Analytical Techniques in Androgen Anabolic Steroids (AASs) Analysis for Antidoping and Forensic Purposes

S. Strano-Rossi*[,1,3], C. Fiore[2], M. Chiarotti[3] and F. Centini[4]

[1]*Antidoping Laboratory, Federazione Medico Sportiva Italiana, L.go G. Onesti, 1, 00197, Rome, Italy*

[2]*Institute of Legal Medicine, University of Foggia, V.le degli Aviatori, 1, 71100, Foggia, Italy*

[3]*Institute of Legal Medicine, Catholic University, L.go F. Vito, 1 00168, Rome, Italy*

[4]*Institute of Forensic Toxicology, University of Siena, V.le Bracci, 53100, Siena, Italy*

Abstract: A survey on the main analytical challenges related to the analysis of Androgen Anabolic Steroids (AASs) is reported. AASs analysis is an issue regarding antidoping analyses as well as forensic toxicology applications. This paper reports an overview of the more recent literature regarding various aspects of sample preparation, analytical techniques and interpretation of results for AASs identification in biological samples. New analytical approaches, mainly for their application to the antidoping field, are reported. The application of AASs analysis in forensic cases is also described, taking into consideration mainly the different biological samples that can be analysed for forensic purposes. Particular attention was played on the application of hair analysis as alternative biological specimen for the determination of AASs abuse.

Keywords: Androgen anabolic steroids analysis, doping, forensic toxicology, analytical techniques.

INTRODUCTION

The analysis of Androgen Anabolic Steroids (AASs) is an issue of main interest in many field of clinical and forensic toxicology. AASs can be used in therapy [1,2], as well as abused to improve sport performance [3-6].

The practice of improving sport performance through the administration of xenobiotics is as old as competitive sport itself [6-7]. Also, Ancient Greek and Ancient Roman gladiators, are known to have enhanced their performance by the intake of herbs and animal extracts.

Cocaine, caffeine, and amphetamines were used as sport enhancing drugs since the 19th century while anabolic steroids misuse in sports began in the middle of 20th century.

The first antidoping tests were performed in the 1960s, under the coordination of individual sport federations (football and cyclism) and, since 1967, of the International Olympic Committee (IOC). At that time, the substances being investigated for were mainly stimulants (amphetamines) and narcotics. Throughout the years, the substances prohibited, and the methods to detect them, have increased in number with the aims of protecting the health of the athletes and guaranteeing the spirit of ethical competitions. In 1976 androgen anabolic steroids (AASs) were added to the IOC list of prohibited substances. In 2004, the World Anti-doping Agency (WADA), founded in 1999, took over the coordination of the fight against doping [7]. For antidoping analyses, the only specimen used is urine (and blood for particular cases such as Gh, Biological passport, new EPO analogues).

AASs toxicity my cause morbidity and mortality; death may occur suddenly or be related to the chronic damage of vital organs. Toxicological investigation is therefore fundamental in those cases of sudden death in subjects suspected of consuming AASs [8-13].

Biological specimens used for toxicological investigation are urine, blood, vitreous humour and hair.

ANTIDOPING

Currently, all professional athletes are subject to antidoping testing under the supervision of WADA. The biological specimen generally used for antidoping testing is urine; the samples are screened for all the substances included in the Prohibited List that WADA revises annually (until 2004 the IOC Medical Commission was responsible for revision of the List). The Prohibited List has been, and still is, continuously revised according to the growing knowledge of illegal sport-enhancing practices. The list is therefore open, and throughout the years, there has been changes of the substances prohibited in sports and, therefore, investigated by the antidoping laboratories. Currently included on the prohibited list are anabolic steroids (subdivided into androgenic steroids and other anabolic agents such as clenbuterol or androgen receptor modulators), hormones and related substances (erythropoietin and analogues, growth hormones and analogues, insulins, corticotrophins), beta-2 agonists, hormone antagonists and modulators, diuretics and other masking agents, stimulants, narcotics, cannabinoids, glucocorticosteroids, and beta-blockers [14]. The total number of substances WADA labs

*Address correspondence to this author at the Antidoping Laboratory, Federazione Medico Sportiva Italiana, L.go G. Onesti, 1, 00197, Rome, Italy; Tel: +390636859631; Fax: +39068076859;
E-mail: sabina.stranorossi@gmail.com

1389-5575/11 $58.00+.00

investigate for is approximately 300. AASs are prohibited in and out of competition, so they must be screened in all samples from antidoping controls.

The base structure of most AASs relevant for antidoping analyses is the testosterone nucleus, an α,β- unsaturated 3-keto steroid. A huge variety of chemically modified steroids have been synthesized from this natural structure, in attempt to maximize anabolic effects and minimize the androgenic ones. Modification of the base testosterone nucleus by the substitution of the 17 α hydrogen with a methyl or ethyl group can also prevent deactivation by first pass metabolism, allowing the oral administration; for parenteral administration, 17 hydroxy group is generally esterified, to allow a slower absorption. Other typical modifications are the removal of the 19 methyl group, as for nandrolone, the attachment of various groups at C2, attachment of methyl group at C1 or C7, attachment of a hydroxyl- or a chlorine at C4, introduction of double bonds at C1, C4, C6, C9.

The majority of AASs undergo to extensive metabolism. E.g. testosterone is metabolised by 5α-reductase to dihydrotestosterone and subsequently, by saturation of the 3-keto group, to the isomers androsterone and ethiocolanolone. AASs with similar base structure undergo to the same metabolic pathway. Other typical metabolic changes are epimerisation, oxidation of the 17ß-hydroxyl group; reduction of carbonyl group or of double bonds; hydroxylation at various positions of the steroid nucleus.

The majority of AASs metabolites undergo to phase 2 metabolism, and are therefore excreted mainly as glucuro-conjugate, and in a minor extent, as sulfo-conjugates. Analytical methods are therefore aimed to detect these substances and/or their main metabolites. They have a poor chromatographic behaviour, so to improve their GC and mass spectrometric properties, need a previous derivatization step. Alternatively, some of them can be detected by LC/MS.

Analytical Techniques

Due to the huge number of substances to be analysed, laboratories develop *ad hoc* screening methods able to preliminary identify the greatest number of substances in a single method. Samples are therefore submitted to a preliminary screening, and if the presence of a prohibited substance is suspected in this phase, the sample is subjected to a confirmation analysis. As in many other fields of analytical chemistry, also in antidoping controls the whole process, both for screening and confirmation, can be subdivided into sample pre-treatment and instrumental analysis.

Sample Pre-Treatment

The majority of AASs are excreted as conjugated, mainly with glucuronic acid and, in a minor extent, as sulphates.

Therefore sample pre-treatment involves a preliminary phase of hydrolysis of conjugates, generally performed with enzymes (β-glucuronidase from E. Coli, helix pomatia or patella vulgata) that are chosen depending on their specific activity, at controlled pH and temperature [15].

The analytes of interest are then extracted from biological samples by liquid/liquid or solid phase extraction under fit-for-purpose conditions of pH and in the presence of organic solvents, or by immunoaffinity purification [16-17].

Times of extraction can be lowered by the assistance of microwaves [18].

To improve their chromatographic and spectrometric characteristics, these substances require a derivatization step prior to their analysis. Derivatization is generally performed to generate trimethyl-silyl derivatives. Various reagents can be used (N, O, bis-trimethylsilyl trifluoroacetamyde (BSTFA), N-methyl, N-trimethylsilyl trifluoroacetamyde (MSTFA), *etc.*) and catalyzers (imidazole, trimethyl-iodosilane, ammonium iodide, dithioerythritol, mercaptoethanol) that, through the formation of a highly reactive intermediate, help the formation of trimethylsilyl esters and ethers also at level of the keto groups [19-22]. Other more laborious derivatizations have been proposed for their application to the confirmation of AASs or to the study of metabolic patterns, to obtain the formation of oxime (hydroxyl ammonium pretreatment) and picolinoyl (mixed anhydride method) derivates, with more characteristic fragmentation patterns [23].

The application of ultrasounds to various phases of sample pre-treatment (hydrolysis, derivatization) can reduce analytical times [22].

Automation of all these phases can be performed in order to diminish manual handling of the samples [24].

Instrumental Analysis: GC/MS and LC/MS

The methods for the determination of AASs are designed to screen the main metabolites and/or their parent compounds, once excreted in urine. The classical methods are generally based on GC-MS [16-30], but also on GC-MS^n or GC-Time-of-Flight (TOF) MS [31-33], after derivatization of urine extracts.

The WADA requires a high detection power for these substances, in some cases down to at least 2 ng/mL. Hence, analyses were generally performed in the Selected Ion Monitoring (SIM) mode. The improvement of equipments that allow the simultaneous SIM/SCAN acquisition, rendered possible this new kind of approach [34]. The advantages are the SIM sensitivity and simultaneously, with the SCAN acquisition, the chance to check possible interferences co-eluting with the peaks of interest, and the possibility to perform a retrospective analysis of the data acquired for each sample (e.g. to search for the presence of new designer drugs through a revision of the data being acquired).

Comprehensive bidimensional GC coupled to TOF-MS has been applied for the screening and confirmation of AASs, complying with the WADA criteria for identification of analytes [35].

Liquid chromatography (LC), coupled to various mass detectors (quadrupoles, triple quadrupoles, ion traps, TOF or –QTOF), is more and more used for the screening of AASs. The lowering of the costs and the versatility of the

instruments render this technique gradually more often used in antidoping field, and the undoubted advantages of sensitivity and versatility are on the rise respect to the disadvantage of a minor reproducibility than GC/MS.

Different conditions of ionisation by adduct formation have been studied to improve the detectability of AASs by LC/MS [36]. LC/MS^n has also been applied to the study of possible endogenous interferences that could hamper the determination of AASs, in order to optimize LC/MS methods [37].

LC/MS analyses are generally performed without the derivatization step, necessary for GC, and can be performed both prior than after the hydrolysis of conjugates.

A main advantage of LC/MS is in fact the possibility to directly analyze the conjugated metabolites of AASs. This has been applied for exogenous AASs [38], or for endogenous ones [39].

The combination of LC/MS^n (triple-quadrupole) and LC/QTOF techniques has been used to elucidate the structure of a huge number of 3keto-steroids, in order to obtain general patterns of fragmentation, useful for the screening of new designer AASs or metabolites [40], also in combination with GC/MS (for the identification of new fluoximesterone metabolites) [41].

Georgakopoulos *et al*. developed a combination of LC and GC orthogonal acceleration TOF methods for the screening of "classical" doping substances and designer steroids [42]. Also Thevis proposed a LC/MS method for the screening of designer steroids, based on the selection of typical transitions common to specific parts of the steroidal structure [43]. The sensitive Orbitrap-MS with in-source collision induced dissociation, coupled to HPLC in APCI mode, has been proposed for the screening of various AASs with Limits of Detection (LODs) up to 50 pg/mL, in urine [44].

A LC/Q-TOF-MS screening, after a simple derivatization of urine extracts with Girard's Reagent P, has been proposed for the determination of the more problematic AASs and metabolites (e.g. THG, epioxandrolone, epimethendiol, stanozolol hydroxyl-metabolites) and for the quantitative confirmation of nandrolone main metabolite 19-norandrosterone [45].

The same analytical approach has been applied for the quantitative confirmation of T/E ratio [46].

The suspected presence of a prohibited substance must be submitted to confirmation analysis in order to provide information on the chemical structure of the substance so that it can be unequivocally identified. As the screening for AASs is generally performed with mass-spectrometric techniques, confirmation techniques can be the same used for screening purposes (*e.g.*, GC-MS, LC-MS) resorting to methods specific for the target compounds, or specific purification steps (sample clean-up by immunopurification or HPLC), extraction with specific solvents, and/or different derivatization techniques capable of generating more characteristic mass spectra.

In some cases it is necessary to use more specific/powerful instrumental techniques. For the differentiation of exogenous administration of anabolic endogenous steroids (testosterone, epitestosterone, dehydroepiandrosterone, dihydrotestosterone, *etc*.), GC-combustion-Isotopic Ratio Mass Spectrometry (IRMS) is the technique of choice to discriminate between endogenous and exogenous substances due to the different amount of stable isotopes ($^{13}C/^{12}C$) present in natural steroids with respect to synthetic ones [47-49]. IRMS allows the different isotopic compositions to be determined in terms of $\delta^{13}C$, *i.e.*, the difference of the isotopic ratio $^{13}C/^{12}C$ between endogenous reference steroids that come from a different metabolic pathway and androgenic steroids of exogenous origin.

High Resolution-MS allows substances at very low amounts in biological samples to be identified on the basis of the precise mass determination (up to the fourth digit). This reduces the background level and enhances specificity and detection power [50-51]. Analyzer can be double focusing magnetic mass spectrometers, TOF, Orbitrap, coupled with GC or LC systems [35, 42, 52-53].

Anabolic steroids at low concentrations (nandrolone, methandienone, stanozolol, and methyltestosterone metabolites) are generally confirmed by this approach [16].

Other Approaches

In an attempt to fool analytical tests, the research on doping continuously goes on by synthesizing new substances with similar chemical structures and with the same activity, or even more active, not included in the routine antidoping tests and the toxicity of which is unknown. Control laboratories are thus prompted to steadily improve their performance and also to monitor possible new doping agents which otherwise would go undetected. Therefore, also new steroids, such as tetrahydrogestrinone (THG), have been included both in the screening and confirmatory methods and research is in continuous progress to develop new antidoping methods and strategies, mainly targeted to the structure/activity relationship [42-43, 54].

Other approaches have been proposed as pre-screening for AASs, such as an assay based on the yeast androgen receptor (AR) reporter gene system to identify anabolic steroids in human urine samples. As biological activity is independent of the chemical structure of androgenic substances, this assay can be suitable to indicate the presence of suspicious substances such as the designer steroids [55]. Another similar approach couples a similar bioassay screening for androgenic activity to Q-TOF analysis for the identification of substances with androgenic activity [56].

Cytochrome P450 enzymes play a central role in drug metabolism. As they are predominantly expressed in the liver, a chimeric mouse model with functional human hepatocytes was evaluated as an alternative model for metabolic studies on AASs. the approval of an ethics committee for administration studies in humans is in fact virtually impossible to obtain. The chimeric mouse can serve as a model for *in vivo* metabolic studies of designer steroids. Metabolism of 4-androstene-3,17-dione [57], methandienone [58], 17-methyl testosterone [59], Stanozolol [60], 19-

norandrosten-dione [61] was studied by the administration of these substances to the humanized mouse, that can be in theory be used for metabolic studies of all classes of AASs, including new designer' ones .

Interpretation of Results: Specific Issues

The presence of some prohibited AASs in biological specimens require particular attention in the interpretation of results, as the presence of these substances/characteristic metabolites can be due to endogenous production/excretion (e.g, 19-norandrosterone can be endogenously produced during pregnancy, under contraceptive treatment, and in very low concentrations, under strenuous fatigue).

The WADA has therefore drafted technical documents as a mandatory guidance to the evaluation of results for nandrolone main metabolite [62], T/E ratio, testosterone epitestosterone and other endogenous androgenic steroids [63]. So, in the case of 19-NA above the decision limit, i.e. cut-off plus a calculated guard band (2.5 ng/mL), the laboratory shall also perform additional mandatory tests: pregnancy and norethisterone metabolite detection in female athletes; if 19-NA concentration is below 10 ng/mL, and the sample shows signs of instability (altered ratio between NA/NE and A/E), a stability test on the urine sample must be performed to exclude demethylation of endogenous steroids; IRMS for 19-NA, in order to determine the exogenous origin of nandrolone metabolites.

Beside the T/E ratio, the evaluation of complete steroid profile is an aid for detection of doping. For instance the decreased ratio androsterone/testosterone (below 20 for men and 40 for women) and increased testosterone concentration (higher than 130 ng/mL for men and 60 ng/mL for women, corrected to a urine density of 1.020) are considered additional parameters which make a urine suspicious for an application of exogenous testosterone, if other influences, e.g. bacterial activities, can be excluded. Various in facts are the parameters that can influence the steroid profile and that should be considered in doping controls [64].

To help the evaluation of the endogenous steroid profile, the screening of an extended number of endogenous steroids and metabolites can provide additional parameters. These can be used to support the atypical findings and can give specific information upon the steroids which have been administered [65]. Anyway, the diagnostic power of such an analytical approach is still limited and the application of population based reference ranges for endogenous anabolic steroids could lead to a misinterpretation of results if steroids are administered at low doses [66].

FORENSIC TOXICOLOGY

The incidence of sudden death in athletes is estimated 2.5 times higher than in non-athletes. It has also been shown that almost every single case of sudden death in athletes was due to an underlying hereditary or acquired heart disease; in only about 1% of he examined cases could drug abuse be confirmed. So even if a possible or probable cause of death is found at the autopsy, there remains doubt about a substance-triggered or facilitated sudden death (e.g., coronary thrombosis with evidence of EPO abuse, stimulants, and channelopathy). Deaths correlated to AASs toxicity have been reported. Main pathologies found out were myocardial injuries (coagulative myocytolysis or contraction bands necrosys) [8-9], also of low entity [10-11], myocardial hypertrophy [12-13]. In other sudden-death cases, pulmonary venous thrombosis and right cerebellar hemorrage were the cause of death [8].

AASs pathological effect is not due to an acute effect, but to a chronic cumulative effect on various vital organs: heart, liver, kidney, brain, uterus, adrenals, testis.

Specimens available in post-mortem toxicology investigations can be numerous and variable, and may be selected based on case history, requests, legal aspects and availability in a given case. Up to date, an harmonized protocol for sampling in suspected poisoning or drug-related death has not been established.

Recommendation on post-mortem sampling have been drawn [67-68].

In particular, constant must be the collection of blood from peripheral sites and heart blood, urine, vitreous humour, bile, cerebrospinal fluid, gastric contents and alternative specimens such as hair, nails or skin samples necessary to extend the chronological window assumption. These samples will always be accompanied by fragments of the major organs.

Anyway, samples generally analysed for the determination of AASs in post-mortem cases are urine, blood, vitreous humour and hair [8-9, 11, 69-70].

Samples pre-treatment and analytical techniques used are the same of those used for confirmatory analyses in antidoping controls. E.g, Fineschi *et al.* extracted blood and urine on C_{18} cartridges after enzymatic hydrolysis, derivatised to obtain trimethylsylil derivatives and analysed the samples by GC/MS in SIM mode for the detection of main nandrolone metabolites [9]. Di Palo *et al.* confirmed stanozolol metabolites in urine by LC/MS [10].

Hair Analysis

Hair analysis has been employed for the past three decades to detect chronic drug use and is gaining attention also for doping agents analysis. Main advantages linked to hair analysis are the easy, non invasive sample collection, that is not easily adulterated, and whose preservation can be performed without specific precautions. Furthermore, hair samples permit a retrospective evaluation of drug use history corresponding to many months before the actual sampling moment, depending on hair length. It allows also to evaluate, by segmental analysis, the approximate period when a certain drug was taken and, likewise, the time of abstinence.

Researches have demonstrated that hair has an isoelectric pH close to 6 and thus favours incorporation of undissociated basic drugs. Parent drug is hence preferentially accumulated in hair matrix (mainly in the melanin) in comparison to its metabolites. As an example, amongst the anabolic steroids, stanozolol gets preferentially distributed into pigmented hairs due to the basic nature of the pyrazole ring present in its chemical structure [71].

Hair analysis is anyway not considered a valid alternative to urine analysis for doping control. Although today is recognized and accepted in the law courts of numerous countries, hair can not be used as a diagnostic specimen for antidoping purposes and this is expressly stated by the WADA.

Hair analysis can anyway provide useful information on the kind of drugs administered (e.g. esters of steroids), and, mainly, can detect substances abused several days before the time of sampling, that is, in antidoping terms, out-of-competition, as is required for Anabolic Agents.

The Society of Hair Testing has drawn a statement reporting the following points about the analysis of hair for doping agents:

1. Hair analysis can essentially contribute to doping analysis in special cases, in addition to urine.
2. Hair specimens are not suitable for general routine control.
3. In the case of positive urine results, the negative hair result cannot exclude the administration of the detected drug and cannot overrule the positive urine result.
4. In case of negative urine result, the positive hair result demonstrates drug exposure during the period prior to sample collection.

Beside this, many studies report the determination of doping agents in hair, especially for its application in forensic toxicology.

Various AASs, such as stanozolol, nandrolone and its esters and metabolites, DHEA, testosterone and its esters, metandienone, methenolone have been investigated in hair [72-80].

As for the "classic" drugs of abuse, the isolation of the drugs from the keratins is a main problem in hair analysis, as it can modify the parent drug/metabolites pattern.

Rambaud *et al*. analyze three main procedures of hydrolysis for the validation of a method for the detection of a wide range of hormonal anabolic compounds in hair [81].

The most efficient hydrolysis procedure used for hair analysis is the hydrolysis of keratins in alkaline conditions, by NaOH digestion at 60–100 ◦C.

Alkaline digestion with NaOH is the approach used more often as sample preparation for AASs analysis [71, 76, 82 - 83].

Segura *et al.* incubate hair with methanol/trifluoroacetic acid, in order not to hydrolyze the steroid esters that have to be detected [84]. Also Thieme evaluates the phase of keratin digestion in order not to alter the parent drug structure (hydrolysis of steroid esters) [85]. The presence of AASs esters can in fact help to evaluate the hexogenous origin of the drug.

After the keratin digestion, an extraction is performed, by liquid/liquid or solid phase extraction or both [73-74, 86].

The method used by Gaillard *et al.* after NaOH hydrolysis, includes a "twin" solid-phase extraction on amino and silica cartridges [87].

The extraction phase is generally followed by derivatization to obtain trimethyl silyl derivatives prior to the analysis in GC/MS. Also derivatization to obtain heptafluorobutyrril derivatives has been used [80].

GC/MS is the technique adopted in the first analyses for the detection of AASs in hair.

Also HR/MS has been used [85]. Successively also Gas chromatography-tandem mass spectrometry (GC-MS/MS) was chosen as detection technique, especially in the SRM mode, because of its specificity and sensitivity, compatible with the target concentration range [73, 82, 84].

Good results were obtained also by the analysis in LC-MS/MS, efficient in detecting steroids in hair even at very low levels, when only about 20 mg hair was processed [71].

Interpretation of Results and Applications

The main advantages of hair analysis, for the analysis of androgenic steroids, is the preferential incorporation of the parent drug into the keratins. Nandrolone and testosterone medicaments are prepared as esters (decanoate, enantate, etc). The determination of their esters in hair can hence help to differentiate the endogenous AASs from the hexogenous ones. Also the presence of nandrolone precursors (norandrostenedione and -diol), previously not included in the WADA prohibited list, can be differentiated from nandrolone intake by hair analysis [74-75].

The wide detection window is another advantage of this biological matrix. Although it is not possible to determine the exact time of ingestion of drugs through hair analysis, it can be useful for epidemiological or prevalence studies for the evaluation of AASs use.

In 2000, Gaillard *et al.* published a study on the compared interest between hair analysis and urine analysis in doping controls [87]. Thirty cyclists were sampled and tested for the two matrices. For corticosteroids, urine sample was preferred whereas for anabolic steroids and amphetamines, hair appeared as a more powerful diagnostic matrix.

Deshmukh *et al.* analyzed hair samples from 180 volunteers for the determination of AASs. 12 stanozolol and one nandrolone positive cases were confirmed. In this study, a preliminary ELISA screening was performed, giving 16 positive results for stanazolol and 3 positive results for nandrolone [71].

Various studies are attempting to evaluate the range of physiological concentrations of the main endogenous AASs in hair in order to distinguish the eventual exogenous intake [83, 88]

Although hair analyses is not accepted for antidoping controls, it has been used in judiciary cases to determine the use of AASs and other doping agents [78, 89].

CONCLUSIONS

Androgen anabolic steroid analysis has become an indispensable and well-established procedure routinely carried out in antidoping laboratories; recently the

determination of AASs constitutes a new analytical challenge also for forensic toxicology laboratories, owing to the increased interest of the forensic sciences in the investigation about "anabolic steroids misuse", particularly in case of sudden death related to anabolic steroids toxicity.

The tools available for the identification of these substances are now several, as well as the analytical methods developed by the laboratories, allowing a very high diagnostic power for the determination of AASs.

Various kinds of mass spectrometers, mainly interfaced to gas chromatography, are powerful analytical tools for the identification of steroids and their metabolites, at sub-nanogram levels, in complex matrices such as biological fluids and tissues.

Tandem mass spectrometry (MS/MS), coupled to gas chromatography or to liquid chromatography, can provide an additional analytical dimension in case of trace analysis or confirmatory purposes. High Resolution-MS allows the identification of substances at very low concentrations, by increasing the signal to noise ratios of the accurate masses characteristic of each analyte. Liquid chromatography, coupled to different kinds of mass spectrometers, is becoming a routinely used technique both in antidoping and forensic toxicology laboratories for the analysis of AASs. Finally, abundance ratios of stable isotopes, as measured by gas chromatographic combustion - mass spectrometry, has been used as unsurpassed pillar to characterize and authenticate the synthetic origin of exogenous steroids in biological samples.

REFERENCES

[1] Bertelloni, S.; Baroncelli, G.I.; Garofalo, P.; Cianfarani, S. Androgen therapy in hypogonadal adolescent males. *Horm. Res. Paediatr.*, **2010**, *74*(4), 292-296.

[2] Geusens, P. Nandrolone decanoate: pharmacological properties and therapeutic use in osteoporosis. *Clin Rheumatol.*, **1995,** *14*(Suppl 3), 32-39.

[3] Wagner, J.C. Enhancement of athletic performance with drugs. An overview. *Sports Med.*, **1991**, *12*, 250-265.

[4] Cowan, D.A.; Kicman, A.T. Doping in sport: misuse, analytical tests and legal aspects. *Clin. Chem.*, **1997**, *43*, 1110-1113.

[5] Hartgens, F.; Kuipers, H. Effects of androgenic-anabolic steroids in athletes. *Sports Med.*, **2004**, *34*(8), 513-554.

[6] Botrè, F.; Pavan, A. Enhancement drugs and the atlete. *Neurol. Clin.*, **2008**, *26*, 149-167.

[7] World Antidoping Agency. A Brief History of Anti-Doping. http://www.wada-ama.org. (Accessed 01/12/2010).

[8] Fineschi, V.; Baroldi, G.; Monciotti, F.; Paglicci Reattelli, L.; Turillazzi, E. Anabolic steroid abuse and cardiac sudden death: a pathologic study. *Arch. Pathol. La.b Med.*, **2001,** *125*(2), 253-255.

[9] Fineschi, V.; Riezzo, I.; Centini, F.; Silingardi, E.; Licata, M.; Beduschi, G.; Karch, S.B. Sudden cardiac death during anabolic steroid abuse: morphologic and toxicologic findings in two fatal cases of bodybuilders. *Int. J. Legal Med.*, **2007**, *121*(1), 48-53.

[10] Di Paolo, M.; Agozzino, M.; Toni, C.; Luciani, A.B.; Molendini, L.; Scaglione, M.; Inzani, F.; Pasotti, M.; Buzzi, F.; Arbustini, E. Sudden anabolic steroid abuse-related death in athletes. *Int. J. Cardiol.*, **2007,** *114*(1), 114-117.

[11] Thiblin, I.; Mobini-Far, H.; Frisk, M. Sudden unexpected death in a female fitness athlete, with a possible connection to the use of anabolic androgenic steroids (AAS) and ephedrine. *Forensic Sci. Int.*, **2009**, *184*(1-3), e7-11.

[12] Luke, J.L.; Farb, A.; Virmani, R.; Sample, R.H. Sudden cardiac death during exercise in a weight lifter using anabolic androgenic steroids: pathological and toxicological findings. *J. Forensic Sci.*, **1990,** *35*(6), 1441-1147.

[13] Hausmann, R.; Hammer, S.; Betz, P. Performance enhancing drugs (doping agents) and sudden death--a case report and review of the literature. *Int. J. Legal Med*., **1998,** *111*(5), 261-264.

[14] World Antidoping Agency. The 2010 Prohibited List International Standard. http://www.wada-ama.org. [Accessed 01/12/2010].

[15] Wakabayashi, M.; Fishman, W.H. The comparative ability of β-glucuronidase preparations (liver, Escherichia Coli, Helix Pomatia and Patella Vulgata) to hydrolyze certain steroid glucosiduronic acids. *J. Biol. Chem.*, **1961**, *236*(4), 996-1001.

[16] Schanzer, W.; Delahaut, P.; Geyer, H.; Machnick, M.; Horning, S. Long-term detection and identification of metandienone and stanozolol abuse in athletes by gas-chromatograpy – high resolution mass spectrometry. *J. Chromatogr. B.*, **1996,** *687*, 93-108.

[17] Dubois, M.; Taillieu, X.; Colemonts, Y.; Lansival, B.; De Graeve, J.; Delahaut, P. GC-MS determination of anabolic steroids after multi-immunoaffinity purification. *Analyst*, **1998**, *123*(12), 2611-2616.

[18] Mazzarino, M.; Riggi, S.; de la Torre, X.; Botrè, F. Speeding up the process urine sample pre-treatment: some perspectives on the use of microwave assisted extraction in the anti-doping field. *Talanta*, **2010**, *81*(4-5), 1264-1272.

[19] Cartoni, G.P.; Ciardi, M.; Giarrusso, A.; Rosati, F. Capillary gas chromatographic-mass spectrometric detection of anabolic steroids. *J. Chromatogr*., **1983**, *279*, 515-522.

[20] Bowers, L.D.; Borts, D.J. Separation and confirmation of anabolic steroids with quadrupole ion trap tandem mass spectrometry. *J. Chromatogr. B.*, **1996,** *687*, 69-78.

[21] Ayotte, C.; Goudreault, D.; Charlebois, A. Testing for natural and synthetic anabolic agents in human urine. *J. Chromatogr. B.*, **1996,** *687*, 3-25.

[22] Galesio, M.; Rial-Otero, R.; Simal-Gándara, J., de la Torre, X.; Botrè, F.; Capelo-Martínez, J.L. Improved ultrasonic-based sample treatment for the screening of anabolic steroids by gas chromatography/mass spectrometry. *Rapid Commun Mass Spectrom.*, **2010**, *24*(16), 2375-2385.

[23] Baranov, P.A.; Appolonova, S.A.; Rodchenkov, G.M. The potential use of complex derivatization procedures in comprehensive HPLC-MS/MS detection of anabolic steroids. *Drug Test. Anal.*, **2010**, *2*(10), 475-488.

[24] Haber, E.; Muñoz-Guerra, J.A.; Soriano, C.; Carreras, D.; Rodriguez, C.; Rodriguez, F.A. Automated sample preparation and gas chromatographic-mass spectrometric analysis of urinary androgenic anabolic steroids. *J Chromatogr. B Biomed. Sci. Appl.*, **2001,** *755*(1-2), 17-26.

[25] Hatton, C.K.; Catlin, D.H. Detection of androgenic steroids in urine. *Clin. Lab. Med*., **1987**, *7*, 655-668.

[26] Massé, R.; Ayotte, C.; Dugal, R. Studies on anabolic steroids. I. Integrated methodological approach to the gas chromatographic-mass spectrometric analysis of anabolic steroid metabolites in urine. *J Chromatogr*., **1989,** *489*, 23-50.

[27] Chung, B.C.; Choo, H.Y.; Kim, T.W., Eom, K.D.; Kwon, O.S.; Suh, J.; Yang, J.; Park, J. Analysis of anabolic steroids using GC/MS with selected ion monitoring. *J. Anal. Toxicol*., **1990**, *14*, 91-95

[28] Saugy, M.; Cardis, C.; Robinson, N.; Schweizer, C. Test methods: anabolics. *Baillieres Best Pract Res Clin Endocrinol Metab*., **2000**, *14*(1), 111-133.

[29] Le Bizec, B.; Gaudin, I.; Monteau, F.; Andre, F.; Impens, S.; De Wasch, K.; De Brabander, H. Consequence of boar edible tissue consumption on urinary profiles of nandrolone metabolites. I. Mass spectrometric detection and quantification of 19-norandrosterone and 19-noretiocholanolone in human urine. *Rapid Commun. Mass Spectrom.*, **2000**, *14*, 1058-1065.

[30] Kokkonen, J.; Leinonen, A.; Tuominen, J.; Seppälä, T. Comparison of sensitivity between gas chromatography-low-resolution mass spectrometry and gas chromatography-high-resolution mass spectrometry for determining metandienone metabolites in urine. *J. Chromatogr. B Biomed. Sci. Appl*., **1999**, *734*, 179-189.

[31] Gambelunghe, C.; Sommavilla, M.; Rossi, R. Testing for nandrolone metabolites in urine samples of professional athletes and sedentary subjects by GC/MS/MS analysis. *Biomed. Chromatogr*., **2002,** *16*(8), 508-512.

[32] Hintikka, L.; Haapala, M.; Franssila, S.; Kuuranne, T.; Leinonen, A.; Kostiainen, R. Feasibility of gas chromatography-microchip

atmospheric pressure photoionization-mass spectrometry in analysis of anabolic steroids. *J. Chromatogr. A.*, **2010**, *1217*(52), 8290-8297.

[33] Georgakopoulos, C.; Vonaparti, A.; Stamou, M.; Kiousi, P.; Lyris, E.; Angelis, Y.S.; Tsoupras, G.; Wuest, B.; Nielen, M.W., Panderi, I.; Koupparis, M. Preventive doping control analysis: liquid and gas chromatography time-of-flight mass spectrometry for detection of designer steroids. *Rapid Commun. Mass Spectrom.*, **2007**, *21*, 2439-2446.

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

[35] Mitrevski, B.S.; Wilairat, P.; Marriott, P.J.. Evaluation of World Anti-Doping Agency criteria for anabolic agent analysis by using comprehensive two-dimensional gas chromatography–mass spectrometry. *Anal. Bioanal. Chem.*, **2010**, *396*, 2503-2511.

[36] Pozo, O.J.; Van Eenoo, P.; Deventer, K.; Delbeke, F.T. Ionization of anabolic steroids by adduct formation in liquid chromatography electrospray mass spectrometry. *J. Mass Spectrom.*, **2007**, *42*, 497–516.

[37] Pozo, O.J.; Deventer, K.; Van Eenoo, P.; Delbeke, F.T. Presence of endogenous interferences in the urinary detection of selected anabolic steroids by liquid chromatography/electrospray tandem mass spectrometry *Rapid Commun. Mass Spectrom.*, **2007**, *21*, 2785–2796.

[38] Hintikka, L.; Kuuranne, T.; Leinonen, A.; Thevis, M.; Schänzer, W.; Halket, J.; Cowan, D.; Grosse, J.; Hemmersbach, P., Nielen, M.W.; Kostiainen, R. Liquid chromatographic-mass spectrometric analysis of glucuronide-conjugated anabolic steroid metabolites: method validation and interlaboratory comparison. *J Mass Spectrom.*, **2008**, *43*(7), 965-973.

[39] Pozo, O.J.; Van Eenoo, P.; Van Thuyne, W.; Deventer, K.; Delbeke, F.T. Direct quantification of steroid glucuronides in human urine by liquid chromatography–electrospray tandem mass spectrometry. *J. Chromatogr. A.*, **2008**, *1183*, 108–118.

[40] Pozo, O.J.; Van Eenoo, P.; Deventer, K.; Grimalt, S.; Sancho, J.V.; Hernandez, F.; Delbeke, F.T. Collision-induced dissociation of 3-keto anabolic steroids and related compounds after electrospray ionization. Considerations for structural elucidation. *Rapid Commun. Mass Spectrom.*, **2008,** *22*, 4009–4024

[41] Pozo, O.J.; Van Thuyne, W.; Deventer, K.; Van Eenoo, P.; Delbeke, F.T. Elucidation of urinary metabolites of fluoxymesterone by liquid chromatography-tandem mass spectrometry and gas chromatography-mass spectrometry. *J. Mass Spectrom.*, **2008**, *43*(3), 394-408.

[42] Georgakopoulos, C.G.; Vonaparti, A.; Stamou, M.; Kiousi, P.; Lyris, E.; Angelis, Y.S.; Tsoupras, G.; Wuest, B.; Nielen, M.W.F.; Panderi, I.; Koupparis, M. Preventive doping control analysis: liquid and gas chromatography time-of-flight mass spectrometry for detection of designer steroids. *Rapid Commun. Mass Spectrom.*, **2007,** *21*, 2439–2446.

[43] Thevis, M.; Geyer, H.; Mareck,U.; Schanzer, W. Screening for unknown synthetic steroids in human urine by liquid chromatography-tandem mass spectrometry. *J. Mass Spectrom.*, **2005,** *40*, 955–962.

[44] Virus, E.D.; Sobolevsky, T.G.; Rodchenkov, G.M. Introduction of HPLC/orbitrap mass spectrometry as screening method for doping control. *J. Mass Spectrom.*, **2008,** *43*, 949–957.

[45] Borges, C.R.; Miller, N.; Shelby, M.; Hansen, M.; White, C.; Slawson, M.H.; Monti, K.; Crouch D.J. Analysis of a Challenging Subset of World Anti-Doping Agency-Banned Steroids and Antiestrogens by LC–MS–MS. *J. Anal. Toxicol.*, **2007**, *31*, 125-131.

[46] Danaceau, J.P.; Morrison M.S.; Slawson, M.H. Quantitative confirmation of testosterone and epitestosterone in human urine by LC/Q-ToF mass spectrometry for doping control. *J. Mass Spectrom.*, **2008,** *43*, 993–1000.

[47] Cawley, A.T.; Flenker, U. The application of carbon isotope ratio mass spectrometry to doping control. *J. Mass Spectrom.*, **2008**; *43*, 854–864.

[48] Cawley, A.T.; Trout, G.J.; Kazlauskas, R.; Howe, C.J.; George, A.V. Carbon isotope ratio (delta13C) values of urinary steroids for doping control in sport. *Steroids*, **2009,** *74*(3), 379-392.

[49] Piper, T.; Mareck, U.; Geyer, H.; Flenker, U.; Thevis, M.; Platen, P.; Schänzer, W. Determination of 13C/12C ratios of endogenous urinary steroids: method validation, reference population and application to doping control purposes. *Rapid Commun Mass Spectrom.*, **2008,** *22*(14), 2161-2175.

[50] Kokkonen, J.; Leinonen, A.; Tuominen, J.; Seppälä, T. Comparison of sensitivity between gas chromatography-low-resolution mass spectrometry and gas chromatography-high-resolution mass spectrometry for determining metandienone metabolites in urine. *J. Chromatogr. B Biomed. Sci. Appl.*, **1999**, *734*, 179-189.

[51] Le Bizec, B.; Gaudin, I.; Monteau, F.; Andre, F.; Impens, S.; De Wasch, K; De Brabander, H. Consequence of boar edible tissue consumption on urinary profiles of nandrolone metabolites. I. Mass spectrometric detection and quantification of 19-norandrosterone and 19-noretiocholanolone in human urine. *Rapid Commun. Mass Spectrom.*, **2000**, *14*, 1058-1065.

[52] Kolmonen, m.; Leinonen, A.; Pelander, A.; Ojanper, I. A general screening method for doping agents in human urine by solid phase extraction and liquid chromatography/ time-of-flight mass spectrometry. *Anal. Chim. Acta.*, **2007**, *585*, 94–102.

[53] Virus, E.D.; Sobolevsky, T.G.; Rodchenkov, G. M. Introduction of HPLC/orbitrap mass spectrometry as screening method for doping control. *J. Mass Spectrom.*, **2008**, *43*, 949-957.

[54] Catlin, D.H.; Sekera, M.H.; Ahrens, B.D.; Starcevic, B.; Chang, Y.C.; Hatton, C.K. Tetrahydrogestrinone: discovery, synthesis, and detection in urine. *Rapid Commun. Mass Spectrom.*, **2004,** *18*, 1245-1249.

[55] Zierau,O.; Lehmann, S.; Vollmer, G.; Schanzer, W.; Diel, P. Detection of anabolic steroid abuse using a yeast transactivation system. *Steroids*, **2008**, *73*, 1143–1147.

[56] Nielen, M.W.F.; Bovee, T.F.H.; van Engelen, M.C.; Rutgers, P.; Hamers, A.R.M.; van Rhijn, J.A.; Hoogenboom L.A.P. Urine Testing for Designer Steroids by Liquid Chromatography with Androgen Bioassay Detection and Electrospray Quadrupole Time-of-Flight Mass Spectrometry Identification. *Anal. Chem.*, **2006**, *78*, 424-431.

[57] Lootens, L.; Van Eenoo, P.; Meuleman, P.; Leroux-Roels, G.; Delbeke, F.T. The uPA(+/+)-SCID Mouse with Humanized Liver as a Model for *in vivo* Metabolism of 4-Androstene-3,17-dione. *Drug Metab. Disposit.*, **2009**, *37*(12), 2367-2374.

[58] Lootens, L.; Meuleman, P.; Pozo, O.J.; Van Eenoo, P.; Leroux-Roels, G; Delbeke, F.T. uPA+/+-SCID Mouse with Humanized Liver as a Model for *In vivo* Metabolism of Exogenous Steroids: Methandienone as a Case Study. *Clin. Chem.*, **2009**, *55*(10), 1783-1793.

[59] Pozo, O.J.; Van Eenoo, P.; Deventer, K.; Lootens, L.; Van Thuyne, W.; Parr, M.K.; Schanzer, W.; Sancho, J.V.; Hernandez, F.; Meuleman, P.; Leroux-Roels, G; Delbeke, F.T. Detection and Characterization of a New Metabolite of 17-Methyltestosterone. *Drug Metab. Disposit.*, **2009**, *37*(11), 2153–2162.

[60] Pozo, O.J.; Van Eenoo, P.; Deventer, K.; Lootens, L.; Grimalt, S.; Sancho, J.V.; Hernandez, F.; Meuleman, P.; Leroux-Roels, G; Delbeke, F.T. Detection and structural investigation of metabolites of stanozolol in human urine by liquid chromatography tandem mass spectrometry. *Steroids*, **2009**, *74*, 837-852.

[61] Lootens, L.; Van Eenoo, P.; Meuleman, P.; Pozo, O.J.; Van Renterghem, P.; Leroux-Roels, G; Delbeke, F.T. Steroid metabolism in chimeric mice with humanized liver. *Drug Test Anal.*, **2009**, *11-12*, 531-537.

[62] World Antidoping Agency. Harmonization Of Analysis And Reporting of 19-Norsteroids Related To Nandrolone, TD 2010NA. http://www.wada-ama.org. [Accessed 01/12/2010].

[63] World Antidoping Agency. Reporting And Evaluation Guidance for Testosterone, Epitestosterone, T/E Ratio And Other Endogenous Steroids. TD2004EAAS. http://www.wada-ama.org. [Accessed 01/12/2010].

[64] Mareck, U.; Geyer, H.; Opfermann, G.; Thevis, M.; Schanzer, W. Factors influencing the steroid profile in doping control Analysis *J. Mass Spectrom.*, **2008**, *43*, 877-891.

[65] Van Renterghem, P.; Van Eenoo, P.; Geyer, H.; Schanzer, W.; Delbeke, F.T. Reference ranges for urinary concentrations and ratios of endogenous steroids,which can be used as markers for steroid misuse, in a Caucasian population of athletes. *Steroids*, **2010**, *75*, 154–163.

[66] Van Renterghem, P.; Van Eenoo, P.; Delbeke, F.T. Population based evaluation of a multi-parametric steroid profiling on administered endogenous steroids in single low dose. *Steroids*, **2010**, *75*, 1047–1057.

[67] Hepler, B.R.; Isenschimid, D.S. Specimen selection, collection, preservation, and security. In: *Drug Abuse Handbook*, Karch, S.B., Ed.; CRC Press, Boca Raton, **1998**, pp. 873-879.

[68] Brinkmann, B. Harmonisation of medico-legal autopsy rules. *Int. J. Legal Med*., **1999**, *113*, 1-14.

[69] Petersson, A.; Garle, M.; Holmgren, P.; Druid, H.; Krantz, P.; Thiblin, I. Toxicological findings and manner of death in autopsied users of anabolic androgenic steroids. *Drug Alcohol Depend*., **2006**, *81*(3), 241-249.

[70] Kintz, P. Testing for anabolic steroids in hair: a review. *Leg. Med. (Tokyo)*., **2003**, 5(Suppl 1), S29-33.

[71] Deshmukh, N.; Hussain, I.; Barker, J.; Petroczi, A.; Naughton, D.P. Analysis of anabolic steroids in human hair using LC-MS/MS. *Steroids*, **2010**, *75*(10), 710-714.

[72] Bresson, M; Cirimele, V.; Villain, M.; Kintz, P. Doping control for metandienone using hair analyzed by gas chromatography-tandem mass spectrometry. *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.*, **2006**, *836*(1-2), 124-128.

[73] Kintz, P.; Cirimele, V.; Dumestre-Toulet, V.; Villain, M.; Ludes, B. Doping control for methenolone using hair analysis by gas chromatography-tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci.*, **2002**, *766*(1), 161-167.

[74] Kintz, P.; Cirimele, V.; Devaux, M.; Ludes, B. Dehydroepiandrosterone (DHEA) and testosterone concentrations in human hair after chronic DHEA supplementation. *Clin Chem*., **2000**, *46*(3), 414-415.

[75] Kintz, P.; Cirimele, V.; Ludes, B. Discrimination of the nature of doping with 19-norsteroids through hair analysis. *Clin Chem*., **2000**, *46*(12), 2020-2022.

[76] Kintz, P.; Cirimele, V.; Jeanneau, T.; Ludes, B. Identification of testosterone and testosterone esters in human hair. *J Anal Toxicol*., **1999**, *23*(5), 352-356.

[77] Kintz, P.; Cirimele, V.; Dumestre-Toulet, V.; Ludes, B. Doping control for nandrolone using hair analysis. *J Pharm Biomed Anal*., **2001**, *24*(5-6), 1125-1130.

[78] Kintz, P.; Cirimele, V.; Sachs, H.; Jeanneau, T.; Ludes, B. Testing for anabolic steroids in hair from two bodybuilders. *Forensic Sci Int*., **1999**, *101*(3), 209-216.

[79] Höld, K.M.; Borges, C.R.; Wilkins, D.G.; Rollins, D.E.; Joseph, R.E. Jr. Detection of nandrolone, testosterone, and their esters in rat and human hair samples. *J. Anal. Toxicol*., **1999**, *23*(6), 416-423.

[80] Cirimele, V.; Kintz, P.; Ludes, B. Testing of the anabolic stanozolol in human hair by gas chromatography-negative ion chemical ionization mass spectrometry. *J. Chromatogr. B. Biomed. Sci. Appl*., **2000,** *740*(2), 265-271.

[81] Rambaud, L.; Monteau, F.; Deceuninck, Y.; Bichon, E.; André, F.; Le Bizec, B. Development and validation of a multi-residue method for the detection of a wide range of hormonal anabolic compounds in hair using gas chromatography-tandem mass spectrometry. *Anal Chim Acta*., **2007**, *586*(1-2), 93-104.

[82] Gambelunghe, C.; Sommavilla, M.; Ferranti, C.; Rossi, R.; Aroni, K.; Manes, N.; Bacci, M. Analysis of anabolic steroids in hair by GC/MS/MS. *Biomed Chromatogr*., **2007**, 21(4), 369-375.

[83] Kintz, P.; Cirimele, V.; Ludes, B. Physiological concentrations of DHEA in human hair. *J. Anal. Toxicol*., **1999**, *23*(6), 424-428.

[84] Segura, J.; Pichini, S.; Peng, S.H.; de la Torre, X. Hair analysis and detectability of single dose administration of androgenic steroid esters. *Forensic Sci Int*., **2000**, *107*(1-3), 347-359.

[85] Thieme, D.; Grosse, J.; Sachs, H.; Mueller, R.K. Analytical strategy for detecting doping agents in hair. *Forensic Sci Int.*, **2000**, *107*(1-3):335-345.

[86] Gambelunghe, C.; Rossi, R.; Ferranti, C.; Rossi, R.; Bacci, M. Hair analysis by GC/MS/MS to verify abuse of drugs. *J Appl Toxicol*., **2005,** *25*(3), 205-211.

[87] Gaillard, Y.; Vayssette, F.; Pépin, G. Compared interest between hair analysis and urinalysis in doping controls. Results for amphetamines, corticosteroids and anabolic steroids in racing cyclists. *Forensic Sci Int*., **2000,** *107*(1-3), 361-379.

[88] Shen, M.; Xiang, P.; Shen, B.; Bu, J.; Wang, M. Physiological concentrations of anabolic steroids in human hair. *Forensic Sci Int.*, **2009,** *184*(1-3), 32-36.

[89] Dumestre-Toulet, V.; Cirimele, V.; Ludes, B.; Gromb, S.; Kintz, P. Hair analysis of seven bodybuilders for anabolic steroids, ephedrine, and clenbuterol. *J Forensic Sci.*, **2002**, *47*(1), 211-214.

Received: December 16, 2010 Revised: January 15, 2011 Accepted: February 07, 2011