and 6.5 in comparison with those of PD, used by WADA as the primary ERC, and the $\delta^{13}C$ of Sofrasolone® previously determined (-17.84 ± 0.18 ‰). Only values within the linearity range (prednisolone: 250-2860 mV; prednisone: 300-3200 mV) were represented. The $\delta^{13}C$ values of THS and PT have also been calculated.

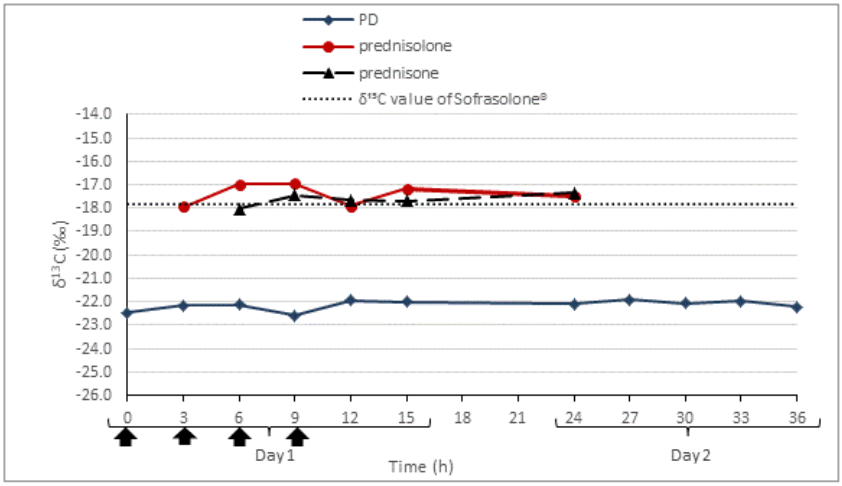


Figure 6.4 Volunteer 1: $\delta^{13}C$ values trend of prednisolone and prednisone after the nasal administration of Sofrasolone® in comparison with those of PD

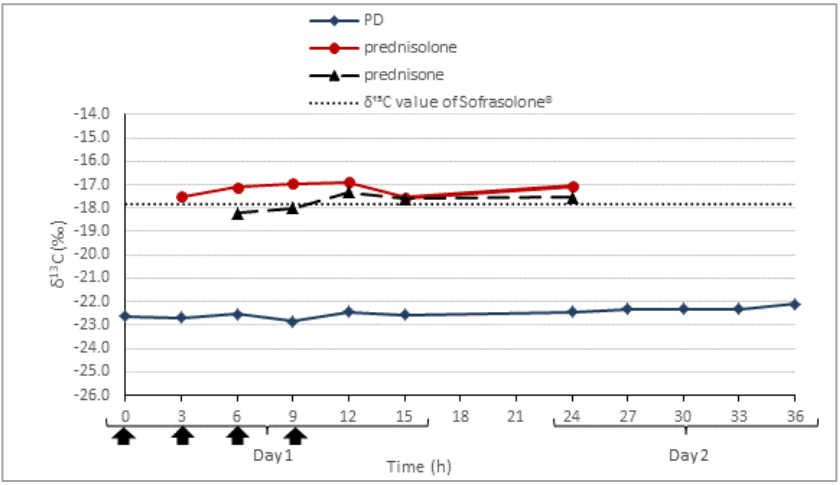


Figure 6.5 Volunteer 2: $\delta^{13}C$ values trend of prednisolone and prednisone after the nasal administration of Sofrasolone® in comparison with those of PD

As shown in Table 6.2, the standard deviation (SD) for each data set obtained with the different ERC was below 0.5 for both volunteers. All the PD, PT, and THS $\delta^{13}C$ values remained unchanged and comparable between them during the entire observational study supporting the high stability of the individual isotopic parameters[30,33].

		Volunteer 1			Volunteer 2		
SAMPLE	**TIME (h)**	**PD**	**PT**	**THS**	**PD**	**PT**	**THS**
Bur	0	-22.47	-21.82	-22.18	-22.63	-22.58	-22.37
Sample 1	3	-22.17	-21.95	-22.74	-22.68	-22.77	-22.15
Sample 2	6	-22.13	-22.68	-22.39	-22.54	-23.15	-22.24
Sample 3	9	-22.59	-21.90	-21.72	-22.84	-22.49	-22.03
Sample 4	12	-21.96	-21.94	-21.98	-22.44	-23.23	-22.18
Sample 5	15	-22.00	-22.52	-21.69	-22.57	-22.78	-22.12
-	18	-	-	-	-	-	-
-	21	-	-	-	-	-	-
Sample 6	24	-22.10	-21.95	-22.22	-22.43	-22.59	-22.22
Sample 7	27	-21.93	-21.23	-22.06	-22.33	-22.49	-22.22
Sample 8	30	-22.07	-22.07	-22.19	-22.31	-22.26	-21.91
Sample 9	33	-21.99	-21.50	-22.22	-22.32	-22.61	-21.97
Sample 10	36	-22.23	-22.54	-22.03	-22.10	-23.15	-21.94
μ ± SD		-22.15 ± 0.21	-21.94 ± 0.41	-22.20 ± 0.28	-22.47 ± 0.21	-22.74 ± 0.32	-22.12 ± 0.47

Table 6.2 $\delta^{13}C$ values of PD, PT and THS after the nasal administration of Sofrasolone®
Average and standard deviation were calculated for each data set
(BUR= blank urine sample)

As expected, $\delta^{13}C$ values of both the glucocorticoids were less negative than the $\delta^{13}C$ values of the three ERC, giving a $\Delta\delta_{(ERC-TC)} > 4$ ‰ in all the specimens analyzed (see Table 6.3a-b).

		Volunteer 1			Volunteer 2		
SAMPLE	**TIME (h)**	**PD-PLONE**	**PT-PLONE**	**THS-PLONE**	**PD-PLONE**	**PT-PLONE**	**THS-PLONE**
Bur	0	-	-	-	-	-	-
Sample 1	3	-4.25	-4.02	-4.82	-5.17	-5.26	-4.63
***Sample 2**	6	-5.13	-5.68	-5.40	-5.42	-6.03	-5.12
***Sample 3**	9	-5.64	-4.96	-4.77	-5.89	-5.54	-5.09
***Sample 4**	12	-4.06	-4.04	-4.08	-5.56	-6.35	-5.30
****Sample 5**	15	-4.84	-5.36	-4.53	-5.02	-5.23	-4.57
-	18	-	-	-	-	-	-
-	21	-	-	-	-	-	-
****Sample 6**	24	-4.61	-4.46	-4.73	-5.37	-5.53	-5.16
Sample 7	27	-	-	-	-	-	-
Sample 8	30	-	-	-	-	-	-
Sample 9	33	-	-	-	-	-	-
Sample 10	36	-	-	-	-	-	-

Table 6.3a $\Delta\delta^{13}C$ for each ERC-PLONE pair

		Volunteer 1			Volunteer 2		
SAMPLE	**TIME (h)**	**PD-PRED**	**PT-PRED**	**THS-PRED**	**PD-PRED**	**PT-PRED**	**THS-PRED**
Bur	0	-	-	-	-	-	-
Sample 1	3	-	-	-	-	-	-
***Sample 2**	6	-4.08	-4.63	-4.35	-4.33	-4.95	-4.04
***Sample 3**	9	-5.13	-4.44	-4.26	-4.85	-4.49	-4.04
***Sample 4**	12	-4.29	-4.27	-4.31	-5.11	-5.90	-4.86
****Sample 5**	15	-4.30	-4.82	-4.00	-4.98	-5.19	-4.53
-	18	-	-	-	-	-	-
-	21	-	-	-	-	-	-
****Sample 6**	24	-4.76	-4.61	-4.88	-4.88	-5.05	-4.68
Sample 7	27	-	-	-	-	-	-
Sample 8	30	-	-	-	-	-	-
Sample 9	33	-	-	-	-	-	-
Sample 10	36	-	-	-	-	-	-

Table 6.3b $\Delta\delta^{13}C$ for each ERC-PRED pair

* Prednisolone concentration between 30 and 60 ng/mL.

** Prednisolone concentration above the limit of quantification of the method (20 ng/mL), but below the reporting level (30 ng/mL)

BUR= blank urine sample before the drug administration

Prednisolone and prednisone ^{13}C content was also measured in the urine samples collected by volunteer 1 after the oral intake of one single Sintredius® vial (-29.28 ± 0.25 ‰) and represented in Figure 6.6.

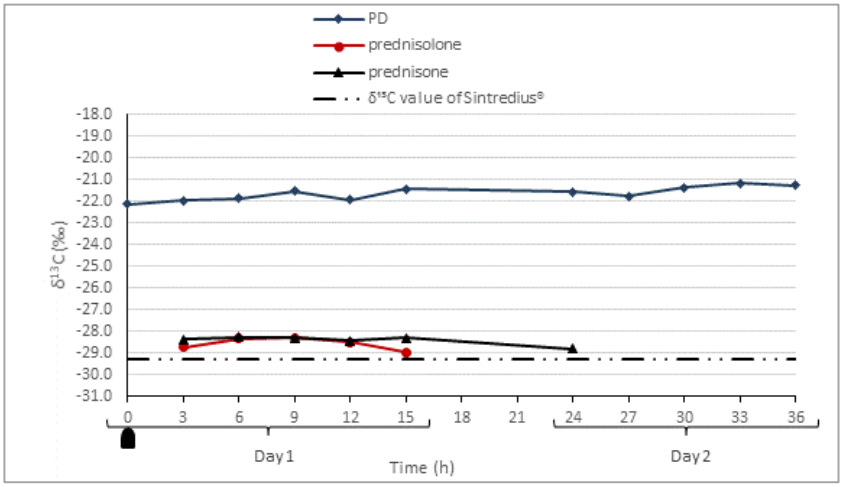


Figure 6.6 Volunteer 1: $\delta^{13}C$ values of prednisolone, prednisone and PD after the oral administration of Sintredius®

Compared to the nasal administration, the isotopic profile of the two TC and the primary ERC, PD, were inverted, as it typically occurs after the administration of exogenous drugs synthetized from natural sources C3 plant derived. In both cases, the $\Delta\delta^{13}C_{(ERC\text{-}TC)}$ were all above 4 ‰ (Tables 6.3a-b and 6.4A), the value we proposed in absolute terms as adequate interpretation criteria for a GC-C-IRMS positive result. WADA established that the $\Delta\delta^{13}C_{(ERC\text{-}TC)}$ are consistent with the exogenous origin of TC if they are > 3 or 4 ‰, depending on the specific ERC-TC couple considered[34]. Such as Sintredius®, most of the steroids commercially available present $\delta^{13}C$ values more negative than the endogenous reference compounds and provide positive $\Delta\delta^{13}C_{(ERC\text{-}TC)}$ values. However, the commercial availability of drugs like Sofrasolone® showing $\delta^{13}C$ value less negative than the selected ERC, should lead to consider the $\Delta\delta^{13}C_{(ERC\text{-}TC)}$ in absolute terms, as it could be positive but also negative.

		A		B	
SAMPLE	**TIME (h)**	**PD-PLONE**	**PD-PRED**	**PD-PLONE**	**PD-PRED**
Bur	0	-	-	-	-
Sample 1	3	6.61	6.26	-4.24	-4.00
Sample 2	6	6.43	6.36	5.43	6.52
Sample 3	9	6.24	6.25	3.73	5.24
Sample 4	12	6.62	6.57	3.32	1.80
Sample 5	15	7.45	6.77	1.54	0.99
-	18	-	-	-	-
-	21	-	-	-	-
Sample 6	24	-	6.99	-2.30	0.29
Sample 7	27	-	-	-0.61	0.23
Sample 8	30	-	-	-0.61	2.02
Sample 9	33	-	-	-	-
Sample 10	36	-	-	-	-

Table 6.4 Volunteer 1: $\Delta\delta^{13}C$ values for each PD-TC pair after the oral administration of Sintredius® (A) and the combined administration of Sofrasolone® and Sintredius® (B)

6.4.4 Combined administration of two prednisolone formulations by different routes

A combined administration of Sofrasolone® (multiple daily administration) with Sintredius® (one single oral vial) was included in this work to consider the possible masking effect that a formulation with an atypical less negative $\delta^{13}C$ value could induce on the IRMS findings. The intake of Sintredius® occurred jointly to the second daily Sofrasolone® nasal puff. The LC-MS/MS and the IRMS analyses were executed on all the samples collected: urinary concentrations and $\delta^{13}C$ values of prednisone and prednisolone are reported in Figures 6.7 and 6.8a-b. Both the TC were detectable in concentration ranging from 5 to 860 ng/mL for prednisolone and from 5 to 450 ng/mL for prednisone. The peak of urinary excretion was reached within 6 hours after dosing. Prednisolone urinary levels dropped below the reporting level after 24 hours, while its metabolite showed a slightly longer excretion tale: prednisone concentration remained above 30 ng/mL until 27 hours after the administration (see Figure 6.7).

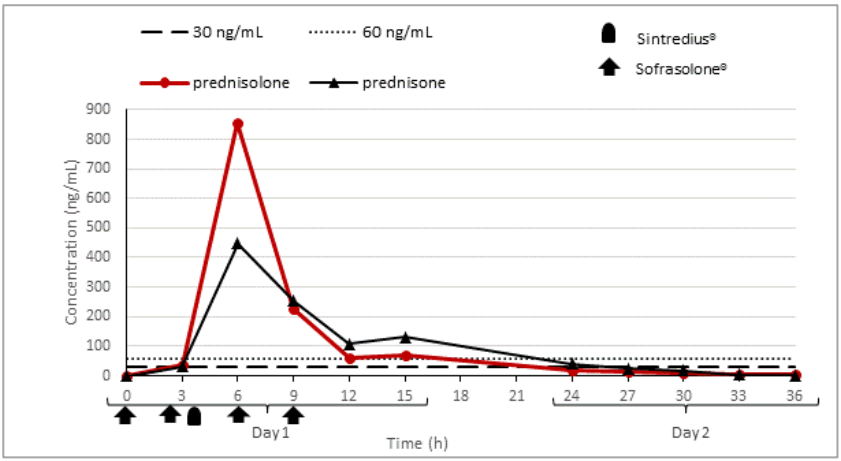


Figure 6.7 Volunteer 1: urinary excretion profile of prednisolone and prednisone after the combined administration of Sofrasolone® nasal spray and Sintredius® oral vial

As regards the isotopic profile, only the $\delta^{13}C$ values within the linearity range and above the LOQ (20 ng/mL) of the method were reported. $\delta^{13}C$ values of the two TC were compared with those of PD, Sofrasolone® (-17.84 ± 0.18 ‰) and Sintredius® (-29.28 ± 0.25 ‰). They were illustrated in Figures 6.8a-b, while the $\Delta\delta_{(ERC-TC)}$ in Table 6.4B.

The initial $\delta^{13}C$ values of both prednisolone and prednisone were consistent with the administration of Sofrasolone®, namely less negative than the $\delta^{13}C$ values determined for the ERC. After the administration of Sintredius®, $\delta^{13}C$ rapidly reduced to values more negative than the endogenous ones. After the last nasal administration of Sofrasolone®, the $\delta^{13}C$ shifted towards values less depleted and overlapping with the PD endogenous delta values. Negative $\Delta\delta_{(ERC-TC)}$ were measured at the beginning of the study after the administration of Sofrasolone®; then they reached positive values due to the influence of the different ^{13}C composition of the two drugs.

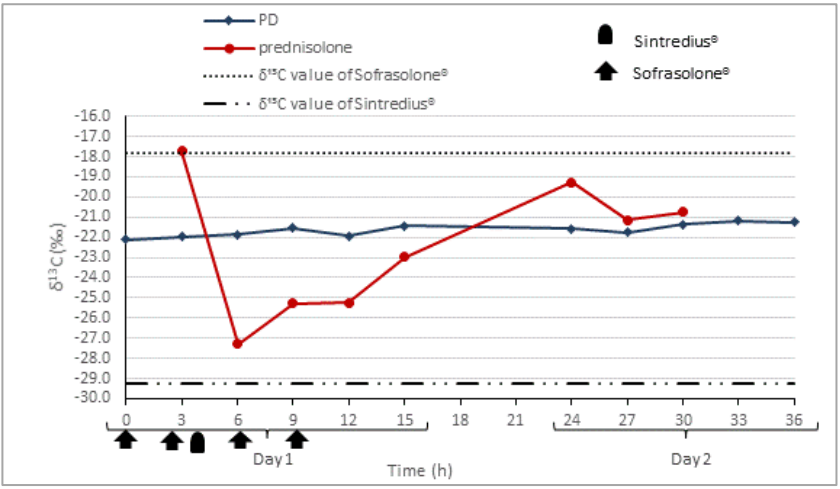


Figure 6.8a Volunteer 1: $\delta^{13}C$ values of prednisolone after the combined administration of Sofrasolone® and Sintredius®

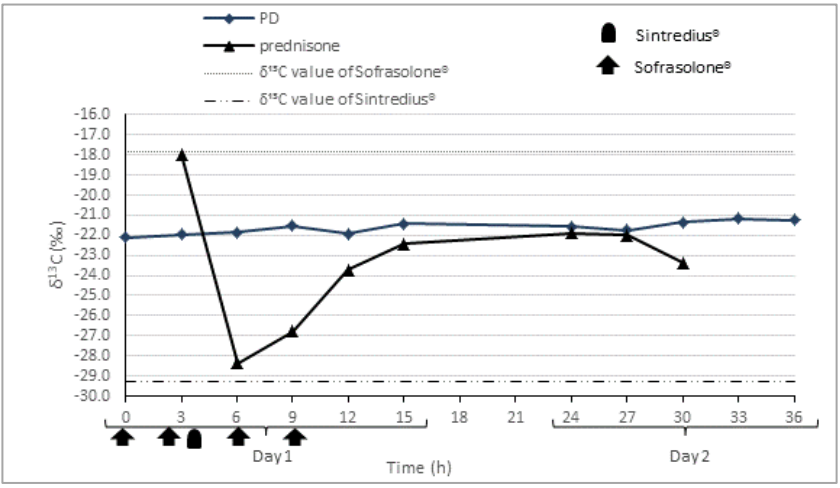


Figure 6.8b Volunteer 1: $\delta^{13}C$ values of prednisone after the combined administration of Sofrasolone® and Sintredius®

The percentage contribution of both pharmaceutical formulations during the whole observational study can be estimated by applying the following formulae:

$$C_{Sint} * \delta_{Sint} + C_{Sof} * \delta_{Sof} = 100 * \delta_{meas} \quad (1)$$

$$100 = C_{Sint} + C_{Sof} \quad (2)$$

By substituting C_{Sint} in (1) with $C_{Sint} = 100 - C_{Sof}$, both contributions can be estimated; where:

C_{Sint} and δ_{Sint} are the percentage contribution and delta value of the Sintredius®,

C_{Sof} and δ_{Sof} are the percentage contribution and delta value of the Sofrasolone®

and

δ_{meas} is the measured delta value of the sample.

The respective trends are presented in Figures 6.9a-b.

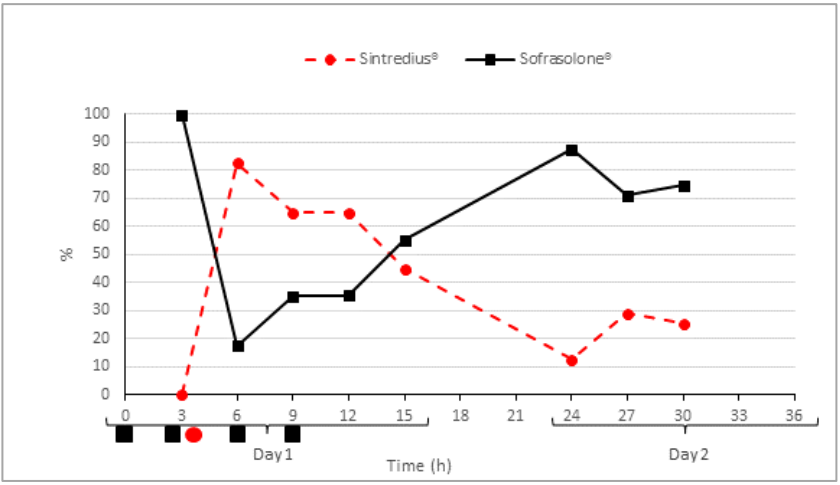


Figure 6.9a Percentage contribution of the two formulations to the resultant $\delta^{13}C$ values of prednisolone after the combined administration

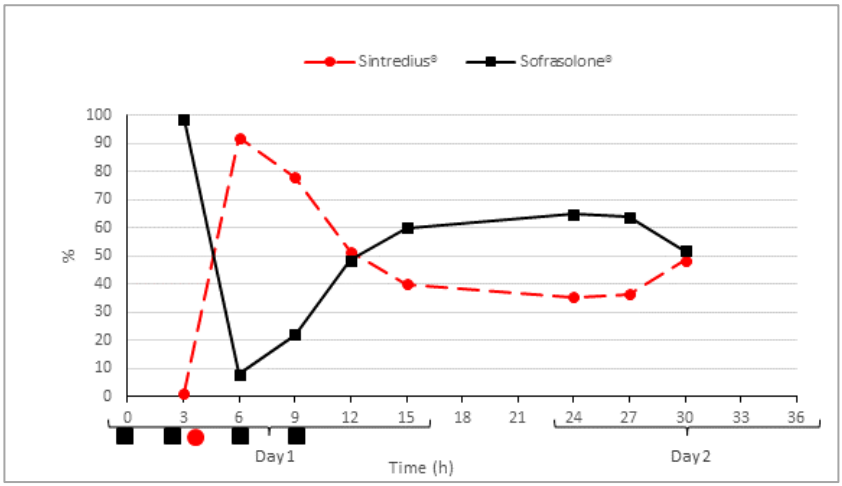


Figure 6.9b Percentage contribution of the two formulations to the resultant $\delta^{13}C$ values of prednisone after the combined administration

In the first specimens collected, 3 hours after the first nasal puff of Sofrasolone®, the contribution of the less depleted $\delta^{13}C$ value of the spray is predominant (100 %). The oral prednisolone formulation significantly influenced the resultant $\delta^{13}C$ value over the next 6 hours, when the high amount of TC excreted after Sintredius® administration overcame that derived from nasal formulation. Twelve-fifteen hours after the administration of Sofrasolone®, the contribution of the two different formulations on $\delta^{13}C$ value was gradually balanced. In the last part of the excretion study, the effect of Sofrasolone® has again become prominent, leading the $\delta^{13}C$ to less negative values. Indeed, as illustrated in Figures 6.8a-b, the last urine samples showed $\delta^{13}C$ values that lay in the endogenous range.

6.5 Conclusions

The carbon isotopic composition of 22 different prednisolone and prednisone pharmaceutical preparations was determined using the GC-C-IRMS method previously validated: one prednisolone nasal formulation (Sofrasolone®) with uncommon delta value (-17.84 ± 0.18 ‰) for a synthetic steroid has been found. The excretion studies after its administration confirmed the applicability of the method to real cases and stressed new critical issues in anti-doping analysis.

The urinary concentrations of prednisolone and prednisone after a single daily use of Sofrasolone® were above the reporting level defined by WADA to discriminate the forbidden administration routes from the allowed ones, even if its nasal administration is not prohibited. More adequate reporting level would be defined to reduce the risk of false positive results caused by the administration of glucocorticosteroids by not banned route.

The exogenous isotopic composition of prednisolone and prednisone after the intake of Sofrasolone® was proficiently detected by GC-CIRMS in Caucasian subjects showing endogenous $\delta^{13}C$ values ranging from -21 to -24 ‰. Conversely, as their endogenous reference compounds possess a less depleted ^{13}C composition (from -16 to -18 ‰), the administration of Sofrasolone® in subjects from the Americas could lead to false negative findings. The commercial availability of a synthetic formulation with $\delta^{13}C$ not distinguishable from the endogenous $\delta^{13}C$ values range of part of the worldwide population suggests possible changes in the pharmaceutical manufacturing processes. The GC-C-IRMS analysis could be no longer sufficient as confirmatory procedure if not combined with a detailed qualitative and quantitative characterization of other diagnostic markers, like 20β-hydroxy metabolite[6], according to the different possible administration routes.

Finally, we have proven that the potential administration of two different prednisolone formulations containing the same active principle, but produced from different natural raw materials, could mask the substance misuse. $\delta^{13}C$ values obtained after the simultaneous intake of the banned oral prednisolone preparation, Sintredius®, with the not banned nasal prednisolone formulation, Sofrasolone®, overlapped with the Caucasian typical ERC values. These results point out worrying limitations on the application of the GC-C-IRMS technique, imposing the activation of follow up, longitudinal studies, considering both the $\delta^{13}C$ values and the urinary concentration trend of the target compounds.

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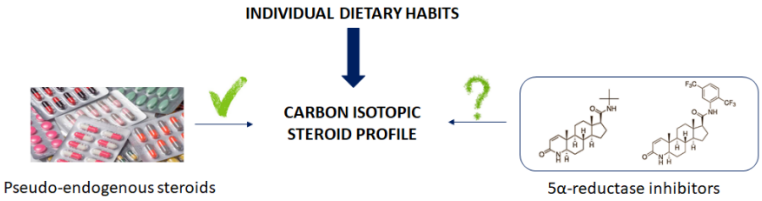
Chapter 7

5α-reductase inhibitors: evaluation of their potential confounding effect on GC-C-IRMS doping analysis



7.1 Abstract

5α-Reductase inhibitors are drugs commonly used in the treatment of benign prostatic hyperplasia, androgenic alopecia and hirsutism: finasteride and dutasteride are the only two pharmaceutical products commercially available for clinical purposes. They are both synthetic 4-azasteroids that suppress the 5α-reductase activity, interfering with the metabolic pathway of the androgenic steroids. Since they may affect the urinary levels of several diagnostic compounds, 5α-reductase inhibitors are considered by the World Anti-doping Agency as potential confounding factors in evaluating the athlete steroid profile.

In this chapter, we investigated the 5α-reductase inhibitors from a different perspective, by verifying their influence on the carbon isotopic composition of the 5α- and 5β- testosterone and nandrolone metabolites.

The analysis was performed through the current GC-MS/MS and GC-C-IRMS protocols in use in our Laboratory on a set of urine samples collected from three male Caucasian volunteers after the acute and chronic administration of finasteride in combination with the intake of 19-norandrostenedione, a nandrolone precursor. The excretion and isotopic profile of androsterone (A), etiocholanolone (Etio) 5α-androstane-3α,17β-diol (5αAdiol) 5β-androstane-3α,17β-diol (5βAdiol) were determined as well as those of 19-norandrosterone (19-NA) and 19-norethiocolanolone (19-NE). $\delta^{13}C$ values of pregnanediol (PD), and pregnanetriol (PT) were also measured to define the endogenous isotopic profile in the IRMS confirmation procedure for detecting the abuse of 19-norsteroids.

7.2 Introduction

Steroid 5α-reductases (5α-R) are membrane-bound enzymes that irreversibly catalyze the reduction of C-19 and C-21 steroids to their corresponding 5α-dihydro-metabolites via NADPH-dependent mechanism[1]. They exist in three isoforms differing in chromosome location, protein structure, optimum pH, tissue distribution and affinity for testosterone[2]. 5α-R type 1 is found throughout the body, including the skin, liver and prostate, while 5α-R type 2 is located mainly in the prostate and other genital tissues[3,4]. 5α-R type 3 is the latest identified one and exerts peculiar role in the *N*-linked glycosylation of proteins[5]. GPSN2 and GPSN2L are two synaptic glycoproteins also included in the 5α-R family: even if structurally different from those previously described, they share similar biochemical functions[2,6]. 5α-Stereoisomers are for most steroid hormones, including progestins and glucocorticoids, biological inactive compounds not able to trigger the transcriptional signaling cascade, but rapidly undergoing the next reduction and glucuro- or sulfo-conjugation reactions. The reduced derivative of testosterone (T), 5α-dihydrotestosterone (5α-DHT), represents a critical exception to this general rule: it is known to be the most potent androgen because of its high binding affinity (about double of that of T) and slow dissociation rate (about a fifth of T) to the androgen receptor. 5α-DHT promotes the sexual differentiation of organs during embryonic life and the development and regulation of male secondary characteristics in adults[7]. An increased DHT synthesis may results in several skin disorders (hirsutism, acne and androgenic alopecia) and benign or neoplastic prostatic hyperplasia, very frequent in men aged over 50 years[8,9]. Accordingly, inhibition of 5α-R enzymes is one of the suitable therapeutic approaches for treating prostatic pathological conditions (BHP, benign prostatic hyperplasia) and male baldness. The 4-azasteroids are the most studied and clinically used 5α-R inhibitors (5-ARIs)[10]: finasteride and dutasteride are the only two pharmaceutical products commercially available and FDA-approved for clinical purposes, even if their safety profile is still under debate[11–19]. They are competitive inhibitors of 5α-R: finasteride is a type 2-selective 5-ARI, whereas dutasteride is a potent dual inhibitor of both 5α-R type-1 and 2[20,21].

In doping control analyses, the possible use of 5-ARIs as masking agents has been widely investigated[22–24]. Androsterone (A), etiocholanolone (Etio), 5α-androstane-

3α,17β-diol (5αAdiol) and 5β-androstane-3α,17β-diol (5βAdiol) and their corresponding ratios (A/Etio; 5αAdiol/5βAdiol) are some of the markers measured by GC-MS^n (gas chromatography coupled to tandem mass spectrometry) to determine the athlete's urinary steroid profile[25]: as 5α- or 5β- androgen metabolites produced from testosterone by 5α-R, they may be all affected by the intake of 5-ARIs. Similarly, the combined administration of 5-ARIs and 19-norsteroids could reduce the urinary levels of 19-norandrosterone (19-NA) under the threshold value of 2.5 ng/mL: neither positive reporting nor supplementary confirmation procedure would be applied, leading to false negative results[22,26–28].

Alterations of the individual steroid profile necessitate a further gas chromatography coupled to isotope ratio mass spectrometry (GC-C-IRMS) analysis to verify the exogenous or endogenous origin of the urinary androgens. Typically, the individual range of variability of delta ^{13}C values is narrower than the one observed for the steroid profile parameters[29,30]: a longitudinal evaluation of the steroids ^{13}C composition has been already proposed as additional Module of the Athlete Biological Passport[31,32].

We herein assessed the impact of 5-ARIs on the stability of the steroids' metabolites delta ^{13}C values; to date, no studies on it may be found in literature.

We carried out a preliminary research on three male subjects after the administration of 19-norandrostenedione, a nandrolone precursor, with the combined use of single or repeating dose of finasteride. $\delta^{13}C$ values of the 5α- and 5β-metabolites of testosterone and their 19-nor analogs commonly evaluated in the GC-C-IRMS analysis for detecting the pseudo-endogenous steroids[33,34] have been determined by the previously published GC-C-IRMS methods[35–37]. 19-Norethiocolanolone (19-NE) has been considered as a supplementary target compound (TC), even if that is not currently required by WADA (World Anti-Doping Agency) in the TD2019NA[34]: compared with 19-NA, it is typically excreted to a lesser extent, but it could become the main metabolite due to the suppressive effect of 5-ARIs on the production of 19-NA. $\delta^{13}C$ values of pregnanediol (PD) and pregnanetriol (PT), selected as ERC, were also examined. Urinary concentrations of the analytes of interest have been estimated by GC-MS/MS through the daily used method in the routine doping controls[38–40]. Resulting values were compared to those already presented after the sole oral intake of 19-norandrostenedione to provide a baseline urine steroid and isotopic profile[37].

7.3 Materials and methods

7.3.1 Standards and reagents

Certified reference standards of steroids employed during the pre-treatment and instrumental analyses have been provided as described before[35–37].

All solvents (n-pentane, acetonitrile, methanol, cyclohexane and isopropanol) and reagents (sodium bicarbonate, potassium carbonate, sodium phosphate, sodium hydrogen phosphate) were of analytical or HPLC grade and supplied by Carlo Erba (Milano, Italy). β-Glucuronidase from *Escherichia coli* K12 was purchased from Roche Diagnostic (Mannheim, Germany). Water was from a Milli Q water purification system (Millipore S.p.A., Milan, Italy).

Certified reference material (CU/PCC 34-3) with $\delta^{13}C$ values traceable to VPDB obtained from Prof. Brenna (Cornell University Certified Reference Material) was used to calibrate the CO_2 reference gas (Solgas, Monza, Italy) of the isotope ratio mass spectrometer for the analysis of underivatized steroids[41].

7.3.2 Excretion studies

Three male volunteers were administrated with finasteride 1 mg (Propecia®, MSD Italy) before the oral intake of 19-norandrostenedione (Genetic Evolutionary Nutrition, Los Angeles) following different protocols. Volunteer 1 (V1, 38 years old, 86 kg) has taken one tablet of finasteride 3 hours before the administration of 10 mg of 19-norandrostenedione and collected the urine samples each 3 hours for more than 4 days (102 hours). Volunteer 2 (V2, 29 years old, 67 kg) has undergone multiple administration of finasteride (one tablet a day for three consecutive days) before the intake of 10 mg of 19-norandrostenedione. Urine specimens were collected each 3 hours for more than 2 days (51 hours). Volunteer 3 (V3, 57 years old, 86 kg) was administered with 5 mg of 19-norandrostenedione after taking finasteride for one month. Urine samples were collected at regular intervals (every 5 hours) for 60 hours after receiving the 19-norsteroid-based formulation.

All samples were stored at -20°C until the analysis.

All participants have signed a written informed consent allowing the use of urine samples for research purposes. The study was conducted in accordance with the recommendations for research involving human subjects described in Declaration of Helsinki[42].

7.3.3 Sample pre-treatment

Urine samples pre-treatment was performed following the two distinct validated and routinely used protocols for the detection of synthetic forms of endogenous anabolic androgenic steroids and 19-norsteroids. We have already described them before[35–37]. Variable volume of urine (3-21 mL) was processed depending on the urinary concentrations of the analytes previously estimated by GC-MS/MS[38–40]. Phosphate buffer (1.5 mL, 0.8 M, pH 7.4) and β-glucuronidase from *E. coli* (100 µL) were added to each urine aliquot to hydrolyze the conjugated steroids (55 °C, 60 min). After incubation, the pH was adjusted to 9-10 with 0.5 mL of carbonate buffer 20 %. Ten mL of *tert*-butyl methyl ether[36] or *n*-pentane[35,37] were added to perform the liquid/liquid extraction (on a mechanical shaker for at least 20 min). Samples were then centrifuged for 2 minutes at 3000 rpm to separate the organic solvent. Organic layers were taken to dryness under nitrogen stream (75 °C); different aliquots from the same sample were previously combined. Fifty µL of a 17α-methyltestosterone solution (MT, internal standard) at 100 µg/mL in water:methanol (50:50) was used to reconstitute the dried residues for the next HPLC purification step.

HPLC purification is necessary to get urinary extracts lacking in interferences able to impact on the accuracy and reliability of the $\delta^{13}C$ values measured during the IRMS analyses. Depending on the method considered, one or two HPLC sequential steps were performed, as already described elsewhere[35–37] and briefly reported here.

7.3.4 Analysis of testosterone 5α- and 5β-metabolites

Fifty µL of the pre-treated samples were injected on an ACE 5 C18 column (CPS Analitica, Milan, Italy) (25 cm, 4.6 mm, 5 µm) and an ACE 5 C18 guard cartridge at 38 °C. Water (A) and acetonitrile (B) as mobile phases were set at constant flow rate of 1 mL/min. Compounds were separated through an isocratic program: 38 % B for 32.50 min then increasing to 55 % B in 0.01 min, then to 65 % B in 1 min and held at 65 % B for 4.49 min. The column was finally flushed for 4 min at 100% B for a total run time of 42 min. Collection intervals (min) of compounds of interest were as follows: 5βAdiol (28.20 ÷ 30.02), 5αAdiol (30.03 ÷ 31.81), Etio (35.70 ÷ 36.26), A (36.27 ÷ 36.90). Each fraction was dissolved in opportune volume of the GC-C-IRMS

internal standard solution (ISTDRI, solution at 10 µg/mL, in cyclohexane:isopropanol 4:1).

7.3.5 Analysis of 19-NA and 19-NE

Sample purification was firstly performed using an Ascentis® phenyl column (Sigma-Adrich, Milan, Italy). 15 cm, 4.6 mm, 5 µm) and an Ascentis® phenyl Supelguard™ guard cartridge (2 cm, 4.0 mm, 5 µm) at 60°C. Water (A) and acetonitrile (B) were selected as mobile phases (constant flow rate at 1 mL/min). An isocratic program was set at 50 % of B for 8.50 min, then increased to 100 % in 0.01 min and held until the end of the ramp, for a total run of 15 min. Fractions containing PT (4.80 ÷ 5.29), 19-NE (5.69 ÷ 6.14), 19-NA (6.17 ÷ 6.73) and PD (6.95 to 8.15 min) were collected. Once dried under nitrogen stream at 75-90 °C, they were reconstituted with ISTDRI or a solution of 5β-estran-17α-ethynyl-3α,17β-diol (NET, at 200 µg/mL in a mixture of methanol:acetonitrile 1:300), if a second HPLC clean-up was requested. 19-NE, 19-NA and PD were further purified in an ACE® EXCEL 5 C18 AMIDE column (25 cm, 4.6 mm, 5 µm) from CPS Analitica (Milan, Italy) at 25 °C though a fast (total run of 15 min) isocratic program with 100 % of acetonitrile. They were collected as follows: 19-NE (4.80 ÷ 5.45), 19-NA (5.20 ÷ 5.70) and PD (11.75 ÷ 12.70) and dissolved in opportune volume of ISTDRI[37].

All the chromatographic separations were performed on an Agilent 1200 Series liquid chromatograph (Agilent Technologies S.p.A., Cernusco sul Naviglio, Milan, Italy).

7.3.6 Sample GC-C-IRMS analysis

The GC-C-IRMS analyses were carried out on Thermo DELTA V™ Advantage or Thermo DELTA V™ Plus isotope ratio mass spectrometers coupled to a Thermo TRACE™ 1310 GC by a Thermo Isolink-Conflo IV Interface (all from ThermoElectron, Bremen, Germany) via a combustion reactor at 940 °C. The analytes were injected in splitless mode (2 - 3 µL); the alternative programmed temperature vaporizing (PTV) injection mode was selected when the injection of large volume (up to 10 µL) was needed. The compounds were separated on a 5 % Phenyl-methylpolysiloxane (30 m x 0.25 mm i.d. x 0.25 µm film thickness) column from J&W Scientific (CPS Analitica, Milan, Italy). The GC chromatographic parameters were set as follows: 50

°C for 1 min, 25 °C/min to 260 °C for 3.6 min, 25 °C/min to 270 °C for 0.9 min, 40°C/min to 290 °C for 1.2 min, 40 °C/min to 310 (1.6 min held) for a total run of 14.10 min (for A, Etio, 5αAdiol, 5βAdiol, PD and 19-NA)[35,36] or 150 °C (1 min held), 35 °C/min to 290 °C for 5 min, 40 °C/min to 310 °C for 4.5 min, for a total run of 15 min (for 19-NE)[37].

7.4 Results and Discussion

In this chapter we explored the influence of the acute (single dose for one or three consecutive days) and chronic (one-month therapy) treatment with 5-ARIs on urinary excretion and carbon isotopic composition of testosterone and nandrolone 5α- and 5β-metabolites. As showed before[37], a chronic administration of dutasteride could affect the 19-norandrostenedione metabolism even if it is suspended for six months, due to its high potency and long-lasting half-life (5 weeks)[21]. Dutasteride is typically prescribed to aged and elderly men affected by prostate enlargement, whereas finasteride to counteract and reduce hair loss mainly in healthy and younger men, making its use more probable also in athletes. Indeed, urine samples from three male volunteers were collected before (at regular intervals of 3 or 5 hours) and after the intake of low dosage of finasteride (1 mg) followed by 5 or 10 mg of 19-norandrostenedione. All the common TC and ERC selected in the pseudo-endogenous steroids' confirmation procedure were examined; only data from compounds (A, Etio, 5αAdiol, 5βAdiol) directly involved in the biosynthetic reactions catalyzed by 5α-R were here presented (Supporting material may be found in the related Annex). Both the metabolites of nandrolone (19-NA and 19-NE) have been evaluated according to the new method for the detection of 19-norsteroids misuse proposed before[37].

7.4.1 Influence of finasteride on testosterone 5α- and 5β-metabolites urinary concentrations

The intake of one single (V1) or repeating dose, three tablets once a day (V2), of finasteride, reduced the production of A and 5αAdiol and doubled the excretion levels of Etio and 5βAdiol, leading to a decrease of their corresponding ratios (A/Etio and 5αAdiol/5βAdiol) below 1. 5αAdiol/5βAdiol ratio was suppressed more permanently compared to that observed for A/Etio; it remained much lower than

1 also in the last samples analyzed: until 102 (V1) and 51 (V2) hours post-administration of finasteride (see Figures 7.1a-b and 7.2a-b and Tables 7.1a-b and 7.2a-b).

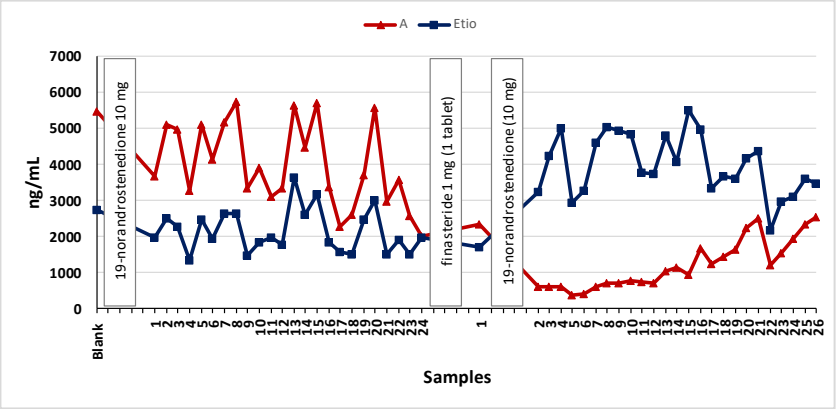


Figure 7.1a Volunteer 1: urinary concentrations trend of A and Etio

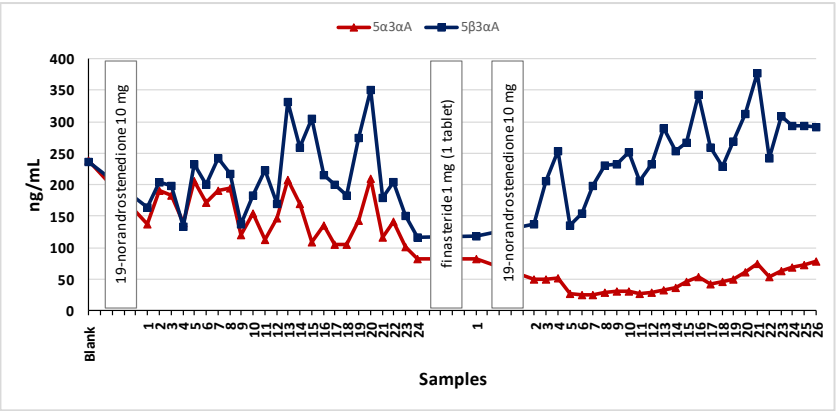


Figure 7.1b Volunteer 1: urinary concentrations trend of 5αAdiol and 5βAdiol

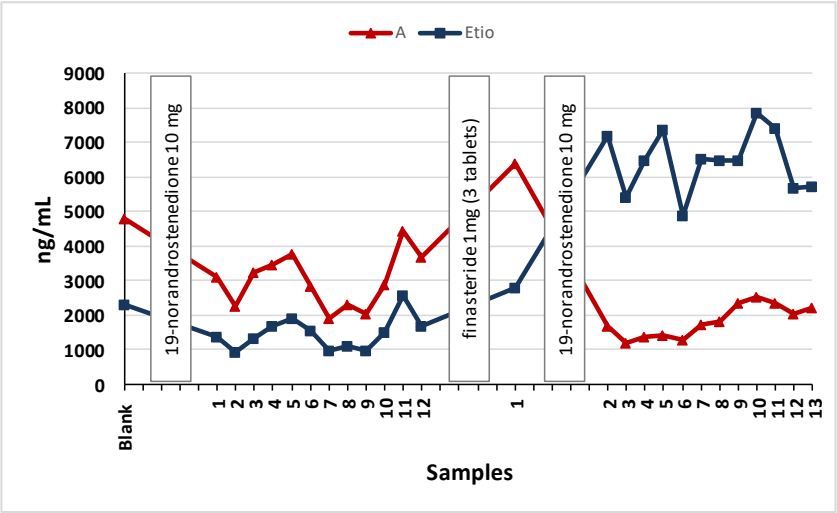


Figure 7.2a Volunteer 2: urinary concentrations trend of A and Etio

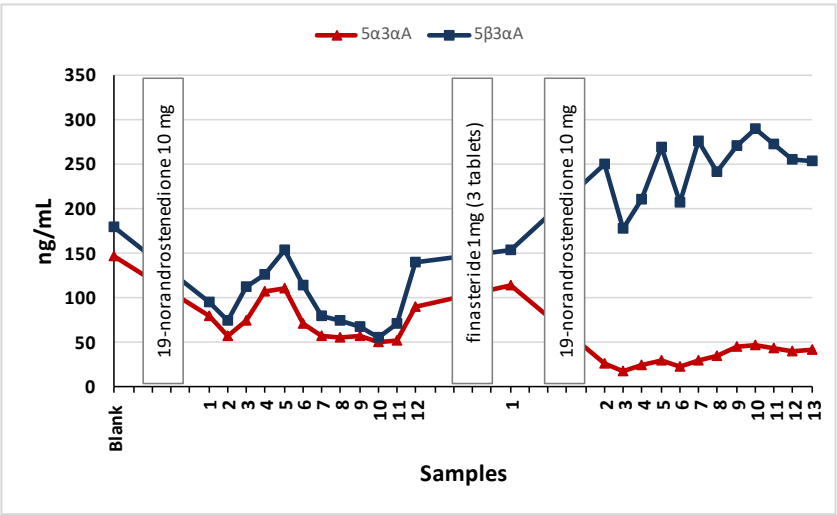


Figure 7.2b Volunteer 2: urinary concentrations trend of 5αAdiol and 5βAdiol

	Time (h)	A/Etio	5αAdiol/5βAdiol	$\Delta\delta_{(A\text{-}Etio)}$	$\Delta\delta_{(5\alpha Adiol\text{-}5\beta Adiol)}$
Blank sample	0	2.01	1.01	0.26	0.06
19-norandrostenedione 10 mg					
Sample 1	3	1.87	0.84	0.67	0.20
Sample 2	6	2.03	0.93	0.87	0.45
Sample 3	9	2.19	0.93	0.73	0.76
Sample 4	12	2.46	1.03	0.50	0.13
Sample 5	15	2.07	0.89	0.38	0.56
-	18				
-	21				
Sample 6	24	2.13	0.86	0.25	0.05
Sample 7	27	1.95	0.79	0.80	0.47
Sample 8	30	2.19	0.90	0.64	0.03
Sample 9	33	2.24	0.88	0.47	0.53
Sample 10	36	2.13	0.85	0.73	0.10
Sample 11	39	1.57	0.51	0.47	-0.37
-	42				
-	45				
Sample 12	48	1.90	0.86	0.49	-0.01
Sample 13	51	1.55	0.63	0.50	0.31
Sample 14	54	1.73	0.65	0.56	0.54
Sample 15	57	1.79	0.36	0.20	-0.09
Sample 16	60	1.83	0.63	0.50	0.29
Sample 17	63	1.46	0.52	0.42	0.33
-	66				
-	69				
Sample 18	72	1.72	0.58	0.54	0.42
Sample 19	75	1.49	0.52	0.26	0.35
-	78				
Sample 20	81	1.85	0.60	-0.04	0.30
-	84				
Sample 21	87	1.96	0.65	0.48	0.26
Sample 22	90	1.86	0.70	0.45	0.13
Sample 23	93	1.71	0.67	-0.06	0.15
Sample 24	96	1.03	0.71	0.24	0.53

Table 7.1a Volunteer 1: urinary concentrations ratios and $\Delta\delta^{13}C$ values before the administration of finasteride of each 5α-, 5β- testosterone metabolites pair

	Time (h)	A/Etio	5αAdiol/5βAdiol	$\Delta\delta_{(A\text{-}Etio)}$	$\Delta\delta_{(5\alpha Adiol\text{-}5\beta Adiol)}$
finasteride 1 mg (one tablet)					
Sample 1	3	1.37	0.70	1.16	0.78
19-norandrostenedione 10 mg					
Sample 2	6	0.18	0.37	0.47	0.11
Sample 3	9	0.14	0.24	0.18	0.23
Sample 4	12	0.12	0.20	0.04	0.46
Sample 5	15	0.12	0.20	-0.05	-
-	18				
-	21				
Sample 6	24	0.12	0.17	-0.13	-
Sample 7	27	0.13	0.13	0.05	0.36
Sample 8	30	0.14	0.12	-0.03	-0.38
Sample 9	33	0.14	0.13	-0.14	-0.29
Sample 10	36	0.16	0.12	-0.21	-0.99
Sample 11	39	0.19	0.13	0.20	-0.24
-	42				
-	45				
Sample 12	48	0.19	0.12	0.06	-0.54
Sample 13	51	0.21	0.11	0.31	-0.80
Sample 14	54	0.28	0.14	0.00	-0.75
Sample 15	57	0.17	0.17	0.40	-0.61
Sample 16	60	0.34	0.15	0.34	-0.71
Sample 17	63	0.37	0.16	1.09	0.01
-	66				
-	69				
Sample 18	72	0.40	0.20	1.26	0.34
Sample 19	75	0.45	0.18	1.56	-0.26
Sample 20	78	0.53	0.20	1.07	-0.32
Sample 21	81	0.57	0.20	1.06	-1.31
Sample 22	84	0.55	0.22	1.48	-0.69
Sample 23	87	0.52	0.20	0.56	-0.47
-	90				
-	93				
Sample 24	96	0.62	0.24	0.72	-1.05
Sample 25	99	0.64	0.25	0.74	-0.22
Sample 26	102	0.73	0.27	0.74	-0.34

Table 7.1b Volunteer 1: urinary concentrations ratios and $\Delta\delta^{13}C$ values after the administration of finasteride of each 5α-, 5β- testosterone metabolites pair

	Time (h)	A/Etio	5αAdiol/5βAdiol	$\Delta\delta_{(A\text{-}Etio)}$	$\Delta\delta_{(5\alpha Adiol\text{-}5\beta Adiol)}$
Blank Sample	0	2.07	0.82	1.72	0.45
19-norandrostenedione 10 mg					
Sample 1	3	2.24	0.84	2.25	-
Sample 2	6	2.38	0.77	1.78	0.79
Sample 3	9	2.42	0.65	1.74	0.02
Sample 4	12	2.04	0.86	1.78	0.11
-	15				
-	18				
Sample 5	21	2.01	0.73	1.50	0.41
Sample 6	24	1.84	0.63	1.69	0.22
Sample 7	27	1.93	0.72	1.92	-
Sample 8	30	2.07	0.75	1.94	0.37
Sample 9	33	2.07	0.84	2.22	-
Sample 10	36	1.93	0.90	2.05	0.52
-	39				
-	42				
Sample 11	45	1.72	0.72	1.58	0.27
Sample 12	48	2.15	0.64	1.57	0.57

Table 7.2a Volunteer 2: urinary concentrations ratios and $\Delta\delta^{13}C$ values before the administration of finasteride of each 5α-, 5-β- testosterone metabolites pair

	Time (h)	A/Etio	5αAdiol/5βAdiol	$\Delta\delta_{(A\text{-}Etio)}$	$\Delta\delta_{(5\alpha Adiol\text{-}5\beta Adiol)}$
finasteride 1 mg (one tablet for three sequential days)					
Sample 1	3*	0.44	0.74	1.80	0.35
19-norandrostenedione 10 mg					
Sample 2	6	0.23	0.10	-0.03	0.06
Sample 3	9	0.22	0.10	0.44	-
Sample 4	12	0.21	0.11	0.66	-
Sample 5	15	0.19	0.10	0.68	-0.58
-	18				
-	21				
Sample 6	24	0.26	0.10	0.31	-0.45
Sample 7	27	0.26	0.10	0.28	-0.60
Sample 8	30	0.28	0.14	0.50	-0.41
Sample 9	33	0.36	0.17	0.70	-0.37
Sample 10	36	0.32	0.16	1.00	-0.92
Sample 11	39	0.32	0.16	0.43	-0.25
-	42				
-	45				
Sample 12	48	0.36	0.15	0.89	-0.48
Sample 13	51	0.39	0.16	-	-

Table 7.2b Volunteer 2: urinary concentrations ratios and $\Delta\delta^{13}C$ values after the administration of finasteride of each 5α-, 5β- testosterone metabolites pair

*Sample collected 3 hours after the intake of the last tablet of finasteride

No data were reported if any analytical issues occurred in the determination of $\delta^{13}C$ value of one or both the TC considered

Similar concentrations trend has been observed in the third study (V3), even if with a greater extent. V3 showed unusual baseline steroid profile, in which both Etio and 5βAdiol have been preferentially produced: it may be attributed to the previous dutasteride therapy to which he was submitted up to six months before the current analyses. The excretion of A and 5αAdiol has been consistently dropped to reach values about ten times less than the starting ones (see Figure 7.3a-b and Table 7.3a-b). A/Etio and 5αAdiol/5βAdiol ratios at the beginning and at the end of the urine collection period were lower compared to studies on V1 and V2.

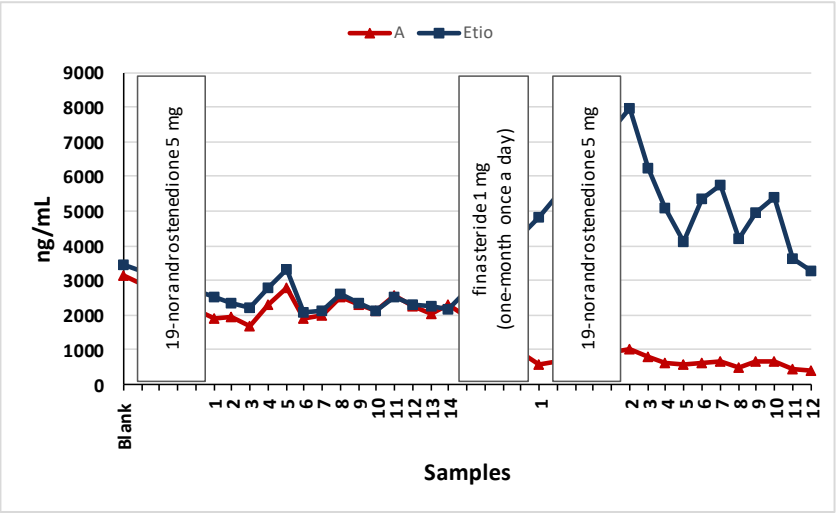


Figure 7.3a Volunteer 3: urinary concentrations trend of A and Etio

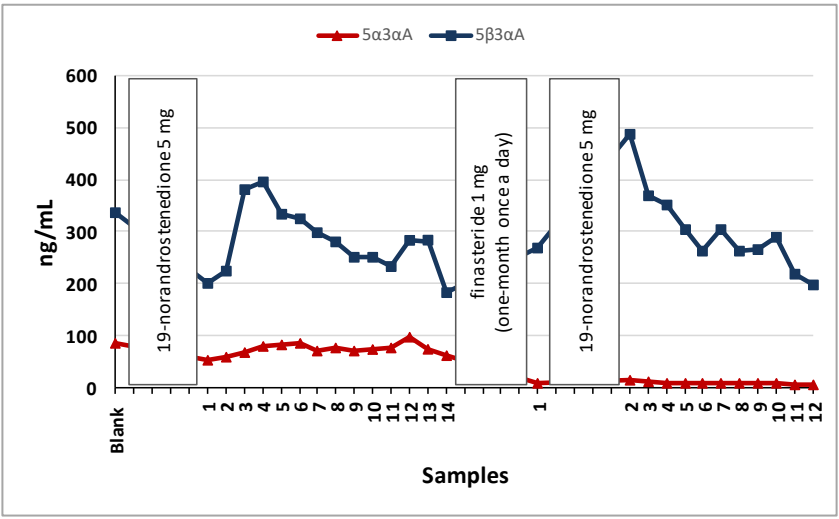


Figure 7.3b Volunteer 3: urinary concentrations trend of 5αAdiol and 5βAdiol

	Time (h)	A/Etio	5αAdiol/5βAdiol	$\Delta\delta_{(A\text{-}Etio)}$	$\Delta\delta_{(5\alpha Adiol\text{-}5\beta Adiol)}$
Blank Sample	0	0.92	0.25	0.91	0.20
19-norandrostenedione 5 mg					
Sample 1	5	0.76	0.26	0.74	0.08
Sample 2	10	0.83	0.26	0.58	-0.05
Sample 3	15	0.75	0.18	0.51	-0.42
-	20				
Sample 4	25	0.83	0.20	0.75	0.22
Sample 5	30	0.84	0.25	1.15	-0.39
Sample 6	35	0.92	0.26	1.07	-0.25
-	40				
Sample 7	45	0.94	0.24	1.02	-0.24
Sample 8	50	0.96	0.27	1.24	-0.45
-	55				
Sample 9	60	0.97	0.28	1.34	-0.30
-	65				
Sample 10	70	0.99	0.29	0.78	-0.21
Sample 11	75	1.02	0.33	0.77	-0.22
Sample 12	80	0.98	0.34	0.84	0.02
-	85				
-	90				
Sample 13	95	0.90	0.26	1.43	-0.29
Sample 14	100	1.07	0.33	0.94	-0.75

Table 7.3a Volunteer 3: urinary concentrations ratios and $\Delta\delta^{13}C$ values before the administration of finasteride of each 5α-, 5β- testosterone metabolites pair

	Time (h)	A/Etio	5αAdiol/5βAdiol	$\Delta\delta_{(A\text{-}Etio)}$	$\Delta\delta_{(5\alpha Adiol\text{-}5\beta Adiol)}$
finasteride 1 mg (one-month therapy)					
Sample 1	5*	0.12	0.03	0.00	-0.99
19-norandrostenedione 5 mg					
Sample 2	10	0.13	0.03	-0.26	-0.99
Sample 3	15	0.13	0.03	-0.24	-0.97
Sample 4	20	0.12	0.03	0.03	-1.03
Sample 5	25	0.14	0.02	-0.14	-0.96
Sample 6	30	0.11	0.03	0.18	-0.38
Sample 7	35	0.11	0.03	-0.11	-0.47
Sample 8	40	0.12	0.04	0.21	-0.84
Sample 9	45	0.13	0.04	-0.24	-0.78
Sample 10	50	0.12	0.03	-0.36	-1.04
Sample 11	55	0.11	0.03	0.06	-0.41
Sample 12	60	0.12	0.03	0.15	-0.61

Table 7.3b Volunteer 3: urinary concentrations ratios and $\Delta\delta^{13}C$ values after the administration of finasteride of each 5α-, 5β- testosterone metabolites pair

*Sample collected 5 hours after the intake of the last tablet of finasteride

7.4.2 Influence of finasteride on testosterone 5α- and 5β-metabolites $\delta^{13}C$ values

The baseline isotopic profile of each volunteer was determined by analyzing a set of urine samples collected before the intake of finasteride, according to the scheme reported in Tables 7.1a, 7.2a and 7.3a. The $\delta^{13}C$ average value ± 3 standard deviations (μ ± 3 SD) was calculated for all the compounds of interest to define the individual reference range and therefore the maximum $\delta^{13}C$ values variation accepted, following the approach already proposed by our Laboratory for the longitudinal evaluation of the isotope ratio mass spectrometric data[32].

Figure 7.4a-b shows the $\delta^{13}C$ values trend obtained from V1. $\delta^{13}C$ values of each pair of metabolites were mostly overlapping among them; Etio had slightly more depleted ^{13}C content than A. In samples collected after the administration of finasteride, the individual fluctuations of A and 5αAdiol did not significantly differ from that seen before, while those of Etio and, mainly, 5βAdiol reached less negative $\delta^{13}C$ values compared to their starting measurements. In samples collected 30 to 102 hours after taking the 5-ARI (from sample 8 to sample 26), $\Delta\delta_{(5\alpha Adiol-\ 5\beta Adiol)}$ values were found to be all negative (except for sample 18), to prove the reversal of trend in 5αAdiol and 5βAdiol ^{13}C abundance (see Table 7.1a-b).

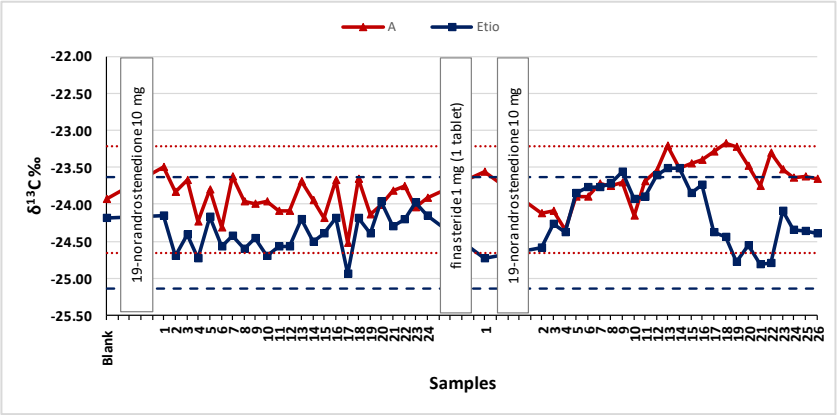


Figure 7.4a Volunteer 1: $\delta^{13}C$ values trend of A and Etio

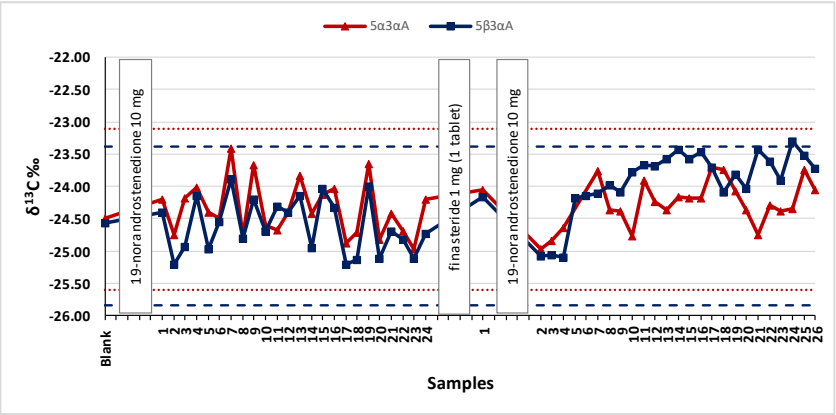


Figure 7.4b Volunteer 1: $\delta^{13}C$ values trend of 5αAdiol and 5βAdiol

$\delta^{13}C$ values resulting from the excretion study on V2 is reported in Figure 7.5a-b. V2 experienced a clear change of the A and 5αAdiol trend towards more negative $\delta^{13}C$ values. The administration of finasteride caused an approximation and even an inversion of $\delta^{13}C$ values of A and Etio and 5αAdiol and 5βAdiol respectively, as can be deduced from their corresponding $\Delta\delta^{13}C$ values (see Table 7.2a-b). Compared to V1, the effect of finasteride in V2 was more noticeable, due to the different individual baseline profile.

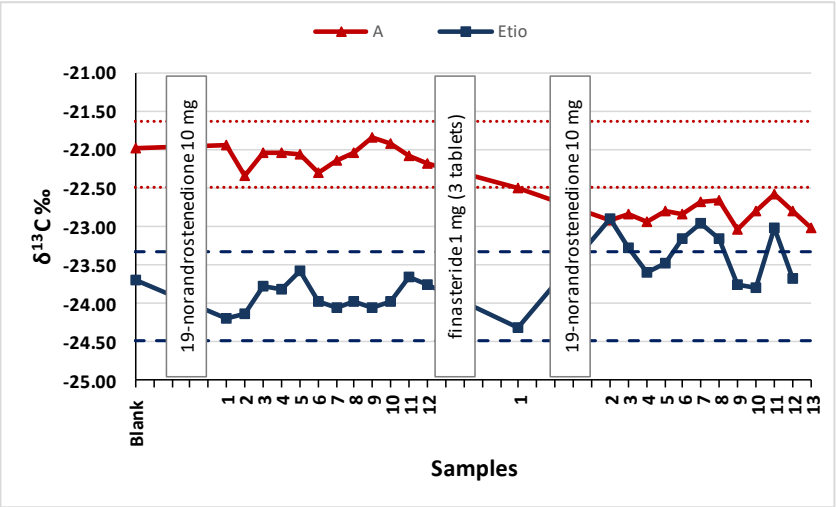


Figure 7.5a Volunteer 2: $\delta^{13}C$ values trend of A and Etio

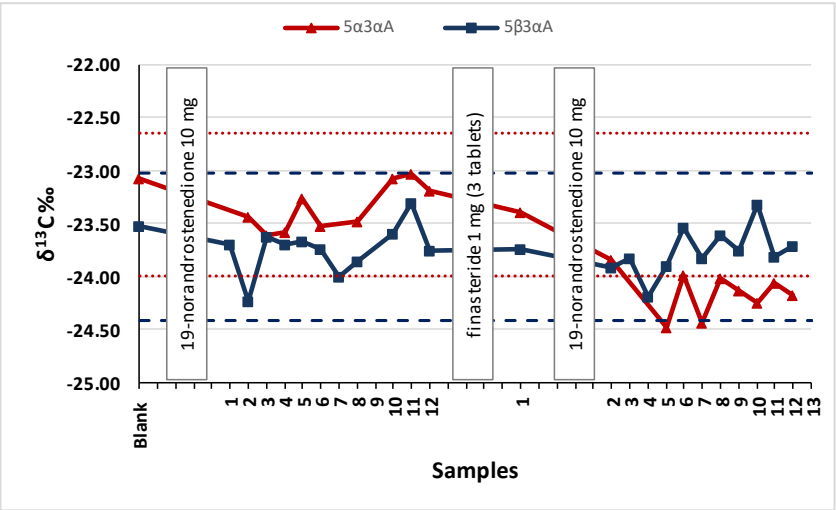


Figure 7.5b Volunteer 2: $\delta^{13}C$ values trend of 5αAdiol and 5βAdiol

Data obtained from urine samples collected by V3 were depicted in Figure 7.6a-b: before the long-term therapy with finasteride, Etio showed $\delta^{13}C$ values more negative than A, whereas 5αAdiol and 5βAdiol displayed analogous $\delta^{13}C$ values range between them. After one month of treatment, A and Etio $\delta^{13}C$ values were mostly overlapped; 5βAdiol has stabilized on less negative $\delta^{13}C$ values compared to 5αAdiol. Their $\Delta\delta^{13}C$ values have changed accordingly (see Table 7.3a-b).

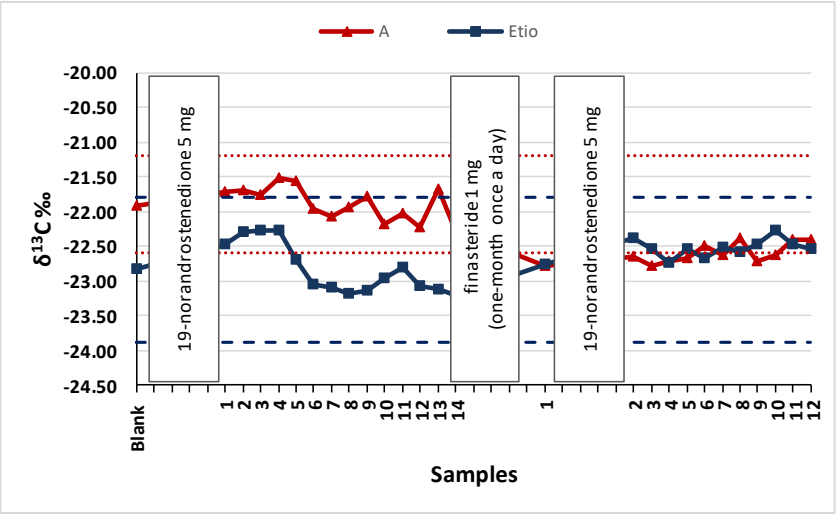


Figure 7.6a Volunteer 3: $\delta^{13}C$ values trend of A and Etio

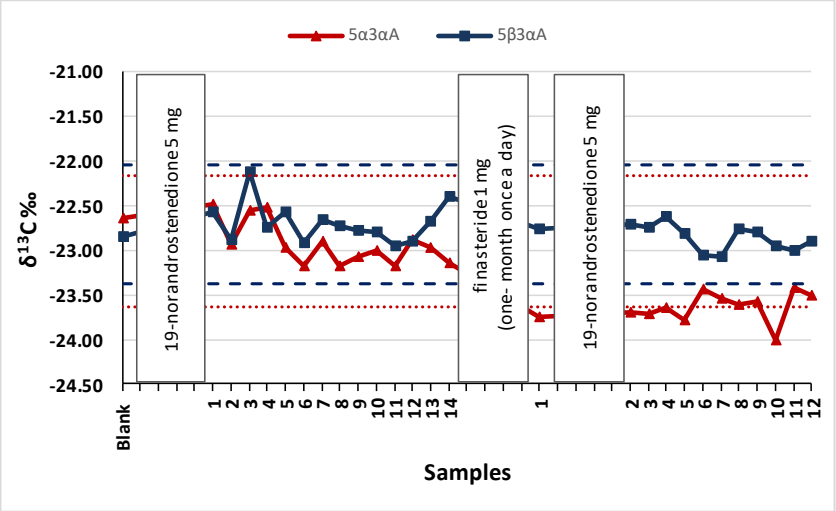


Figure 7.6b Volunteer 3: $\delta^{13}C$ values trend of 5αAdiol and 5βAdiol

Visible deviations from the baseline isotopic profile have been detected in all the studies analyzed. Several $\delta^{13}C$ values outside the limits of the individual reference range (calculated for both the 5α- and 5β-metabolites as μ ± 3SD and defined by dotted lines, red or blue respectively, in the graphs above) have been measured after multiple dosage.

7.4.3 Influence of finasteride on 19-NA and 19-NE urinary concentrations

The excretion profile of 19-NA and 19-NE after the intake of 5 or 10 mg of 19-norandrostenedione has been already analyzed[37](Tables 7.4a, 7.5a, 7.6a). 19-NA was the main produced metabolite throughout the study, even if the urinary levels of 19-NE exceeded those of 19-NA in the late excretion phase of volunteer 3 (V3) periodically subjected to chronic dutasteride therapy (as shown in Table 7.6a). Once verified by GC-MS/MS that no traces of both metabolites were detectable, V1, V2 and V3 were treated again with one tablet of 19-norandrostenedione (5 or 10 mg) after dosing finasteride at 1 mg via different administration schedule.

Data obtained from samples collected by V1 are reported In Table 7.4b. 19-NE urinary levels overcame those of 19-NA for a period of 51 hours (19-NA/19-NE < 1).

Then, the ratio 19-NA/19-NE has settled to values greater than 1, but lower than those measured when no finasteride has taken. The administration of multiple doses of finasteride (V2) affected the TC trends in a shorter time window (see Table 7.5b). 19-NE peaked the maximum urinary concentration after 3 hours from the intake of 19-norandrstenedione and remained the main excreted metabolite for 30 hours. In the late excretion phase, urinary levels of 19-NA and 19-NE approximately balanced each other; after 48 hours both 19-NA and 19-NE were no longer detectable. Under a chronic treatment (one-month, one tablet a day) with finasteride (V3), the excretion of 19-NA was suppressed in a more meaningful and relevant way. As reported in Table 7.6b, the typical ratio between 19-NA and 19-NE (in which 19-NA > 19-NE) was not restored throughout the study, not even in the late excretion phase (55 hours following the administration of 5 mg of 19-norandrostenedione).

	Time (h)	19-NA	19-NE	19-NA/19-NE
Blank sample	0	-	-	-
19-norandrostenedione 10 mg				
Sample 1	3	4965	1098	4.52
Sample 2	6	5194	775	6.70
Sample 3	9	1409	237	5.93
Sample 4	12	229	44.2	5.18
Sample 5	15	88.8	22.0	4.03
-	18			
-	21			
Sample 6	24	71.1	12.1	5.89
Sample 7	27	52.7	8.97	5.87
Sample 8	30	40.9	5.36	7.62
Sample 9	33	36.7	3.43	10.7
Sample 10	36	16.9	1.92	8.8
Sample 11	39	33.0	2.85	11.6
-	42			
-	45			
Sample 12	48	12.1	1.23	9.88
Sample 13	51	19.3	1.97	9.81
Sample 14	54	24.5	1.99	12.3
Sample 15	57	48.8	3.71	13.2
Sample 16	60	16.8	-	-
Sample 17	63	6.07	-	-
-	66			
-	69			
Sample 18	72	6.88	-	-
Sample 19	75	8.20	-	-
-	78			
Sample 20	81	11.6	-	-
-	84			
Sample 21	87	5.27	-	-
Sample 22	90	4.83	-	-
Sample 23	93	2.30	-	-
Sample 24	96	-	-	-

Table 7.4a Volunteer 1: 19-NA and 19-NE urinary levels and their corresponding ratios before the administration of finasteride

	Time (h)	19-NA	19-NE	19-NA/19-NE
finasteride 1 mg (one tablet)				
Sample 1	3	-	-	-
19-norandrostenedione 10 mg				
Sample 2	6	3166	20094	0.16
Sample 3	9	514	2371	0.22
Sample 4	12	147	837	0.18
Sample 5	15	39.8	182	0.22
-	18			
-	21			
Sample 6	24	20.0	79.3	0.25
Sample 7	27	27.4	83.7	0.33
Sample 8	30	29.4	56.9	0.52
Sample 9	33	32.7	51.8	0.63
Sample 10	36	27.7	39.1	0.71
Sample 11	39	21.1	29.0	0.73
-	42			
-	45			
Sample 12	48	15.5	20.9	0.74
Sample 13	51	15.4	16.9	0.91
Sample 14	54	13.9	12.4	1.12
Sample 15	57	14.6	14.0	1.05
Sample 16	60	12.4	13.6	0.91
Sample 17	63	7.0	6.82	1.03
-	66			
-	69			
Sample 18	72	5.76	4.53	1.27
Sample 19	75	5.54	3.45	1.61
Sample 20	78	6.52	3.61	1.80
Sample 21	81	7.12	3.65	1.95
Sample 22	84	2.96	-	-
Sample 23	87	3.70	-	-
-	90			
-	93			
Sample 24	96	3.94	-	-
Sample 25	99	3.29	-	-
Sample 26	102	2.45	-	-

Table 7.4b Volunteer 1: 19-NA and 19-NE urinary levels and their corresponding ratios after the administration of finasteride

	Time (h)	19-NA	19-NE	19-NA/19-NE
Blank Sample	0	-	-	-
19-norandrostenedione 10 mg				
Sample 1	3	7010	3094	2.27
Sample 2	6	610	144	4.24
Sample 3	9	501	227	2.21
Sample 4	12	404	232	1.74
-	15			
-	18			
Sample 5	21	173	82.5	2.09
Sample 6	24	34.9	13.0	2.68
Sample 7	27	7.24	2.55	2.84
Sample 8	30	9.26	3.43	2.70
Sample 9	33	3.62	1.26	2.88
Sample 10	36	10.4	2.29	4.54
-	39			
-	42			
Sample 11	45	11.2	3.10	3.62
Sample 12	48	1.29	-	-

Table 7.5a Volunteer 2: 19-NA and 19-NE urinary levels and their corresponding ratios before the administration of finasteride

	Time (h)	19-NA	19-NE	19-NA/19-NE
finasteride 1 mg (one tablet for three sequential days)				
Sample 1	3*	-	-	-
19-norandrostenedione 10 mg				
Sample 2	6	2747	7411	0.37
Sample 3	9	878	1587	0.55
Sample 4	12	199	287	0.70
Sample 5	15	47.9	155	0.31
-	18			
-	21			
Sample 6	24	30.1	83.4	0.36
Sample 7	27	6.72	16.6	0.41
Sample 8	30	4.14	8.11	0.51
Sample 9	33	2.00	2.05	0.97
Sample 10	36	1.52	1.46	1.04
Sample 11	39	2.57	2.35	1.09
-	42			
-	45			
Sample 12	48	3.38	2.56	1.32
Sample 13	51	-	-	-

Table 7.5b Volunteer 2: 19-NA and 19-NE urinary levels and their corresponding ratios after the administration of finasteride

*Sample collected 3 hours after the intake of the last tablet of finasteride

	Time (h)	19-NA	19-NE	19-NA/19-NE
Blank Sample	0	-	-	-
19-norandrostenedione 5 mg				
Sample 1	5	4666	2642	1.77
Sample 2	10	1462	528	2.77
Sample 3	15	82.5	79.0	1.04
-	20			
Sample 4	25	49.8	73.9	0.67
Sample 5	30	23.5	60.7	0.39
Sample 6	35	14.4	31.6	0.46
-	40			
Sample 7	45	14.3	25.3	0.56
Sample 8	50	9.31	8.27	1.13
-	55			
Sample 9	60	5.48	3.15	1.74
-	65			
Sample 10	70	3.00	-	-
Sample 11	75	2.62	-	-
Sample 12	80	2.93	-	-
-	85			
-	90			
Sample 13	95	1.91	-	-
Sample 14	100	1.03	-	-

Table 7.6a Volunteer 3: 19-NA and 19-NE urinary levels and their corresponding ratios before the administration of finasteride

	Time (h)	19-NA	19-NE	19-NA/19-NE
finasteride 1 mg (one-month therapy)				
Sample 1	5*	-	-	-
19-norandrostenedione 5 mg				
Sample 2	10	4298	19275	0.22
Sample 3	15	2831	10520	0.27
Sample 4	20	275	1046	0.26
Sample 5	25	35.7	137	0.26
Sample 6	30	10.5	34.7	0.30
Sample 7	35	8.25	20.7	0.40
Sample 8	40	4.37	10.0	0.44
Sample 9	45	4.96	12.7	0.39
Sample 10	50	3.12	7.81	0.40
Sample 11	55	1.18	2.50	0.47
Sample 12	60	-	-	-

Table 7.6b Volunteer 3: 19-NA and 19-NE urinary levels and their corresponding ratios after the administration of finasteride

*Sample collected 5 hours after the intake of the last tablet of finasteride

Values not available in the Tables above, refer to samples in which 19-NA and 19-NE were not present (sample 1) or < LOQ (1 ng/mL or 2.50 respectively) of the GC-MS/MS method.

7.4.4 Influence of finasteride on 19-NA and 19-NE $\delta^{13}C$ values

$\delta^{13}C$ values of both 19-NA and 19-NE (if > 2.0 ng/mL, LOQ of the GC-C-IRMS procedure) after the combined administration of finasteride and 19-norandorstenedione (-29.70 ± 0.30 ‰)[35] were determined according to the previous proposed method and compared to those obtained after the sole oral intake of the nandrolone precursor[37]. Carbon isotopic composition of PD and PT were measured to define the endogenous isotopic profile of each volunteer and to evaluate if the resulting $\Delta\delta_{(ERC-TC)}$ values were influenced or not by the 5-ARIs. Androsterone has not been considered as ERC, since, as evidenced before, its $\delta^{13}C$ values could be affected by the administration of finasteride.

$\delta^{13}C$ values of 19-NA and 19-NE determined for V1 and V2 were comparable (Figures 7.7 and 7.8): they were mostly overlapped without finasteride and moved to fewer negative values with finasteride. In the late excretion phase of V1, when 19-NE showed a more ^{13}C depleted composition against 19-NA, a slight reversal of the initial trend was achieved.

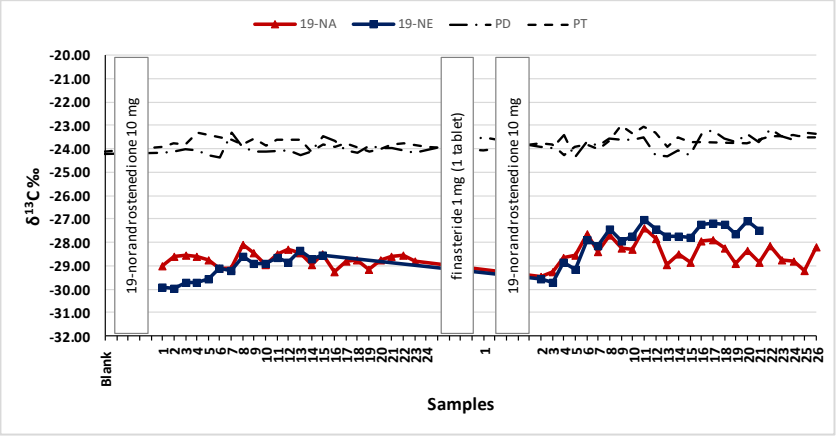


Figure 7.7 GC-C-IRMS confirmation analysis for detecting the 19-norsteroids abuse: TC and ERC $\delta^{13}C$ values of V1

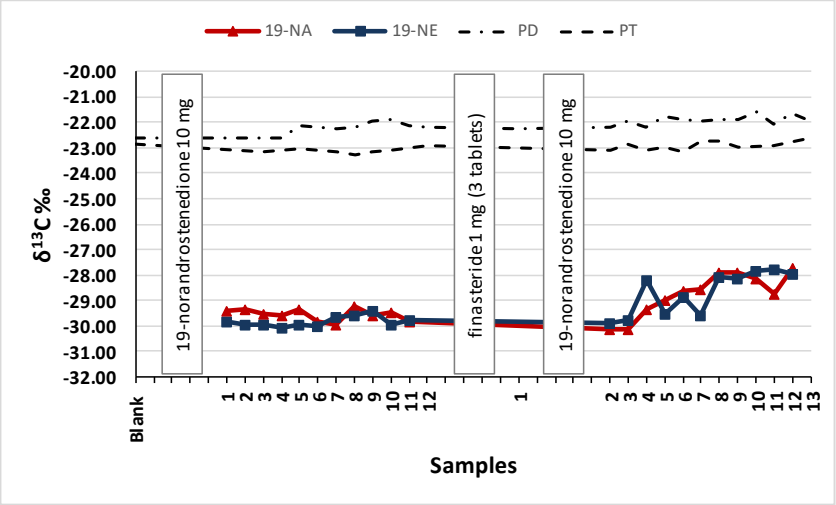


Figure 7.8 GC-C-IRMS confirmation analysis for detecting the 19-norsteroids abuse: TC and ERC $\delta^{13}C$ values of V2

Data obtained from V3 is depicted in Figure 7.9 and revealed that the administration of finasteride caused a visible carbon isotopic fractionation of both TC towards less negative $\delta^{13}C$ values. 19-NE was influenced by 5-ARIs with a greater extent compared to its 5α-counterpart: it displayed a peculiar trend non only after the administration of finasteride, but also before, due to the potential effect of dutasteride-based therapy stopped six months earlier.

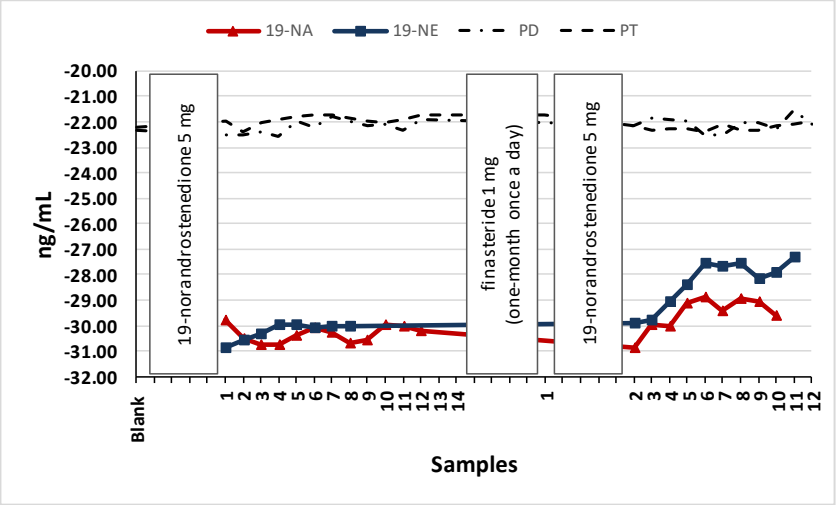


Figure 7.9 GC-C-IRMS confirmation analysis for detecting the 19-norsteroids abuse: TC and ERC $\delta^{13}C$ values of V3

In all the samples collected by the three selected Caucasian male volunteers, the $\Delta\delta_{(ERC-TC)}$ for each ERC-TC pair, remained > 3 ‰, the limit required by WADA for reporting an adverse analytical findings in the GC-C-IRMS confirmation procedure of 19-norsteroids abuse.

7.5 Conclusions

In this work we have evaluated the effect of 5-ARIs on both the urinary levels and the carbon isotopic composition of testosterone and nandrolone 5α- and 5β-metabolites commonly detected in the screening and confirmation doping control analyses. The results herein presented specifically refer to finasteride, but it may be easily extended to dutasteride, the other commercially available 5-ARI showing more powerful and lasting action. We confirmed that the excretion profile is influenced by finasteride (already in acute dosage), supporting the need to monitor the 5-ARIs as confounding factors in defining the athlete steroid module. Moreover, we proved that a treatment with finasteride, especially if chronic, could lead the $\delta^{13}C$ values of the selected TC outside the individual isotopic range. If a longitudinal evaluation were to be required by WADA as additional module to the Athlete

Biological Passport (ABP), the use of 5-ARIs should be confirmed or excluded to avoid any misinterpretation of the IRMS outcomes.

Data from the combined administration of finasteride and 19-norandrostenedione displayed that the suppressive effect of 5-ARIs on 19-NA should be not overlooked in detecting the 19-norsteroids abuse. 19-NE urinary levels are currently considered only when results of the IRMS analysis inconclusive or consistent with an endogenous origin of 19-NA have been obtained. However, we showed that in case of chronic therapy with finasteride, 19-NE is found in urine as the main metabolite until the late excretion phase. The alternative determination of 19-NE would be helpful both in screening and confirmation procedures to avoid inconclusive and false negative results when the measurement of 19-NA is prevented by the impact of 5-ARI on the enzymatic pathway of nandrolone. For this purpose, the application of the new GC-C-IRMS method involving both 19-NA and 19-NE as target compounds became crucial[37].

In this study we also established that the use of finasteride, especially if long-lasting, may influence the carbon isotopic composition of the nandrolone metabolites: indeed, in the late excretion phases, 19-NA and 19-NE reached less depleted ^{13}C content. Their $\delta^{13}C$ values were still distinguishable from those measured for the ERC in all the samples collected from our Caucasian volunteers, but they could not ensure a $\Delta\delta_{(ERC-TC)} > 3$ in case of Nordic population showing different endogenous range reference values[43].

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nandrolone, boldenone, and testosterone preparations seized in Norway compared to those of endogenously produced steroids in a Nordic reference population. *Drug Test Anal*. 2014;6:1163-1169.

General conclusions

Conclusions

The pseudo-endogenous steroids are substances widely abused by athletes due to their anabolic and myotrophic effects. They share the same chemical structure and functions of the androgenic steroid hormones produced in the body, by which they differ for a more ^{13}C depleted content. The pharmaceutically produced compounds are typically synthetized from raw materials (phytosterols and sapogenins) derived from C3 plants showing a $\delta^{13}C$ range from 24 to 32 ‰. The carbon isotopic composition of endogenous steroids arises from the individual eating habits Instead: specific endogenous reference range (between-16 to -26 ‰) has been identified in the worldwide population according to a C3 or C4 more enriched diet.

The GC-C-IRMS is the tool used in doping control analyses to disclose the endogenous from the synthetic origin of urinary steroids and then to detect the abuse of pseudo-endogenous steroids. It is a highly sensitive technique but requires laborious and time-consuming samples pre-treatment procedures.

Novel approaches have been here implemented in order to comply with the new requirements of the World Anti-doping Agency (WADA), to simplify, standardize the current operating protocols, and reduce the risk of misinterpreting the analytical findings.
More specifically:

I. the benefits of performing a large volume injection by a programmed temperature vaporizer inlet instead of the traditional splitless injection mode at constant temperature have been proved in analyzing steroids, showing low urinary levels reliable data (nandrolone metabolites, boldenone and its metabolite and prednisolone and prednisone);
II. a method to disclose the endogenous or *ex vivo* production of prednisolone and prednisone has been developed and fully validated: a LOQ of 20 ng/mL (< WADA reporting level, 30 ng/mL) for both the target compounds has been guaranteed;
III. the current method for the detection of the abuse of 19-norsteroids has been further optimized, improving the purification step of 19-norandrosterone (19-NA) and selecting 19-norethiocolanolone (19-NE) as additional target compound. The proposed protocol could be useful in several scenarios, in which the $\delta^{13}C$ determination of the primary

nandrolone metabolite is prevented. The LOQ of 2 ng/mL has been established for both 19-NA and 19-NE.

As a proof of concept, the new GC-C-IRMS methods herein presented have been applied to real cases, emphasizing some weakness of the IRMS technique and of the current WADA criteria:

i. the GC-C-IRMS analysis could be no longer fully adequate as confirmatory procedure if not combined with a detailed qualitative and quantitative characterization of other diagnostic markers when pharmaceutical formulations with $\delta^{13}C$ values not distinguishable from the endogenous $\delta^{13}C$ values range have been administered;
ii. a more adequate reporting level would be defined to reduce the risk of false positive results caused by the administration of glucocorticoids by not banned route (nasal);
iii. the high stability of $\delta^{13}C$ values of the individual isotopic profile could be affected by exogenous factors (as the intake of 5α-reductase inhibitors) interfering with the androgenic steroids metabolic pathway;
iv. the 5α-reductase inhibitors could induce a carbon isotopic fractionation in the metabolism of nandrolone and its precursors, potentially masking their misuse.

This thesis has yielded a detailed overview of the GC-C-IRMS technique in anti-doping analysis, stressing its strong points as well as its drawbacks and proposing, whenever possible, suitable troubleshooting.

Annex I

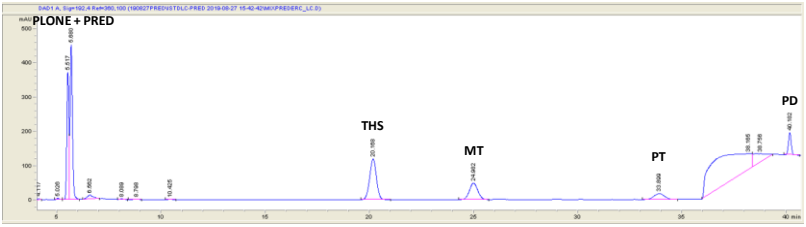


Figure A.1: 1st HPLC chromatogram of TC (PLONE, PRED) and ERC (THS, PT and PD) in the pre-treatment procedure to detect prednisolone and prednisone abuse
MT is the internal standard

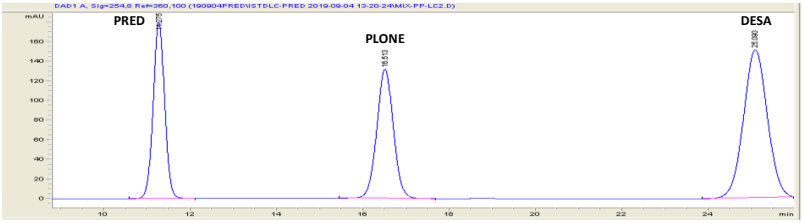


Figure A.2: 2nd HPLC chromatogram of TC (PRED, PLONE) in the pre-treatment procedure to detect prednisolone and prednisone abuse
DESA is the internal standard

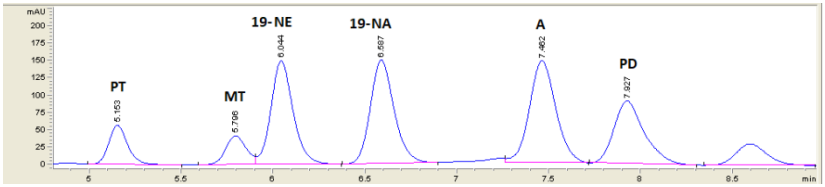


Figure A.3: 1st HPLC chromatogram of TC (19-NE and 19-NA) and ERC (PT, A and PD) in the new proposed pre-treatment procedure to detect 19-norsteroids abuse
MT is the internal standard

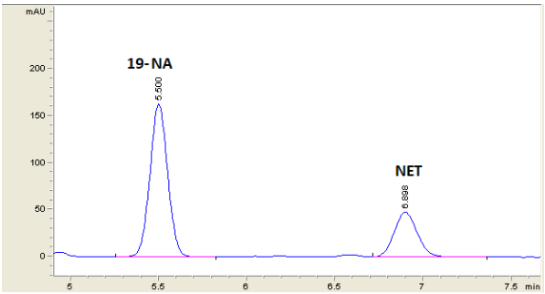


Figure A.4: 2nd HPLC chromatogram of 19-NA
in the new proposed pre-treatment procedure to detect 19-norsteroids abuse
NET is the internal standard

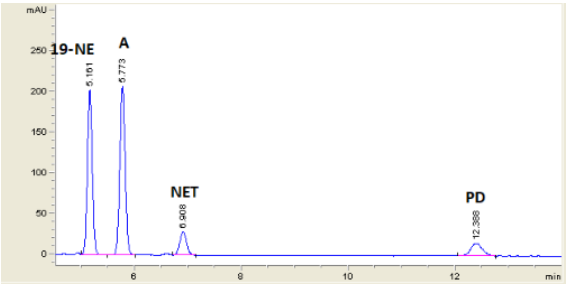
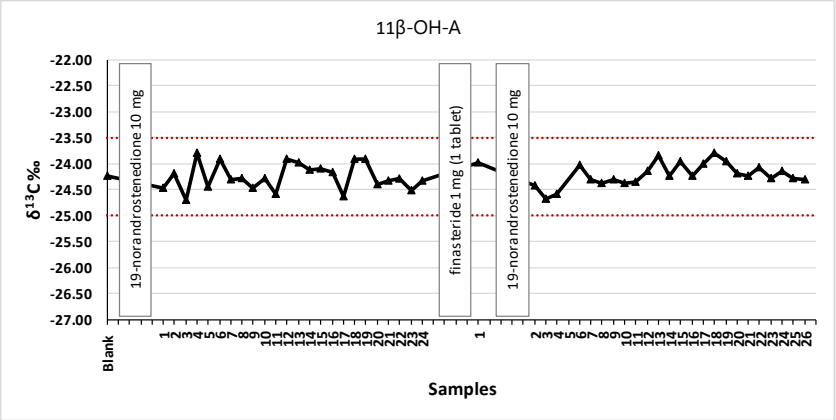
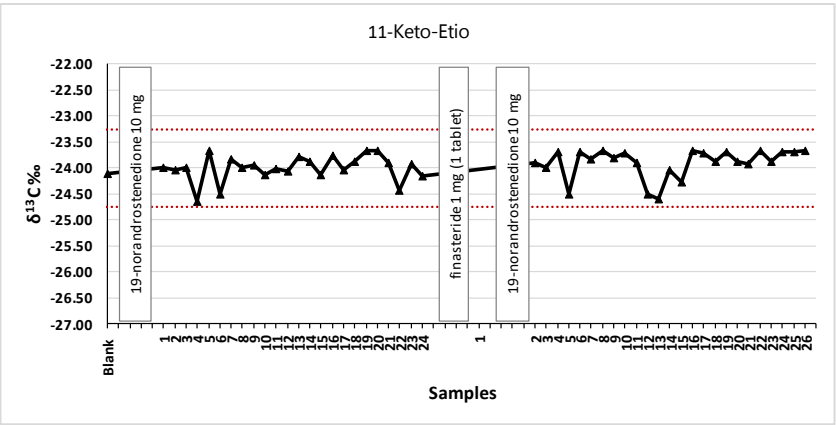
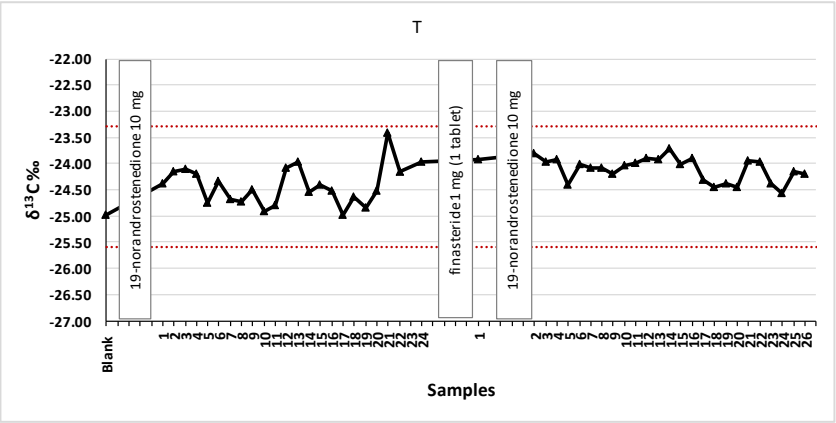
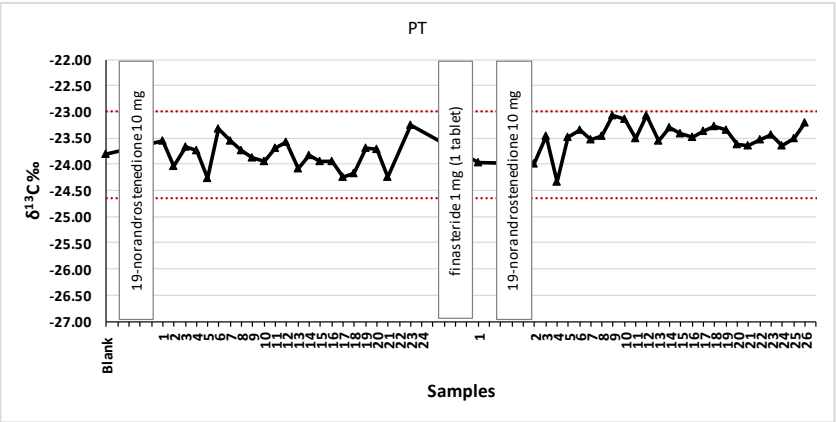


Figure A.5: 2nd HPLC chromatogram of 19-NE, A and PD
in the new proposed pre-treatment procedure to detect 19-norsteroids abuse
NET is the internal standard


11β-OH-A
δ13C‰
-22.00
-22.50
-23.00
-23.50
-24.00
-24.50
-25.00
-25.50
-26.00
-26.50
-27.00
19-norandrostenedione 10 mg
finasteride 1 mg (1 tablet)
19-norandrostenedione 10 mg
Blank
Samples


11-Keto-Etio
δ13C‰
-22.00
-22.50
-23.00
-23.50
-24.00
-24.50
-25.00
-25.50
-26.00
-26.50
-27.00
19-norandrostenedione 10 mg
finasteride 1 mg (1 tablet)
19-norandrostenedione 10 mg
Blank
Samples


T
δ13C‰
-22.00
-22.50
-23.00
-23.50
-24.00
-24.50
-25.00
-25.50
-26.00
-26.50
-27.00
19-norandrostenedione 10 mg
finasteride 1 mg (1 tablet)
19-norandrostenedione 10 mg
Blank
Samples


PT
δ13C‰
-22.00
-22.50
-23.00
-23.50
-24.00
-24.50
-25.00
-25.50
-26.00
-26.50
-27.00
19-norandrostenedione 10 mg
finasteride 1 mg (1 tablet)
19-norandrostenedione 10 mg
Blank
Samples

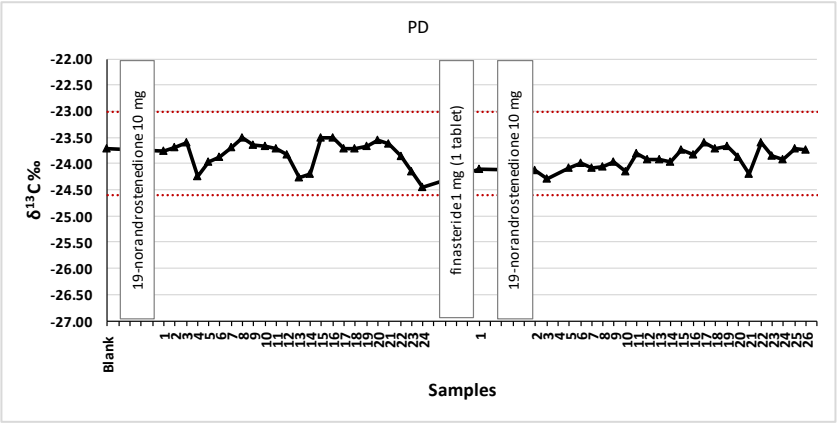
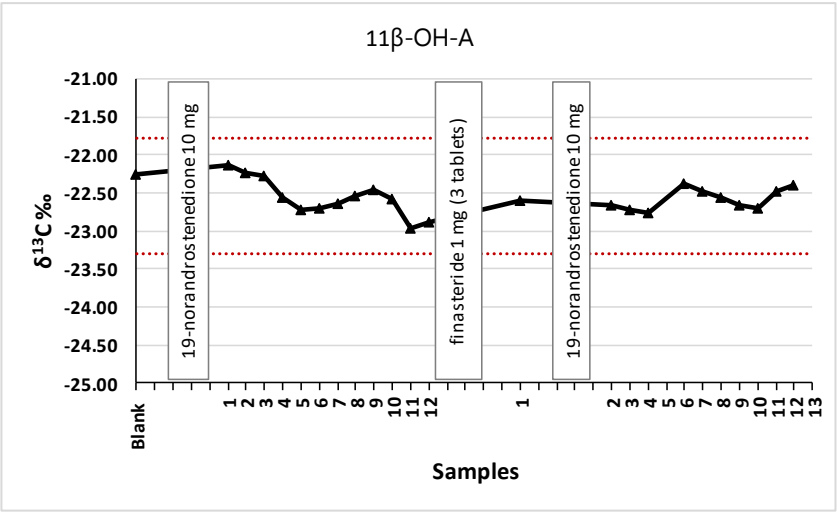
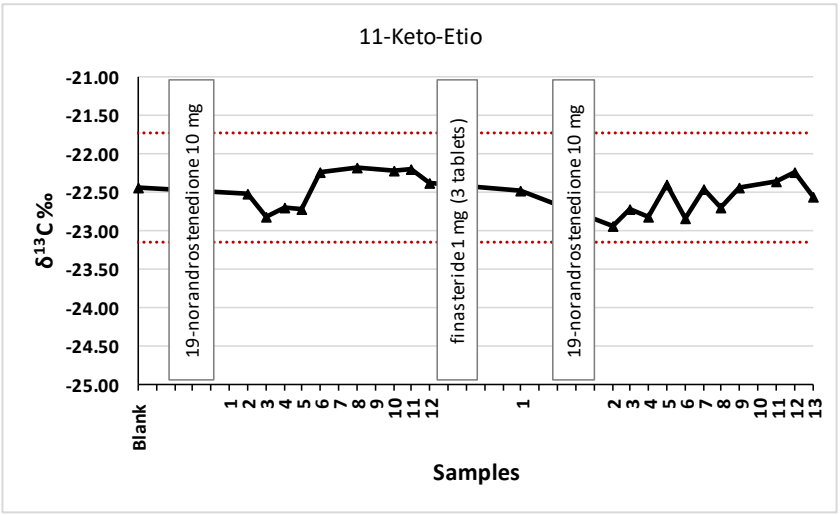
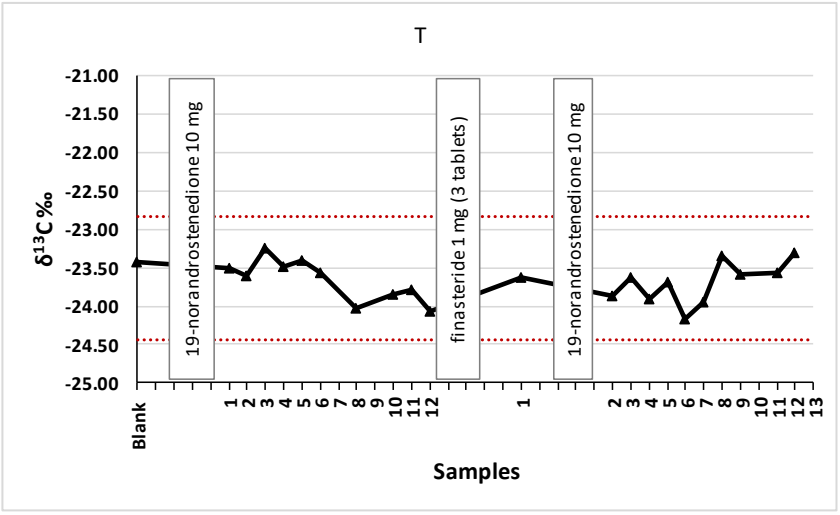


Figure A.6: Volunteer 1: carbon isotopic composition of other compounds (11-β-OH-A; 11-Keto-Etio; T; PT and PD) commonly determined to detect the abuse of pseudo-endogenous steroids
Dotted lines define the individual reference range (μ ± 3SD)




11-Keto-Etio
δ13C ‰
-21.00
-21.50
-22.00
-22.50
-23.00
-23.50
-24.00
-24.50
-25.00
19-norandrostenedione 10 mg
finasteride 1 mg (3 tablets)
19-norandrostenedione 10 mg
Blank
1 2 3 4 5 6 7 8 9 10 11 12
1
2 3 4 5 6 7 8 9 10 11 12 13
Samples


T
δ13C ‰
-21.00
-21.50
-22.00
-22.50
-23.00
-23.50
-24.00
-24.50
-25.00
19-norandrostenedione 10 mg
finasteride 1 mg (3 tablets)
19-norandrostenedione 10 mg
Blank
1 2 3 4 5 6 7 8 9 10 11 12
1
2 3 4 5 6 7 8 9 10 11 12 13
Samples

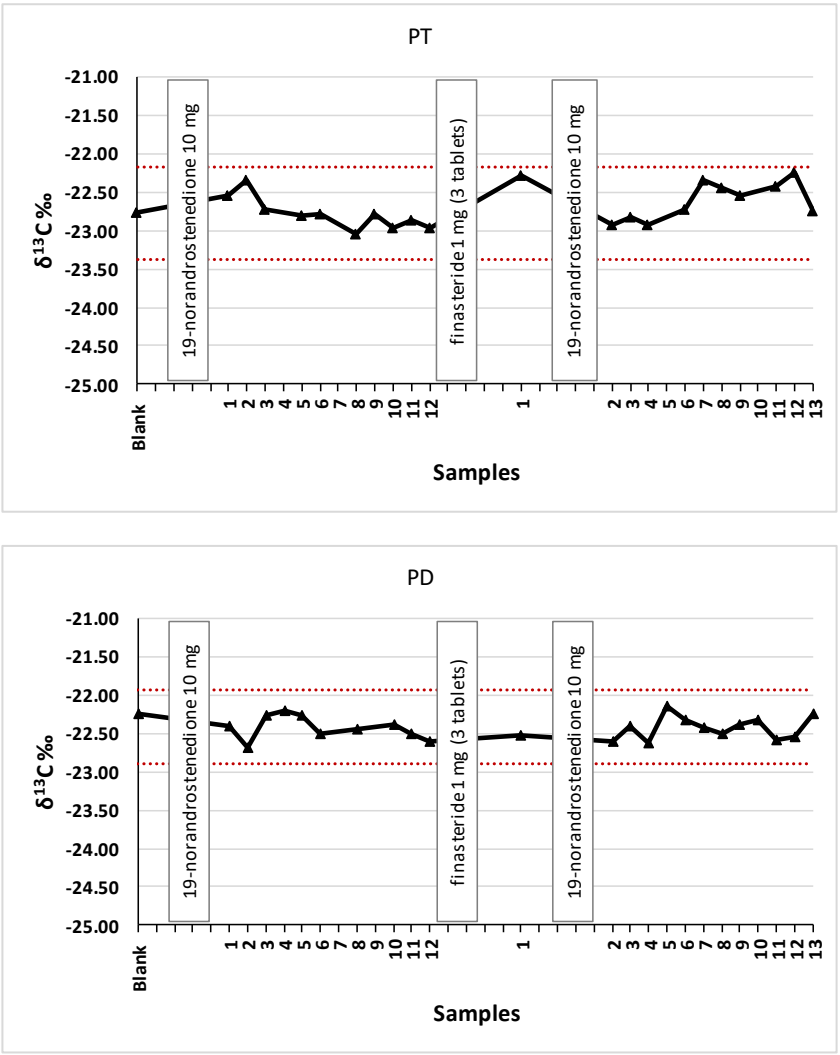
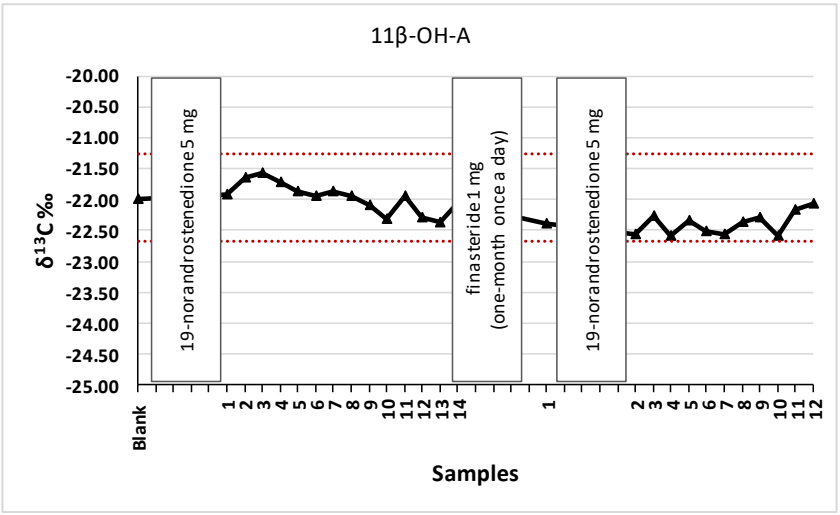
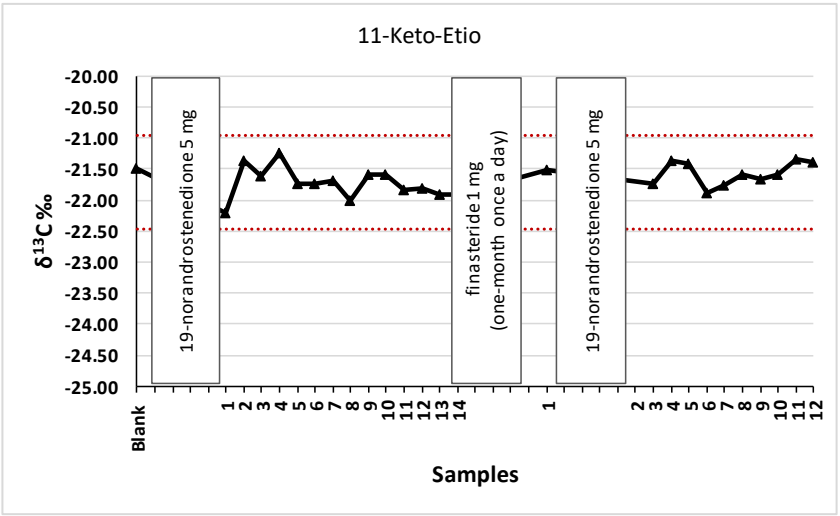
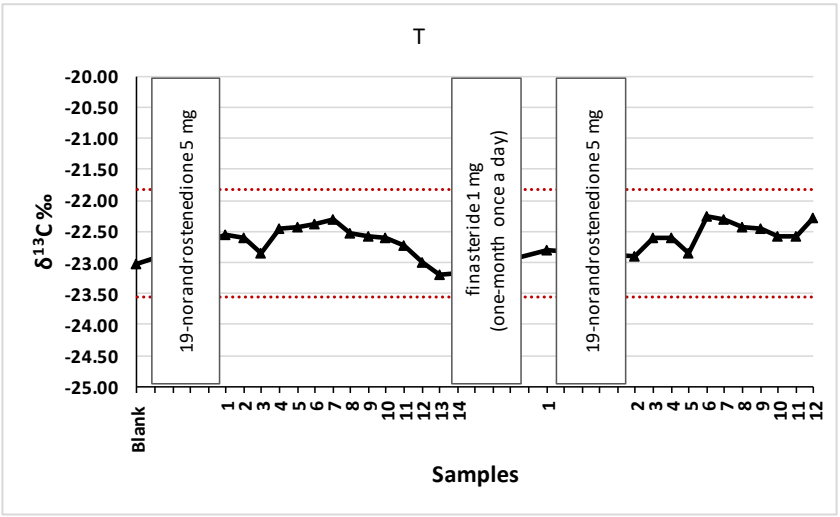
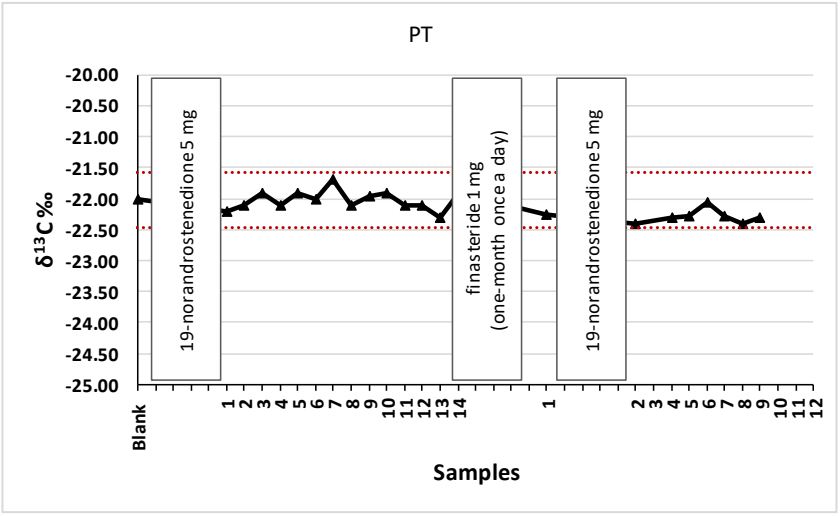


Figure A.7: Volunteer 2: carbon isotopic composition of other compounds (11-β-OH-A; 11-Keto-Etio; T; PT and PD) commonly determined to detect the abuse of pseudo-endogenous steroids
Dotted lines define the individual reference range (μ ± 3SD)


11β-OH-A
δ13C ‰
-20.00
-20.50
-21.00
-21.50
-22.00
-22.50
-23.00
-23.50
-24.00
-24.50
-25.00
19-norandrostenedione 5 mg
finasteride 1 mg
(one-month once a day)
19-norandrostenedione 5 mg
Blank
1 2 3 4 5 6 7 8 9 10 11 12 13 14
1 2 3 4 5 6 7 8 9 10 11 12
Samples


11-Keto-Etio
δ13C ‰
-20.00
-20.50
-21.00
-21.50
-22.00
-22.50
-23.00
-23.50
-24.00
-24.50
-25.00
19-norandrostenedione 5 mg
finasteride 1 mg
(one-month once a day)
19-norandrostenedione 5 mg
Blank
1 2 3 4 5 6 7 8 9 10 11 12 13 14
1 2 3 4 5 6 7 8 9 10 11 12
Samples


T
δ13C ‰
-20.00
-20.50
-21.00
-21.50
-22.00
-22.50
-23.00
-23.50
-24.00
-24.50
-25.00
19-norandrostenedione 5 mg
finasteride 1 mg
(one-month once a day)
19-norandrostenedione 5 mg
Blank
1 2 3 4 5 6 7 8 9 10 11 12 13 14
1
2 3 4 5 6 7 8 9 10 11 12
Samples


PT
δ13C ‰
-20.00
-20.50
-21.00
-21.50
-22.00
-22.50
-23.00
-23.50
-24.00
-24.50
-25.00
19-norandrostenedione 5 mg
finasteride 1 mg
(one-month once a day)
19-norandrostenedione 5 mg
Blank
1 2 3 4 5 6 7 8 9 10 11 12 13 14
1
2 3 4 5 6 7 8 9 10 11 12
Samples

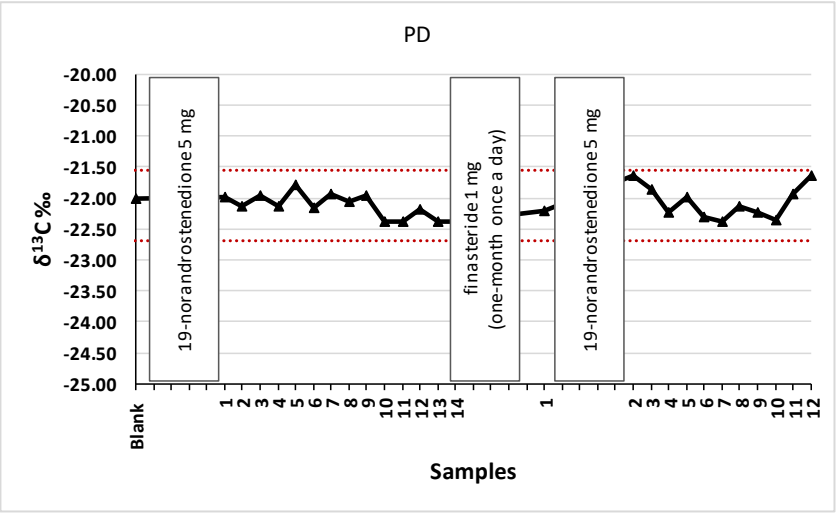


Figure A.8: Volunteer 3: carbon isotopic composition of other compounds (11-β-OH-A; 11-Keto-Etio; T; PT and PD) commonly determined to detect the abuse of pseudo-endogenous steroids
Dotted lines define the individual reference range (μ ± 3SD)

List of publications and contributions

Scientific articles and manuscripts in preparation

L. Iannella, C. Colamonici, D. Curcio, F. Botrè, X. de la Torre (2019)
Development and validation of a method to confirm the exogenous origin of prednisone and prednisolone by GC-C-IRMS
Drug Test Anal. 11: 1615-1628

L. Iannella, F. Botrè, C. Colamonici, D. Curcio, C. Ciccarelli, M. Mazzarino, X. de la Torre (2020)
Carbon isotopic characterization of prednisolone and prednisone pharmaceutical formulations: implications in antidoping analysis
https://doi.org/10.1002/dta.2876

L. Iannella, C. Colamonici, D. Curcio, F. Botrè, X. de la Torre (2020)
Detecting the abuse of 19-norsteroids in doping controls: a new GC-C-IRMS method for the analysis of 19-norandrosterone and 19-noretiocholanolone
Accepted for publication in Drug Testing and Analysis

L. Iannella, C. Colamonici, D. Curcio, F. Botrè, X. de la Torre
5α-reductase inhibitors: evaluation of their potential confounding effect on GC-C-IRMS doping analysis
In preparation

Oral communications

L. Iannella, F. Botrè, C. Colamonici, D. Curcio, C. Ciccarelli, X. de la Torre (2020)
Potential masking effect of different prednisolone based pharmaceutical formulations on IRMS confirmation analysis
XXXVIII Cologne Workshop on Doping Analysis

L. Iannella, C. Colamonici, D. Curcio, F. Botrè, X. de la Torre (2019)
The IRMS anti-doping analysis: applications and current challenges
7th Conference of the Forensic Isotope Ratio Mass Spectrometry Network, Trento

L. Iannella, C. Colamonici, D. Curcio, F. Botrè, X. de la Torre (2019)

Development and validation of a GC-C-IRMS method to confirm the exogenous origin of urinary prednisolone and prednisone
XXXVII Cologne Workshop on Doping Analysis

L. Iannella, C. Colamonici, D. Curcio, F. Botrè, X. de la Torre (2018)
Isotopic metabolic fractionation of endogenous steroids by 5α-reductase inhibitors
2nd Isotope Ratio MS Day, Messina

Posters

L. Iannella, C. Colamonici, D. Curcio, F. Botrè, X. de la Torre (2019)
Prednisone and Prednisolone Detection in Urine Samples: a GC-C-IRMS Method to Discriminate their Exogenous or Endogenous Origin.
6th European Congress & Exhibition MSACL2019, Salzburg

L. Iannella, C. Colamonici, D. Curcio, F. Botrè, X. de la Torre (2019)
Natural steroidal precursors: a critical issue in anti-doping IRMS analysis. The prednisolone and prednisone case study
3rd MS NatMed Day - MASSA 2019, Arezzo

L. Iannella, C. Colamonici, D. Curcio, F. Botrè, X. de la Torre (2018)
Large volume injection: improving the detection capacity of endogenous anabolic steroids by GC-C-IRMS
XXII International Mass Spectrometry Conference, Firenze

Research Grant

Avvio alla Ricerca 2018
Isotopic metabolic fractionation of endogenous steroids by 5α-reductase inhibitors