BIOANALYSIS ZONE

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Review

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Bioanalysis

Applications and challenges in using LC–MS/MS assays for quantitative doping analysis

LC–MS/MS is useful for qualitative and quantitative analysis of 'doped' biological samples from athletes. LC–MS/MS-based assays at low-mass resolution allow fast and sensitive screening and quantification of targeted analytes that are based on preselected diagnostic precursor–product ion pairs. Whereas LC coupled with high-resolution/high-accuracy MS can be used for identification and quantification, both have advantages and challenges for routine analysis. Here, we review the literature regarding various quantification methods for measuring prohibited substances in athletes as they pertain to World Anti-Doping Agency regulations.

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Zhanliang Wang[‡,1], Jianghai Lu[*,‡,2], Yinong Zhang[1], Ye Tian[2], Hong Yuan[2] & Youxuan Xu[**,2]

[1]Food & Drug Anti-doping Laboratory, China Anti-Doping Agency, 1st Anding Road, ChaoYang District, Beijing 100029, PR China

[2]National Anti-doping Laboratory, China Anti-Doping Agency, 1st Anding Road, ChaoYang District, Beijing 100029, PR China

*Author for correspondence:
Tel.: +86 108 437 6282
Fax: +86 106 498 0526
lujianghai@chinada.cn

**Author for correspondence:
xuyouxuan@chinada.cn

‡Authors contributed equally

LC–MS/MS can be used to measure trace compounds with high sensitivity and selectivity, and it is suitable for quantitation and identification of banned substances in biological samples. Operation of the technique in multiple reaction monitoring (MRM) mode permits rapid screening of multiple [1] small molecules in a single run, so LC–MS/MS is a preferred technique for routine athletic doping analysis.

Advances in measuring high molecular weight peptides and proteins for doping control have been made: high-resolution MS enhances the signal-to-noise ratio and specificity for compound identification by providing elemental fragment composition. Thus, isobaric fragments with different elemental compositions from different molecular portions can be differentiated. Based on the exact mass detected, high-resolution MS (HRMS)/MS was confirmed to be well suited for quantification and offers a better signal-to-noise ratio compared with low-resolution LC–MS/MS.

The World Anti-Doping Agency (WADA) Technical Document 2014 DL (decision limits for threshold substances) [2] established that ten substances had urinary thresholds for competitive sports: 19-norandrosterone (19-NA), 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (THCA), salbutamol, formoterol, glycerol, morphine, cathine (d-norpseudoephedrine), ephedrine, methylephedrine and pseudoephedrine. If found in greater concentrations than WADA limits (threshold + $U_{c\ Max}$ – maximum acceptable combined standard uncertainty values), such compounds are reported as an 'adverse analytical finding' (AAF). Based on global WADA accredited laboratories and relevant rounds of the WADA External Quality Assessment Scheme, the $U_{c\ Max}$ represents a 95% CI finding. Additionally, to ensure that all WADA-accredited laboratories can uniformly report prohibited substances, metabolite(s) or marker(s) as well as provide detection within thresholds, standardized testing methods have established. The WADA Technical Document 2015 minimum required performance levels (MRPL) [3] requires that all laboratories attain a minimal capacity for screening and

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confirmation of doping compounds by establishing thresholds for AAFs. Therefore, as required by WADA TD2014DL and WADA Technical Document 2015 MRPL, quantitative analysis of the banned substance against a threshold and estimation of the substance concentrations (semiquantification) are used for doping tests. As defined by the WADA International Standard for Laboratories (§5.2.4.4), any testing results obtained from hair, nails, oral fluid or other biological material shall not be used to counter AAFs or atypical findings in the urine. Thus, this review is chiefly focused on methods to assay urine samples (see Table 1 for methods).

Quantification of banned substances with & without thresholds

19-norandrosterone

Confirmation of illicit use of nandrolone or other 19-norsteroids is based on confirming that the main urinary metabolite 19-NA (derived from hydrolysis with β-glucuronidase) exceeds 2 ng/ml [4]. The presence of 19-NA in the range of 2–10 ng/ml or as high as 15 ng/ml can be confirmed by GC–MS/(MS) and LC–MS/MS, GC/C/IRMS is employed to distinguish the origination of 19-NA which is endogenous or exogenous. Normally, quantification of 19-NA includes a deuterated internal standard (e.g., d_4-19-NA-glucuronide), a calibration curve that includes the estimated sample concentration or a single calibration point at 10 or 15 ng/ml plus appropriate negative and positive quality control samples. Although required by WADA Technical Document 2014 for Decision Limits [2] that 'the threshold concentration is based on the sum of the glucuronide conjugate (expressed as the free drug) and free drug concentrations,' some suggest that 19-NA sulfates and glucuronides can be measured in human urine [5–9] but that these methods have less LOQ at the ng/ml level and omit hydrolysis. In addition, these methods show the detection difficulty in free form 19-NA. Thus, the ratio between the glucuro- and sulfoconjugate derivatives of 19-NA and 19-noretiocholanolone (19-NET) do not offer more information about the origin of 19-NA or 19-NET. To discriminate endogenous versus exogenous 19-NA or 19-NET [5] ion suppression for each exceeds 50%, with higher RSD. Meanwhile 19-NA and 19-NET separation was not preferred even with a relatively reasonable retention time of 13.57 and 14.02 min, respectively. Thus, screening may not be problematic with sufficient sensitivity for MRPL, but for quantification, ion suppression and separation may increase data variation.

THCA

LC–MS/MS methods for THCA quantification in urine, plasma, hair and oral fluids are summarized in Table 2. THCA and its glucuronide conjugate are major urinary cannabinoid metabolites and the WADA threshold for THCA is 150 ng/ml. Dilute-and-shoot method development and validation for LC–MS/MS to measure urinary THCA has been described in the literature [10–12] and this approach permitted more rapid sample preparation with fewer steps prior to LC–MS/

Table 1. Decision limits for the confirmatory quantification of threshold substances.

Threshold substance	Threshold (T) Nonthreshold (MRPL)	Max. combined standard uncertainty (uc Max) at T		Decision limit (DL)	Ref.
		Absolute	Relative		
19-Norandrosterone	2.0 ng/ml	0.3 ng/ml	15	2.5 ng/ml	[4–9]
THCA	150 ng/ml	15 ng/ml	10	180 ng/ml	[10–21]
Salbutamol	1.0 μg/ml	0.1 μg/ml	10	1.2 μg/ml	[22–24]
Formoterol	40 ng/ml	6.0 ng/ml	15	50 ng/ml	[10,25–27]
Glycerol	4.3 mg/ml	0.65 mg/ml	15	5.3 mg/ml	[28–30]
Morphine	1.0 μg/ml	0.15 μg/ml	15	1.3 μg/ml	[31–41]
Cathine	5.0 μg/ml	0.5 μg/ml	10	6.0 μg/ml	[22]
Ephedrine	10 μg/ml	0.5 μg/ml	5	11 μg/ml	[42–51]
Methylephedrine	10 μg/ml	0.5 μg/ml	5	11 μg/ml	
Pseudoephedrine	150 μg/ml	7.5 ug/ml	5	170 μg/ml	
Clenbuterol†	0.2 ng/ml				[67–73]
Glucocorticoids†	30 ng/ml				[74–76]

†Nonthreshold substances.
MRPL: Minimum required performance levels.

Table 2. Summary of assay characteristics dedicated to THCA from urine, blood, hair and oral fluid.

Analytes	Matrix	IS	Sample preparation	Stationary phase	Mobile phase	Quantification mode	LOD (ng/ml)	Linearity range (ng/ml)	Ref.
THCA	Urine	D_9-THCA	Hydrolysis Dilution	C18, RP, 5 μm	0.1% formic acid/ 0.1% formic acid ACN	ESI+ MRM	6.1	50–400	[10]
THCA	Urine	D_9-THCA	Hydrolysis Dilution	C8, RP, 5 μm	0.001%HAc,1 mM NH_4OAc/ 0.001%HAc,1 mM NH_4OAc MeOH	ESI+, ESI- MRM	0.5	5–40	[11]
THCA	Urine	D_3-THCA	Hydrolysis Filtration	C18, RP, 1.7 μm	0.1% formic acid/ 0.1% formic acid MeOH	ESI- MRM	10	5–3000	[12]
THCA THCAG 5 others	Urine	D_9-THCA	SLE THC column	C18, RP, 5 μmm	10 mM NH_4OAc, pH = 6.15/ 15% MeOH-ACN	ESI- MRM	0.5 0.5	1–100 5–500	[13]
THCA THC	Whole blood	D_3-THC D_3-THCA	PP SPE	C18, RP, 5 μmm	0.1% formic acid/ 0.1% formic acid ACN	ESI+/- MRM	0.1	0.25–50	[14]
THCA 2 others	Whole blood	D_3-THCA	PP On-line SPE	C18, RP,	ACN/water/formic acid, 30/70/0.01, v/v	ESI+ MRM	1.5	2.5–100	[15]
THCA 2 others	Whole blood	D_3-THCA	SPE	C18, RP, 1.8 μm	2 mM NH_4CO_2,0.1% formic acid/ MeOH	ESI+ MRM	0.5	0.5–100	[16]
THCAG THCA 4 others	Whole blood	D_9-THCA	PP SPE	C18, RP, 5 μm	10 mM NH_4OAc, pH = 6.15/ 15% MeOH-ACN	ESI- MRM	1 1	5–250 1–100	[17]
THCA	Hair	D_3-THCA	MPE	C18, RP, 3 m	5 mM NH_4OAc 0.5% formic acid/ ACN	ESI- MRM	0.1†	0.1–5†	[18]
THCA-derivative	Oral fluid	D_3-THC	SPE	C18, RP, 1.8 μm	5 mM NH_4CO_2, pH = 6.4/ 0.5% formic acid ACN	ESI+ MRM	0.01	0.01–1	[19]
THCA 3 others	Oral fluid	D_3-THCA	PP SPE	C18, RP, 3 μm	10 mM NH_4OAc, pH = 6/ ACN	HRMS HESI-II	0.015	0.015–0.5	[20]
THCA	Oral fluid	D_9-THCA	SPE	C18, RP, 3 μm	0.01% acetic acid/ 0.01% acetic acid MeOH	ESI- MRM	0.009	0.012–1.02	[21]

†pg/mg.

+: Positive ionization; -: Negative ionization; ACN: Acetonitrile; HESI: Heated electrospray ionization source; HRMS: High-resolution mass spectrum; MeOH: Methanol; MPE: Micropulverized extraction; MRM: Multiple reaction monitoring; PP: Protein precipitation; SLE: Supported-liquid extraction; SPE: Solid-phase extraction; THC: Δ9-tetrahydrocannabinol; THCA: 11-nor-9-carboxy-THC; THCAG: THCA-glucuronide; THCG: THC-glucuronide.

MS analysis. Also, there were no absolute matrix effects (ME) for universal columns (96–101%) (Bond Elut Plexa columns) whereas Styre Screen THC columns had substantial ion suppression (37–56%), suggesting that universal sorbent allowed fewer matrix components to pass through [12]. Scheidweiler [13] reported a method to quantify Δ^9-tetrahydrocannabinol (THC), 11-hydroxy-THC (11-OH-THC), THCA, cannabidiol, cannabinol, THC-glucuronide and THCA-glucuronide in 0.5 ml of human urine via supported-liquid extraction. Compared with analysis of urine, plasma THCA sample preparation is less complicated. SPE is required for purification [14–17] and protein precipitation [14,15,17] is used for sample clean-up. Schwope *et al.* [17] developed and validated a sensitive and specific LC–MS/MS method for simultaneous detection of free and glucuronidated cannabinoids in human whole blood. Linearity ranged from 1.0 to 100 µg/l THCA and 5.0–250 µg/l THCA-glucuronide. Both methods [13,17] were well validated but a lack of a commercially available deuterated THC-glucuronide and THCCOOH-glucuronide to use as an internal standard or for quality control may limit implementation. For long-term detection of cannabinoids abuse, an LC–MS/MS method [18] for hair analysis was developed and the LOQ was validated for THCA at 0.2 pg/mg using a pretreatment method with alkaline dissolution and micropulverized extraction with a stainless bullet. Three LC–MS/MS methods reported in the literature have been used to measure THCA in human oral fluids (LOQs of 10, 15 and 12 pg/ml, respectively) [19–21] and one of the methods required derivatization to achieve LOQ but the other two methods did not [20,21]. All three methods required an isotope internal standard for quantification to decrease ME.

Salbutamol

The salbutamol threshold is set 1000 ng/ml [2] and a simple and sensitive LC–MS/MS [22] method has been developed and validated for measuring seven threshold substances including salbutamol in human urine. For this method, urine samples were diluted with acetonitrile (8:2, v/v) and centrifuged and supernatants were injected into an LC–MS/MS system. For measuring salbutamol and other banned substances, however, an additional enzymatic hydrolysis with β-glucuronidase from *Escherichia coli* was performed prior to sample dilution as required by WADA [3] and because the concentration is the sum of the glucuronide conjugate and the free drug. Another highly sensitive LC–MS/MS assay [23] for measuring salbutamol in plasma and in urine was developed and validated over concentrations 0.05–100 ng/ml in plasma and from 0.18 to 135 ng/ml in urine. A sensitive chiral LC–MS/MS method [24] was developed for quantifying salbutamol enantiomers in human plasma and urine but when separation is not required the chiral inversion is not an issue.

Formoterol

The threshold of formoterol is 40 ng/ml [2]. Recently, several papers focused on formoterol quantification in human urine and this included enzymatic hydrolysis of the samples which were then subjected to liquid–liquid extraction (LLE) [25] or to dilution with acetonitrile [10,26,27]. Inhaled formoterol was detected in the urine up to 72 h [25] and ion suppression averaged 30% (RSD 19%) across the six urine samples. This high average may be explained by a relatively large matrix extracted by the mixture of diethyl ether/isopropanol. After correcting formoterol with formoterol-d_6, ion suppression was reduced to -3.4% (RSD 3.5%). Therefore, the deuterated internal standard was applied to formoterol-d_6 [26,10] and $^{13}C,^{2}H_3$-formoterol [27]. Applying an LC–MS/MS system with an aqueous urinary matrix allowed analysis of urinary formoterol, requiring no extraction or preconcentration steps [10,26,27]. Acetonitrile was used for protein precipitation in the dilution step and the maximum urinary concentration detected was 15 ng/ml [26] (free + glucuronide) after enzymatic hydrolysis which does not approach the MRPL established in 2010, so the MRPL for formoterol was modified to 30 ng/ml in 2012 and this is presently 40 ng/ml.

Glycerol

Glycerol can be used as a plasma volume expander to mask doping so WADA has prohibited its use in competitive sports. In September 2013, a glycerol threshold was set at 4.3 mg/ml [2] because the previous threshold (1.3 mg/ml) was more prone to false positives [28]. Glycerol is not expected to have an AAF and an accurate, precise and sensitive method with LC–MS was proposed for measuring it using simple urine sample preparation including a 10-fold urine dilution followed by injection into the LC coupled with high-resolution/high-accuracy MS (LC–HRMS) (dilute-and-shoot). [29] This assay offered more rapid run time, easy workup and expanded linearity ranges as the internal isotope standard (D_5-glycerol) calibration curves had linear calibration ranges of 1–1000 µg/ml. The ME for the analyte was not significant using LC–MS/MS and study of the application [30] describes screening by LC–HRMS based on monitoring metal adducts $[M+Na]^+$ and confirmation using GC-EI-MS. Based on the maximum endogenous urinary concentration which was previously encountered in blank urines (99.9 percentile of male and female athletes, the

cutoff for the screening method was set to 1.5 mg/ml). Quantitative confirmatory analysis of the real case sample by GC–MS showed a final concentration of 12.6 mg/ml which is significantly greater than the DL.

Morphine

WADA takes the position [2] that the presence of morphine is an AAF and this is quantified as morphine, morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) in urine that exceeds 1 µg/ml unless it is arises from permissible codeine use. Without hydrolysis and derivatization LC–MS methods for doping detection can directly quantify intact urinary glucuronides after one-step dilution as a sample preparation step [22,31–34] (Table 3). For plasma and serum, protein precipitation and an automatic 96-well plate SPE [35–38] are needed for sample purification. DBS [39,40] can be used as well and they require a re-extraction step with a methanol and water solution. LC-separation of morphine, M3G and M6G is typically performed with reverse-phase (RP) C18 columns in some WADA accredited laboratories but this method is limited by the fact that peaks shapes influence integration in quantification. Kolmonen's group [41] reported a hydrophilic interaction LC-time-of-flight MS method for measuring and confirming morphine, codeine, M3G, M6G and codeine-6-glucuronide. Selectivity and baseline separation of glucuronide conjugates was achieved with HILIC and retention of polar compounds was correlated with analyte elution order. That is, more polar glucuronide conjugates eluted last so codeine and morphine eluted prior to morphine glucuronides and had better separation and peak shapes.

Cathine, ephedrine, methylephedrine & pseudoephedrine

Ephedrine, pseudoephedrine, norpseudoephedrine (cathine) and methylephedrine appear on the WADA Prohibited List with specific threshold values of the 10, 150, 5 and 10 µg/ml, respectively for doping analysis (Table 4). To achieve baseline chromatographic separation of pseudoephedrine and its metabolite, norpseudoephedrine, which are ephedrine and norephedrine diastereomers, respectively, only one dilution step is needed for LC–MS or LC–MS/MS [22,42,43]. For plasma and serum samples, protein precipitation requires dilution with methanol [44] or acetonitrile [45,46,49], or LLE [47,48]. RPLC is a method of choice for urine [22,43,50], plasma and serum ephedrine separations [43–45,49]. An insufficient diastereomeric separation may decrease quantification accuracy so for complete separation, mobile phases contain small amounts of formate to improve retain polar bases or organic additives for attaining acceptable peaks. Thus, retention time drifts or ion suppression/enhancement caused by stationary phase dewetting appeared with highly aqueous mobile phases. Due to both hydrophilicity and alkalinity, ephedrines require complex separation of isomers and related substances. Poor analyte retention and large tailing factors previously discussed complicate identification of the peak end, confounding reliable peak integration and quantification. Gray's group [50] investigated the use of high pH (9.8) for simultaneous quantification of urinary ephedrines using LC–MS/MS and better chromatographic performance was noted: symmetric and smooth peak shapes and good retention permitted preferred chromatographic resolution of the diastereoisomers. Also, [51] this group compared high-pH RP and HILIC conditions for accurate and robust quantitative LC–MS analysis of ephedrines for doping assays. HILIC columns offered better peak shape and enhanced sensitivity with ESI-MS detection but HILIC has expensive solvents and a lengthy system balance requirement to acquire repeatable results.

Testosterone & endogenous androgenic anabolic steroids

Testosterone (T) is an endogenous anabolic, androgenic steroid (EAAS) [52] and belongs to class S1.1 b of WADA prohibited substances. Despite restrictions, T is one of the most commonly used performance-enhancing drugs. Epitestosterone (E) is an endogenously produced biologically inactive epimer of T (not a metabolite) so it is an analytical challenge for doping assays. The testosterone/epitestosterone ratio (T/E) is the most well-established biological marker for T abuse and T/E ratios exceeding than 4 are considered indicative of T abuse and require subsequent confirmation. The T/E ratio has been traditionally measured with GC–MS after enzymatic hydrolysis, extraction and derivatization in WADA accredited laboratories. LC–MS/MS is an alternative method, using the negative ionization mode due to the acidity of the glucuronide and sulfate moiety [53,54] or the positive ionization mode because of the proton affinity of the steroid keto functional group (T and ET) [55,56], as shown in Table 5. Different extraction steps have been described for LLE [54], or SPE [53,55,56]. Badoud's group [53] reported a sensitive and selective method for simultaneous quantification of major urinary metabolites after T intake using ultra high-pressure LC coupled to quadrupole time-of-flight mass spectrometer. Wudy's group [55] reported a novel method for quantification of 11 intact steroid sulfates in human serum using LC–MS/MS and the assay offered linearity ($R^2 > 0.99$) and suitable recovery for all compounds, with LOQs ranging between 1 and

Table 3. Summary of assay characteristics dedicated to Morhine from urine, serum, plasma and DBS.

Analytes	Matrix	IS	Sample preparation	Stationary phase	Mobile phase	Quantification mode	LOD (ng/ml)	Linearity range (ng/ml)	Ref.
M and 6 others	Urine	D_3-M and 3 others	Dilution	C18, RP, 5 μm	0.1% formic acid/ 0.1% formic acid ACN	ESI+ MRM	32	100–2000	[22]
M, M3G M6G	Urine	D_3-M, D_3-M3G D_3- M6G	Dilution	C8, RP, 5 μm	0.1% HOAc,1 mM NH_4OAc / MeOH	ESI+ MRM	50	50–2000	[31]
M, M3G M6G, 17 others	Urine	D_6-M, D_3-M3G D_3- M6G and 17 others	Dilution	C18, RP,1.8 μm	0.1% formic acid, 2 mM NH_4OAc / 0.1% formic acid ACN	ESI+ MRM	10 50 50	10–1000	[32]
M and 6 others	Urine	D_3-M and 6 others	Hydrolysis Dilution	C18, RP, 5 μm	10 mM NH_4CO_2, 0.5 ml formic acid/ MeOH	ESI+ MRM	60	60–30,000	[33]
M3S M6S	Urine Plasma	D_3-M	Dilution PP	C18, RP,1.8 μm	0.1% formic acid/ MeOH	ESI+ MRM	50, 45.4 5, 4.54	50–5000, 45.4–4544; 5–500, 4.5–454	[34]
M, M3G M6G, 2 others	Serum	D_6-M, D_3-M3G D_3- M6G	PP 96-well	PFP, RP, 2.6 μm	0.1% formic acid/ 0.1% formic acid MeOH	ESI+ MRM	5	5–1000	[35]
M, M3G M6G, 3 others	Plasma	D_3-M	SPE	C18, RP, 5 μm	0.1% formic acid/ ACN	ESI+ MRM	3.5	3.5–700	[36]
M, M6G, 2 others	Whole blood	D_6-M, D_3- M6G	SPE	Chiral column 5 μm	10 mM NH_4OAc, pH 7.0/ ACN, 22:78 v/v	TOFMS+	0.005 0.003	0.02–1 0.04–0.8	[37]
M, M3G M6G	Plasma	D_3-M	PP 96-well		0.1% formic acid/ 0.1% formic acid MeOH	ESI+ MRM	2.4 1.2 2.5	2.5–500 1.25–250 2.5–500	[38]
M, M3G M6G	Plasma DBS	D_3-M, D_3-M3G D_3- M6G	PP Extraction solvent	C18, RP, 2.6 μm	0.1% formic acid/ 0.1% formic acid ACN	ESI+ MRM	0.1, 0.5 0.25, 0.5	0.25–1000, 1–1000 1–1000, 2.5–1000	[39]
M	DBS	D_3-M	Extraction solvent	C18, RP,3 μm	5mM NH_4CO_2, 0.1% formic acid/ACN	ESI+ MRM	0.4	4–1000	[40]
M, M3G M6G, 2 others	Urine	D_3-M, D_3-M3G D_3- M6G, 2 others	SPE	HILIC, 3.5 μm	10 mM NH_4CO_2, pH = 6.4/ ACN	TOFMS+	50	50–5000	[41]

+: Positive ionization; -: Negative ionization; ACN: Acetonitrile; GP: Firard reagent; HILIC: Hydrophilic interaction liquid chromatography; M3G: Morphine-3-glucuronide; M6G: Morphine-6-glucuronide; M3S: Morphine-3-sulfate; M6S: Morphine-6-sulfate; MeOH: Methanol; MRM: Multiple reaction monitoring; PP: Protein precipitation.

Table 4. Summary of assay characteristics dedicated to ephedrines from urine, serum and plasma.

Analytes	Matrix	IS	Sample preparation	Stationary phase	Mobile phase	Quantification mode	LOD (µg/ml)	Linearity range (µg/ml)	Ref.
EP, PEP, NPE, MEP	Urine	D_3-EP	Dilution	C18, RP, 5 µm	0.1% formic acid/ 0.1% formic acid ACN	ESI+ MRM	0.94 3.21 0.44 0.91	5–25 50–500 1–20 5–25	[22]
EP, PEP, NPE, MEP 11 others	Urine	D_3-Octopamide	Dilution	HILIC column, 3 µm	Water/ACN/200 mM NH_4OAc, 0.15% glacial acetic acid	Orbitrap HESI-II+ Full scan and t-HCD	10×10^{-3}	$10–200 \times 10^{-3}$	[42]
EP, PEP	Urine Plasma	D_3-Diphenhydramine	Dilution LLE	C18, RP, 2.7 µm	5mM NH_4CO_2, 0.1% formic acid Water-MeOH(8%)/ACN	ESI+ MRM	0.5×10^{-3} 0.2×10^{-3}	$1–100 \times 10^{-3}$ $1.5–200 \times 10^{-3}$	[43]
EP PEP NPE MEP	Whole blood	D_3-NPE	PP Filter	C18, RP, 5 µmm	0.1% formic acid/ 0.1% formic acid MeOH	ESI+ MRM	0.03	0.03–1.2	[44]
PEP	Plasma	Chlorpheniramine	PP	C18, RP,	0.1% formic acid/ MeOH	ESI+ MRM	0.01	0.078–5	[45]
EP and 11 others	Plasma	Reserpine	Dilution PP	C18, RP,3 µm	0.1% formic acid,5 mM NH_4OAc/ 0.1% formic acid ACN	ESI+ MRM	0.1	0.1–10	[46]
PEP NPE MEP	Whole blood	D_3-PEP D_3-NPE	LLE	C18, RP, 5 µm	50 mM NH_4CO_2, 0.1% formic acid/ 0.1% formic acid ACN	ESI+ MRM	0.01	0.01–1	[47]
PEP and 4 others	Plasma	Diphenhydramine	LLE	C18, RP, 5 µm	Formic acid:10 mM NH_4OAc:MeOH (1:40:60, v/v)	ESI+ MRM	0.00025	$0.25–100 \times 10^{-3}$	[48]
EP, PEP, NPE, MEP, 4 others	Rat plasma	Diphendydramine puer	PP	C18, RP, 1.8 µm	0.1% formic acid/ ACN	ESI+ MRM	0.0025 0.0013 0.0025 0.0005	0.0025–1.012 0.0013–0.506 0.0025–0.1004 0.0005–0.2072	[49]
EP, PEP, NPE, MEP	Urine	D_3-norephedrine	Dilution	C18, RP, 2.5 µm	10 mM NH_4OAc, pH = 9.8/ 10 mM ammonium bicarbonate pH = 9.8 in 60% MeOH	APCI+ MRM	2.5 25 1 2.5	2.5–40 25–400 1–20 2.5–40	[50]
EP, PEP, NPE, MEP	Urine	D_3-norephedrine	Dilution	HILIC Column 1.7 µm	ACN:water:200 mM NH_4OAc, 40:55:5, pH = 5/ ACN:200 mM NH_4OAc, 95:5, pH = 5	TOFMS+	2.5 37.5 1.25 2.5	2.5–20 37.5–300 1.25–10 2.5–20	[51]

+: Positive ionization; -: Negative ionization; ACN: Acetonitrile; EP: Ephedrine; HILIC: Hydrophilic interaction liquid chromatography; LLE: Liquid–liquid extraction; MEP: Methylephedrine; MeOH: Methanol; MRM: Multiple reaction monitoring; NPE: Norpseudoephedrine; PEP: Pseudoephedrine; PP: Protein precipitation.

Table 5. Summary of assay characteristics dedicated to testosterone and endogenous anabolic, androgenic steroid from urine, serum, plasma, saliva and hair.

Analytes	Matrix	IS	Sample preparation	Stationary phase	Mobile phase	Quantification mode	LOD (ng/ml)	Linearity range (ng/ml)	Ref.
TG, TS and 9 others	Urine	D_3-TG, D_3-TS and 7 others	SPE HLB	C18, RP, 1.7 μm	0.1%formic acid/ ACN	TOFMS-	1.0	2–200	[53]
TG and 3 others	Urine	D_3-TG and 3 others	LLE acidic	C18, RP, 1.8 μm	1 mM ammonium acetate, 0.1% formic acid/ MeOH	ESI+ MRM	0.05	1.25–200	[54]
TS and 10 others	Serum	D_3-TS and 5 others	SPE HLB	C18, RP, 2.6 μm	10 mM ammonium acetate/ MeOH-ACN	ESI- MRM	1.0	1–500	[55]
TG and 5 others	Urine	D_3-TS and 5 other	SPE	C18, RP, 1.7 μm	1 mM ammonium acetate, 0.01% formic acid/ MeOH	ESI+ MRM	0.2	1–100	[56]
T and 3 others	Urine	Methandienone	LLE alkaline	C18, RP, 1.8 μm	1 mM ammonium acetate, 0.01% formic acid/ MeOH	ESI+ MRM	0.006	0.05–500	[57]
T	Serum	D_3-T	LLE	C18, RP, 2.5 μm	0.1% formic acid/ MeOH-ACN	ESI+ MRM	0.01	0.02–20	[58]
T and 4 others	Serum	D_3-T and D_3-DHT	PP 96-well	C18, RP, 1.7 μm	0.1%formic acid/ MeOH	ESI+ MRM	0.4	1–250	[59]
T and 4 others	Plasma Serum	D_3-T and D_3-DHT	LLE 96-well	C18, RP, 3 μm	0.4 mM formic acid MeOH-water/ 0.4 mM formic acid MeOH	ESI+ MRM	0.003	0.006–3.75	[60]
T and 44 others	Equine Plasma	D_3-T and D_3-Stanozolol	LLE	C18, RP, 1.9 μm	1 mM ammonium formate pH = 3.5/ MeOH	H-ESI+ MRM	0.1	0.1–5	[61]
T and 29 others	Hair	D_9-THC	MeOH	C18, RP, 1.8 μm	0.1% formic acid/ ACN	TOFMS+	75†	250–25000†	[62]
T and ET	Hair	D_3-Stanozolol	LLE Alkali digestion	C18, RP, 1.8 μm	Water/ ACN	ESI+ MRM	0.1†	0.25–100†	[63]
T	Saliva	D_5-T	LLE	C8, RP, 2.6 μm	2 mM ammonium acetate /2 mM ammonium acetate MEOH	ESI+ MRM	5‡	5–260‡	[64]
GP-T and 4 others	Follicular fluid	D_5-GP-T	LLE	C18, RP, 1.6 μm	0.1% formic acid/ ACN	ESI+ MRM	9‡	100–10000‡	[65]
T	Serum DBS	D_3-T	96-well LLE	C18, RP, 3 μm	0.1% formic acid/ ACN	ESI+ MRM	0.001 0.04	0.001–5 0.04–10	[66]

†pg/mg.
‡pmol/l.
+: Positive ionization; -: Negative ionization; ACN: Acetonitrile; GP: Firard reagent; LLE: Liquid–liquid extraction; MeOH: Methanol; MRM: Multiple reaction monitoring, PP: Protein precipitation; TG: Testosterone-glucuronide; TS: Testosterone-sulfate.

80 ng/ml. An UHPLC–MS/MS method for direct quantification of two T metabolites (3a-glucuronide-6b-hydroxyandrosterone and 3a-glucuronide-6b-hydroxyetiocholanolone) was developed and validated by Pozo's group [54] and this was accomplished with an UHPLC–MS/MS method because three interfering compounds were noted during GC–MS/MS analysis with the most intense ion transitions.

For the free T or its conjugates and other EAAS after hydrolysis, LC–MS/MS methods are available for quantification [57] in urine, plasma and serum [58–61], hair [62,63] and saliva [64]. Fabregat's group [57] described a rapid, accurate and sensitive LC–MS/MS method for measuring T fractions and its metabolites released under basic conditions. Urine samples (5 ml) were alkalinized and released analytes were extracted with LLE and this method was linear from 0.05 to 50 ng/ml for T. Different procedures such as LLE [57,58,60,61], protein precipitation [59] or SPE [59–61] could be implemented for preconcentrating plasma and serum T. For hair specimens, an alkaline step is necessary [62,63]. Feng's group [65] reported a method for the accurate and sensitive detection of seven steroid hormones in the biosynthetic pathway from cholesterol to T using a pair of isotopes as labeling reagents, Girard reagent P and d_5-Girard reagent P. Michal's group [66] presented a method that required a derivatization step involving a novel, permanently charged, quaternary amino-oxy reagent or an MS-tag that reacts to the ketone functionality of T and significantly enhances its ESI-MS/MS sensitivity. For quantification of T and EAAS, LC–MS/MS choices to assay multiple specimens based on target analytes are available.

T misuse is currently monitored by quantifying T, its epimer epitestosterone (E), dihydrotestosterone (DHT) and selected endogenous steroids in human urine using GC–MS or GC–MS/MS. Confirmation analysis by GC-C-IRMS is performed when a urinary T/E >4 threshold is found but this can be influenced by sex, route of T administration, diet, physical exercise and race. Thus, a novel method for monitoring T misuse was to longitudinally measure serum T and E [58,59]. A UPLC–MS/MS method was used with isotopic internal standards to eliminate ion suppression. For some cases, deviation from the urinary steroidal profile given in the Athlete Biological Passport by GC–MS or GC–MS/MS were judged as AAF.

Clenbuterol

With the MRPL of clenbuterol changed from 2 ng/ml to 0.2 ng/ml in the 2013 MRPL TD, the AAFs of clenbuterol have accounted for an explosive increase in 'wada_2013_anti-doping_testing_figures _ report.' Although clenbuterol is not a threshold substance defined by WADA, it occurs too frequently due to food contamination and some AAFs are also caused by the inadvertent doping. Moreover, the relative antidoping authority should try to distinguish different situations based on the estimated clenbuterol concentrations. Thus, the laboratory is often asked to provide the clebuterol concentration, even at the trace level. Clenbuterol can be identified with sensitive assays using LC–MS/MS, particularly with high-resolution/high-accuracy MS. Detection limits of 1 pg/ml were achieved for clenbuterol [67–70] and sensitivity was improved for AAFs of small amounts of clenbuterol after MRPL was modified to 0.2 ng/ml [3]. Because clenbuterol has a history of use in animals, [71] consumption of meat tainted with clenbuterol can produce AAF so food-introduced drug must be differentiated from performance enhancing use, such as confirming clenbuterol enantiomeric composition [72] with LC–MS. Doping use of clenbuterol formulations cause equally abundant isomer pairs at least up to 24 h [73], and the presence of R-(-)-clenbuterol depleted enantiomers corroborate contaminated food ingestion (Figure 1).

Glucocorticoids

Glucocorticoids have no threshold and are prohibited substances by WADA if they exceed 30 ng/ml, the threshold at which each glucocorticoid and its metabolite is reported as an AAF. All glucocorticosteroids are prohibited in competition by all routes of administration other than inhalational. Drug concentration measurement is not required but semiquantification can be used to confirm that the amount does not exceed the reporting limit. An LOQ of at least 15 ng/ml is required for each glucocorticoid and quantification of urinary metabolites of betamethasone [74], budesonide [75] and triamcinolone acetonide [76] have been measured with LC–MS/MS to provide more information about the excretion profile of glucocorticoids given by systemic and nonsystemic routes. The urine excretion of betamethasone metabolites is critical for discriminating between legal and illegal use [74]. This work involved the investigation of markers to discriminate between permitted and forbidden use of betamethasone in doping control analyses. Four metabolites of budesonide (budesonide, 6-hydroxy-budesonide and 6α-hydroxy-budesonide) were used to quantify urinary excretion of orally administered and inhaled treatment to ensure no false-positives after inhalational use or false-negatives after oral intake. Comparing excretion profiles of triamcinolone acetonide and eight metabolites excreted as free and conjugated drug in urine samples helped to define criteria for distinguishing between administration routes as well.

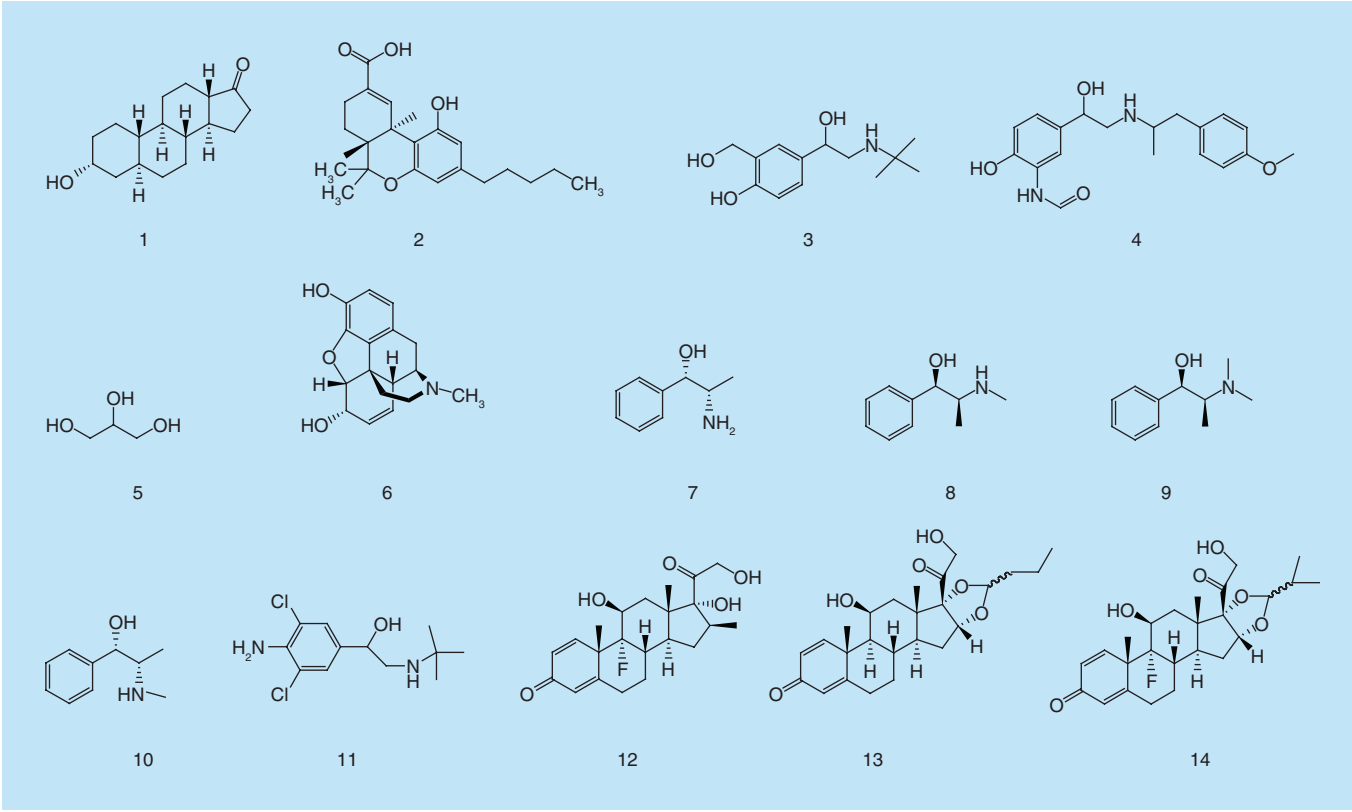

Figure 1. Chemical structure of threshold and nonthreshold substances, 19-norandrosterone (1), THCA (2), salbutamol (3), formoterol (4), glycerol (5), morphine (6), cathine (7), ephedrine (8), methylephedrine (9), pseudoephedrine (10), clenbuterol (11), betamethasone (12), budesonide (13) and triamcinolone acetonide (14).

Challenges in LC–MS/MS

LC–MS/MS allows unambiguous identification and quantification of many low or high molecular weight doping agents in a single run, and significantly reduces cost and time. A precursor/fragment ion pair is referred to as an MRM transition and monitoring unique MRM transitions can greatly increase method sensitivity. Urinary analytes in the low pg/ml range can be achieved depending on the instrument and methodology. Currently, LC–MS/MS has been used to measure doping agents from class S1 to S9 but some limitations for this method remain.

Specificity of interest molecules

For separation of prohibited substances RPLC is the chiefly used method but due to challenges with RP materials, highly polar and charged compounds are difficult to assay. HILIC may help solve this problem because compared with RPLC, polar and hydrophilic analytes are more greatly retained on an HILIC column but few studies exist describing quantification of hydrophilic target analytes like morphine and codeine and their glucuronide [41], cathinone-designer drugs (11 chemicals) [77], etilefrine and oxilofrine [78], ephedrines [79,51] and narcotics [80]. The few studies reported suggest that HILIC requires simple sample preparation, offers good sensitivity and robustness and only requires adequate column equilibration and precondition time. Thus, HILIC may be more frequently applied in doping control laboratories test. Also, HILIC combined with HRMS may be even more useful for detecting highly polar metabolites and biomarkers.

Recently, quantification of Phase II metabolites by LC–MS/MS has been widely used for doping assessment. Specifically, free, glucuronidated and sulfated drug are quantified separately in one run. Phase II metabolites contain an ionizable group that is sensitive in the ESI+ and ESI- modes. However, Phase II metabolites lack quantitative standards because targets have multiple positions for forming metabolites. For example, morphine metabolites M3G and M6G are the same after hydrolysis and this also occurs in steroid metabolites which have more than two conjugation positions. Presently, the best choice for quantification of exogenous and endogenous steroids is GC–MS/MS which is used by WADA laboratories. The LC–MS/MS-based quantification of steroid Phase II metabolites is a direct method and metabolites do not alter the analysis. Routine tests, however, are costly when standards are available and Phase II metabolite assays are usually 5–10-times more

expensive than measuring free drug standards. Few applications for Phase II metabolite quantification is used routinely but with better technology, they will be more commonly measured in the future.

Sample preparation

Skipping the preconcentration sample preparation step with dilute and shoot (DS)-LC–MS/MS depends on two prerequisites. First, compounds with efficient ionization are more easily analyzed with DS-LC–MS/MS. For example, stimulants and opiates contain an easily ionizable nitrogen atom; and diuretics usually include sulfonamide groups; Phase II glucuronide and sulfate-conjugated metabolites, carboxylic acids or amines that can be easily deprotonated or protonated. Next, a relatively high DL or MRPL is required. DS-LC–MS/MS applications have been described in the literature for measuring β_2-agonists, diuretics, hormone and metabolic modulators, stimulants, narcotics, THCA, glucocorticoids and β-blockers in urine and plasma. DS-LC–MS/MS allows direct quantification of the glucuronide conjugate, such as for morphine and THCA. DS-LC–MS/MS does have limited sensitivity due to a dilution step and DS-LC–MS/MS is difficult to use with low DL or MRPL. Thus, decreasing sample preparation steps may improve LC–MS/MS methods.

ME are direct or indirect alterations or interferences due to the presence of unintended analytes (for analysis) or other interfering substances in the sample [81]. ME can either suppress or enhance ions and compromise quantification accuracy. Ion suppression may, at worst, cause false-negative results. A commonly reported main source of interference is endogenous substances such as organic or inorganic molecules in the sample that are retrieved in the final extract. Another source of influence is the presence of exogenous substances from external sources during sample preparation. Presently ME is essential to validation of any LC–MS/MS-based methods that are widely accepted. ME can be assigned to a specific part of the analytical process during sample preparation or ionization steps. Four sets of solutions are prepared for each analyte at the MRPL concentration and ME is calculated [1] which offers a reliable solution with standards spiked in ultrapure water, extracted urine, urine before the extraction step or a neat solution before extraction. The following equation is used to solve this issue:

Process efficiency (PE) = c/a; matrix effect (ME) = b/a; extraction recovery (RE) = c/b; extraction yield (EY) = d/a.

In total, 103 analytes were classified by the type of ME and the signal of most compounds was influenced by the matrix at 66 and 19% of analytes were not altered by coeluting compounds in the matrix during the entire process.

One solution for overcoming ME is to improve chromatographic separation and shift the analyte retention time away from the area affected by suppression. Also, improving sample preparation to reduce interferences in the final extract is helpful. Usually interfering substances coelute with the analytes. Finally, an internal isotope standard is suitable for limiting interference. The deuterated reference substance as internal standards can be used for measuring doping agents with LC–MS or LC–HRMS and accurate product mass may decrease interference. ME cannot be so easily removed, so combining different methods should yield a stable, sensitive and robust quantification method.

Applications of LC–MS/MS quantification should involve a/multi stable-isotope-labeled internal standards to eliminate procedure step effects, including ME, to achieve linear calibration. An ideal internal standard for MS-based methods, SIL analogs of the analytes, are frequently used for quantification. Due to their identical physicochemical properties, SIL analogs can compensate for variability arising from sample preparation or instrumental analysis especially under the influence of ME. If available, at least three deuterium atoms are used as SIL analogs and they have the same extraction behavior and ionization efficiency as the respective undeuterated analytes. For example, D_2-testosterone is not suitable for urinary testosterone quantification [58]. Spiking patient samples with D_2- versus D_3-testosterone significantly increased the ion count of unlabeled testosterone in the linearity test. Quantification methods should have essential tests for selection of stable-isotope-labeled internal standards and its effect on ion suppression or enhancement. Otherwise, these tests must be performed using matrix-based samples from different sources. Only SIL analogs with no observable suppression and enhancement effects should be selected and it not commercially available, so SIL analogs can be synthesized for qualification and quantification analysis to decrease ME. The analysis of JWH-018 in human urine by LC–MS/MS [82] is an example of this technique.

Detection

At present, the MRM-based LC–MS/MS method is the first choice for targeted doping marker quantification. The two-stage MRM is sensitive to low molecular weights and specific to structure. Dwell time hampered MRM quantification methods for simultaneous measurements of many targeted analytes in the past and better instruments with more rapid MRM dwell times have improved this, allowing many MRMs in

one run and sufficient data to acquire a smooth chromatographic peak for precise quantification of more compounds without loss of sensitivity. Rarely, two analytes with 10 or more orders of magnitude of difference in concentration may reduce quantification accuracy when each analyte has a similar retention time. In combination with sample preparation and chromatographic separation, multiple targets will be measured simultaneously. Another challenge is that predefined MRM transition lacks the flexibility of removing endogenous isobaric interference from the matrix. To address this, dominant product ions unique to the analyte must be chosen for the analysis or an adequate chromatographic separation must occur between analytes. Therefore, with good analyte separation MRM-based LC–MS/MS quantification can offer reliable sensitive doping substance quantification.

Quadrupole MS and ion trap MS are considered low resolution (or unit resolution), offering a resolution of 1000–4000 full-width at half-maximum. If the HRMS instrument is properly calibrated and applied, high-mass accuracy (e.g., <5 ppm) can be obtained by HRMS instruments. Then, MS peak assignment is improved and ambiguity is reduced due to coeluting compounds with similar but not identical mass-to-charge ratio. Compound identification confidence is with the isotopic abundance patterns used in mass analysis of complex biological samples. Currently, two widely used HRMS technologies, TOF and orbitrap are used for doping analysis [20,37,42,53,62] and TOFMS can be used to measure long-lasting metabolites by mass spectra. These long detection windows have been used for exogenous steroid detection. Time-to-digital converter detectors have been improved but the limited dynamic range typical of TOF technology reduces its use. Another typical HRMS instrument, orbitrap, which uses heated drying gas as the ESI source and has been applied for doping measurement [42] and data were obtained with high-resolution/accuracy in targeted MS/MS mode by applying individually optimized collision energies to decrease interference and increase accuracy. However, linearity range limits HRMS and slow acquisition speed for MS/MS scans, limited sensitivity and dynamic range reduces LC–HRMS quantification use compared with MRM-based LC–MS/MS methods. Modern orbitraps have improved sensitivity, mass accuracy and linearity range. Thus, doping analysis may more frequently include LC–HRMS/MS [73]. HRMS suffers from interference from compounds with the same mass-to-charge ratio (such as isomers of analytes) so analytes cannot be distinguished. Thus, more widespread combination of chromatographic systems with HRMS instrumentation for doping analysis will be more frequently used in antidoping laboratories.

Conclusion

Faster and more reliable methods have increased confidence in doping assessments. MRM-based LC–MS/MS is a first choice quantifying doping agents

Executive summary

Introduction

- This review introduces the progress of LC–MS/MS quantification in the sport drug.

Quantification of banned substances with & without thresholds

- LC–MS/MS is used to identify and quantify banned sport drugs such as 19-norandrosterone, carboxy-tetrahydrocannabinol (THCA), salbutamol, formoterol, glycerol, morphine, d-norpseudoephedrine, ephedrine, methylephedrine and pseudoephedrine, testosterone, clenbuterol and glucocorticoids.

Challenges in LC–MS/MS

- Analysis of highly polar and charged compounds; matrix effects are common existing in LC–MS/MS quantification method, nowadays situation is how to decrease the effects and acquiring a repeatable quantification results; high-resolution MS in quantification analysis should be an alternative choice in the near future especially in HRMS/MS mode; dilute and shoot represent a tendency for omitting the sample preparation step and short data acquired time; stable-isotope-labeled internal standards are the perfect choice in quantification method especially in LC–MS; phase metabolites as the targets analytes should be a tendency in near future; LC–MS/MS-based multiple reaction monitoring is the first choice for targeted doping markers quantification.

Conclusion

- The multiple reaction monitoring based LC–MS/MS method has become the leading choice for targeted doping agents in quantification, their metabolites or their markers in urine and blood specimen. With the development of modern instrument the direct analysis or simple steps in sample preparation combined with LC–HRMS/MS hold promise for the quantification of doping agents in the near future.

Future perspective

- This review presents the perspective development of LC–MS/MS in the future.

or their metabolites or markers in urine and blood and LC–HRMS/MS holds promise for quantification of doping agents in the future.

Future perspective

Aside from improvements in LC–MS/MS, issues remain with assessing suspected biological samples – such as rapid quantification, appropriate sensitivity and accuracy and the ability to confirm Phase II metabolites as target analytes for doping control. Also, threshold substances should be expanded as should their metabolites. In 2016, MRPL for peptide detection has been effective so including peptides for doping assessment may be used in the future.

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Review

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Bioanalysis

Current status and recent advantages in derivatization procedures in human doping control

Derivatization is one of the most important steps during sample preparation in doping control analysis. Its main purpose is the enhancement of chromatographic separation and mass spectrometric detection of analytes in the full range of laboratory doping control activities. Its application is shown to broaden the detectable range of compounds, even in LC–MS analysis, where derivatization is not a prerequisite. The impact of derivatization initiates from the stage of the metabolic studies of doping agents up to the discovery of doping markers, by inclusion of the screening and confirmation procedures of prohibited substances in athlete's urine samples. Derivatization renders an unlimited number of opportunities to advanced analyte detection.

Ioanna Athanasiadou[1,2], Polyxeni Kiousi[3], Nasia Kioukia-Fougia[3], Emmanouil Lyris[3] & Yiannis S Angelis*[,3]

[1]Laboratory of Biopharmaceutics & Pharmacokinetics, Faculty of Pharmacy, National & Kapodistrian University of Athens, Greece

[2]Anti-Doping Lab Qatar, Sports City Road, Aspire Zone, P.O. Box 27775, Doha, Qatar

[3]Doping Control Laboratory of Athens, Olympic Athletic Center of Athens (OAKA), 15123 Maroussi, Greece

*Author for correspondence:
Tel.: +30 210 686 8412
Fax: +30 210 683 4021
y_angelis@yahoo.gr

Since the introduction of chromatographic methods in the determination of xenobiotics in biological fluids – a field related to veterinary drug analysis, forensic science and doping control analysis – and together with the implementation of chromatographic methods coupled with different types of mass spectrometers for the unquestionable analyte identification in routine analysis, derivatization procedures have played a central and critical role.

The term derivatization is used to describe the procedures in which chemical groups (e.g., protons from protic sites) are replaced by groups that modify physical and chemical properties of analytes, like molecular formula, thermal stability, volatility, polarity, chromatographic behavior, light absorption, ionization efficiency, mass spectra fragmentation, protection of labile groups, etc, thus resulting in more sensitive and robust detection. For a successful derivatization, a single and stable derivative should be formed for each compound and the derivatization reaction should be simply and rapidly performed under mild conditions with a high and reproducible yield.

Excellent handbooks and recent reviews are available on the general advantages of derivatization procedures [1–6].

Hundreds of chemically different drugs are included in the List of Prohibited Substances [7] issued by the World Anti-Doping Agency (WADA). The Prohibited List is revised annually and comprises substances grouped according to their pharmacological action. The demanding task of doping control laboratories is to develop analytical methods able to screen a large number of substances, minimizing by the same token, sample preparation and costs.

Primarily, doping control analysis requires the use of chromatographic methods [8–10], particularly GC–MS and LC–MS. Application of GC–MS and LC–MS for screening purposes is complementary, depending on the properties of the compounds like volatility, polarity, molecular weight, ionization properties etc.

GC–MS has played major role in the doping control analysis for several decades, despite the increasing use of LC systems in doping control laboratories. The screening benefits from GC–MS (e.g., ionization, capillary column chromatographic resolution, etc.) are significant, especially for the low ng/ml detection of anabolic androgenic steroids (AAS), since LC–MS ionization for AAS lacking at least a conjugated keto-

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Key terms

Doping control: Drug testing for performance enhancing substances.

Derivatization: Process of chemical modification of a substance to improve its detection.

Anabolic androgenic steroids: Synthetically produced analogs of the principal male sexual hormone testosterone.

Adverse analytical finding: Report from a WADA-accredited laboratory that identifies the presence of a prohibited substance and/or its metabolites or markers or evidence of the use of a prohibited method in an athlete's sample.

moiety, is not adequate [10]. Moreover, reproducible databases with the mass spectral information of GC electron ionization (EI)/MS of derivatized analytes are widely used [11]. Additionally, the development of quantitative structure–retention relationships for the prediction of relative retention times of different classes of substances including designer steroids and/or their metabolites, stimulants, narcotics as well as β_2-agonists after trimethylsilyl (TMS)-derivatization has been proven useful for the detection of newly synthesized substances, or those that are not available as reference materials for doping control purposes [12].

Over the last decades, LC–MS(/MS) has been evolved into a dominant analytical technique owing this to its high selectivity, sensitivity and versatility [13,14]. In doping control analysis, LC–MS(/MS) has enabled the determination of prohibited substances that are barely detectable or even undetectable by conventional analytical techniques such as GC–MS or immunoassays [15]. Complex and time-consuming sample preparation procedures have been minimized and replaced by faster, more comprehensive and robust methods. Numerous analytical assays based on LC–MS(/MS) detection have been developed and applied for different classes of prohibited substances (e.g., anabolic steroids, diuretics, stimulants, narcotics, β_2-agonists, β-blockers, peptide hormones and proteins) in order to enhance their screening and confirmation analysis.

Derivatization is considered a key step for the enhancement of chromatographic behavior and mass spectrometric detection of analytes. Its application on the screening and confirmatory analysis as well as on metabolic studies can lead to the broadening of the detectable range of doping substances. There are several examples with most of them presented herein of the improvement that derivatization can offer on the analyte detection, even in LC–MS analysis, where derivatization is not a mandatory step. However, due to the laborious character of the derivatization procedures many efforts are also being made toward avoiding its use where applicable and not at the cost of the detection sensitivity. The aim of the present review is to unravel the current status including the advantages and disadvantages of the derivatization procedures specifically applied in the field of doping control analysis. Initially, the main derivatization procedure applied in doping control screening analysis of endogenous and exogenous AAS is presented. An extended description focused on the derivatization procedures implemented on the GC–MS analysis of different classes of doping agents will follow. In this part, a special emphasis has been given on the coverage of new developments in the whole range of doping control activities from the stage of markers discovery of doping agents to the stage of confirmation of a prohibited substance in athlete's samples. Finally, the developments on derivatization procedures for LC–MS doping control analysis are discussed.

Doping control screening procedure & derivatization

Current doping control analysis requires the development of unified screening methods characterized by minimum sample preparation steps and costs, including the maximum number of substances. As the number of analytes, that doping control laboratories have to screen, is constantly increasing [7], comprehensive screening procedures including the vast majority of small-molecule drugs (less than 800 amu) have to be developed. The procedure dedicated to the detection of AAS [16], the group of substances with the highest number of adverse analytical findings (AAF) [17], was mined in order to cover along with steroids, different classes of prohibited substances under one screening method. This method mainly focuses on those analytes and metabolites that are excreted free or as glucuronides into the urine and can be extracted either by LLE or SPE.

As shown in Figure 1, sample preparation aliquots are hydrolyzed enzymatically using β-glucuronidase and after extraction and concentration of analytes from the matrix, sample extract is split for detection, using either LC–MS(/MS) or GC–MS(/MS) [16]. For the sample fraction that is intended for GC–MS systems, a derivatization step is performed prior to analysis. Unfortunately, this major screening method cannot be applied to the detection of substances with very low extraction recovery like glycerol or other plasma expanders, AICAR etc. For the latter analytes, other screening methods are applied. For example, dilute-and-shoot methods for substances that are LC–MS(/MS) amenable [18], or GC–MS methods that employ sample evaporation and derivatization with *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) (for substances that are not ionized in

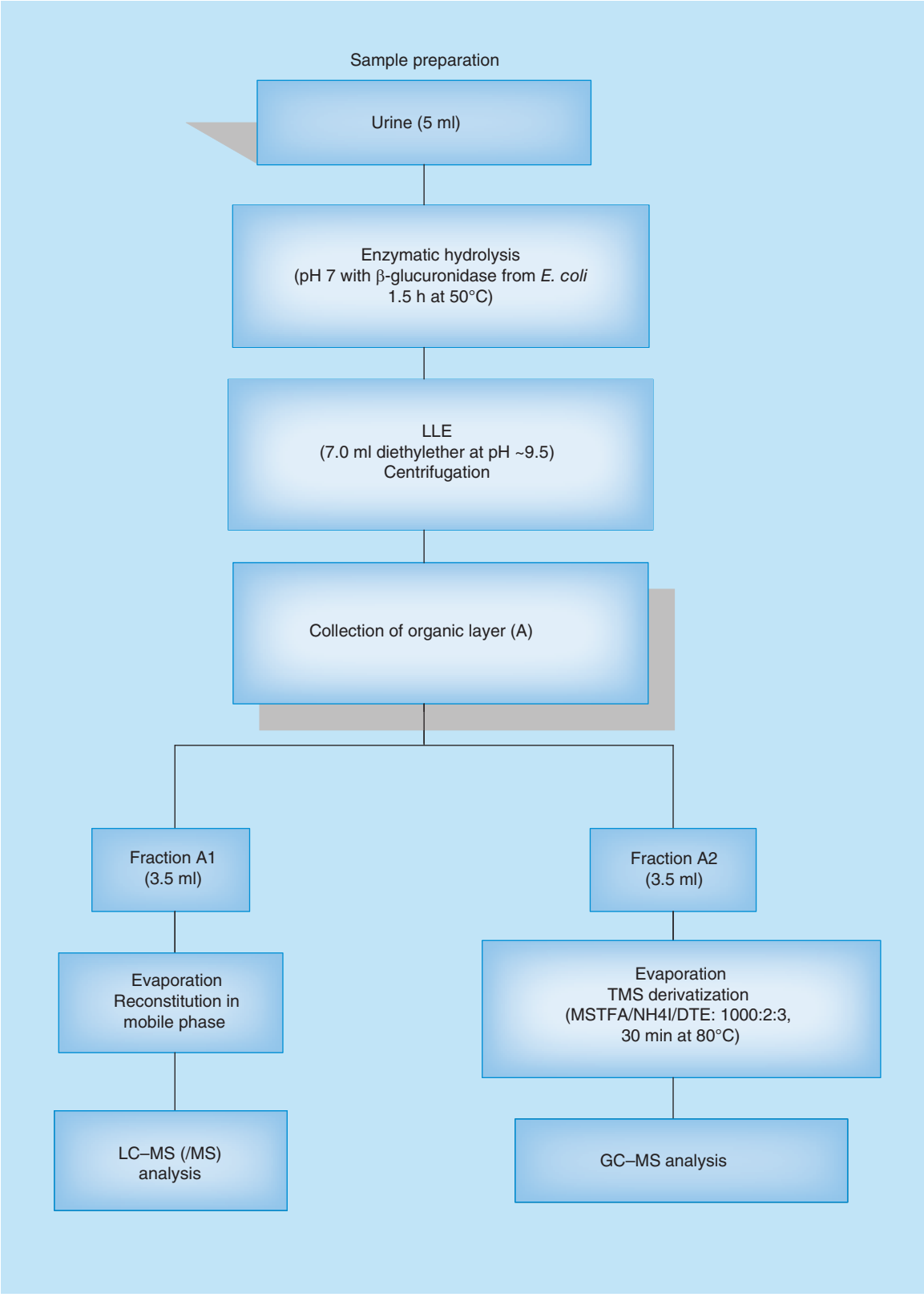

Figure 1. Representation of the major sample preparation procedure applied in doping control screening analysis.
DTE: Dithioerythritol; MSTFA: *N*-methyl-*N*-trimethylsilyltrifluoroacetamide; NH4I: Ammonium iodide; TMS: Trimethylsilylation.

Key term

Derivatization artifacts: Formation of unexpected products through the reaction of derivatization reagents ingredients with the analytes to be derivatized.

LC–electrospray (ESI)–MS(/MS) ion sources, like glycerol) [19].

The main screening method of doping control laboratories is based more or less on the same derivatization procedure developed initially by Donike *et al.* [20] for the simultaneous derivatization of hydroxyl and keto-groups of the steroids as TMS-ether and TMS-enol-ether, respectively. The derivatization solution is a mixture of three components: MSTFA, a strong trimethylsilyl donor; a catalyst, trimethylsilyliodide or NH_4I that creates the active trimethylsilyliodide *in situ* and a preservative that inhibits the oxidation of iodide (I^-) to iodine (I_2) such as dithioerythritol, ethanethiol, propanethiol, 2-propanethiol etc.

While the reaction between the trimethylsilyl donor and a hydroxyl-group is considered as nucleophilic in nature (SN_2), the reaction between the trimethylsilyliodide and a steroidal keto-group is a two-step process with the enolization to be the rate determining step. Enolization is straightforward and leads, in most cases, to one isomer coming from the transition state, where the most stabilized carbocation is located (Figure 2) [21]. Where alternative enolization directions exist, the preferred direction is dependent on the type of substitution and stereochemistry [22]. For example, in 5β-3-keto steroids C_3–C_4 enolization predominates while in 5α-3-keto steroids a mixture of C_2–C_3 and C_3–C_4 enolization can sometimes be observed. The efficiency of derivatization is controlled in every sample by monitoring the ratio of androsterone mono-TMS area, which ideally should be absent relative to the androsterone di-TMS area, which is usually one of the most abundant analytes in this procedure.

Although derivatization for the vast majority of analytes gives rise to the desired derivatives in quantitative yield, there are cases where the formation of by-products or artifacts can be observed. Artifacts can be obtained either from incomplete derivatization, especially for substances with multifunctional groups and/or from the incorporation of unexpected ingredients coming from the derivatization reagents mixture into the analyte. Uncontrolled formation of unexpected derivatives can be produced, if the reaction conditions are not well established. In order to avoid or minimize artifact formation, recommendations have been reported [23] based on the optimization of reaction time and concentration of derivatization reagent, selection of a different derivatization reagent or type of derivatization reaction. As an example, 4,9,11-trien steroids are derivatized poorly in typical doping control screening procedure and hence, another derivatization has been proposed for their GC–MS detection [24].

The reactivity of both hydroxyl and keto-groups in a steroidal structure is highly dependent on the stereochemical environment, hence, harsh reaction conditions are strongly advised to ensure complete derivatization of all hydroxyl-groups in steroids with several derivatizable functional groups [25]. A typical example of uncompleted **derivatization artifacts** is 9α-fluoro-17α-methylandrost-4-ene-3α,6β,11β,17β-tetrol, one of the major fluoxymesterone metabolites that can be found in almost 50% analogy equally of tri- and tetra-OTMS under standard derivatization procedures for GC–MS doping control analysis of steroids. A new approach in the determination of the derivatization degree of methyltestosterone silylation, used as internal standard, has been reported recently [26]. Multipeak analytes caused from incomplete derivatization are commonly observed [27] also in nitrogen-containing analytes including but not limited to stimulants, β-blockers and β_2-agonists. The low binding energy between nitrogen-atoms and silyl-

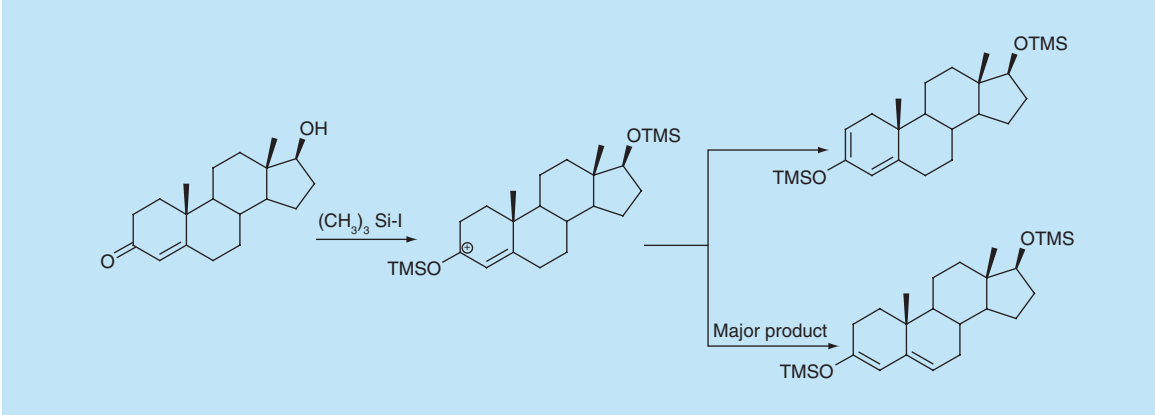

Figure 2. Enolization of testosterone after the reaction of the steroidal keto-group with trimethylsilyliodide.

groups along with the stereochemical hindrance due to the introduction of a first TMS group on a primary amine or in a vicinal to amine hydroxyl-groups results in mixtures of analytes by the introduction of a second TMS group [1–6].

The incorporation of ethanethiol moiety from derivatization mixture has been reported for substances like oxandrolone and epioxandrolone. This artifact is always observed and its abundance seems to depend on the time of analysis [28]. Quality control samples analyzed at the beginning of the GC–MS sequence show low abundance of this artifact, however, artifacts can be prominent at the end of a long sequence. The simultaneous monitoring of both steroids, along with their artifacts, minimizes the risk of false-negative results. Minor incorporation of ethyl-thio group into steroidal backbone has also been observed for other steroids under prolonged storage with high concentration of ethanethiol but the concentration of these artifacts can be marginal and thus, they cannot affect the analysis of steroids [29].

The incorporation of a whole MSTFA molecule in analyte structure that has been reported [30] mainly as artifact of aldehydes and ketones can also be observed in doping control analysis. The derivatization of ethacrynic acid with the aforementioned mixture results in both the expected mono-TMS derivative and an artifact that can be explained by a 1,4-conjugated addition of trifluoromethyl acetamide to the double bond and the protection of the keto-group of ethacrynic acid as enol-TMS. This artifact is very stable and allows the low ng/ml detection of ethacrynic acid abuse. Fur-

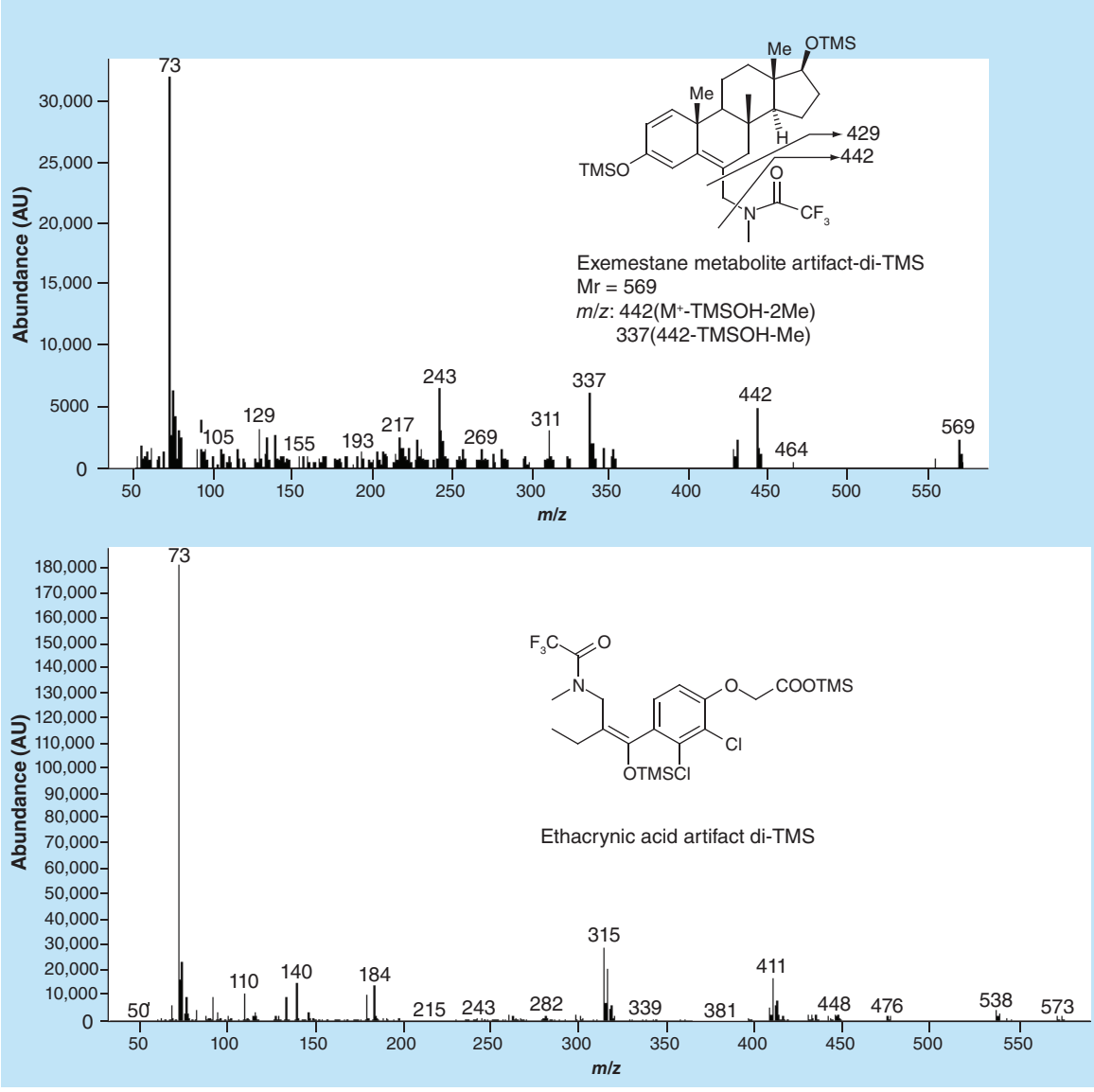

Figure 3. Full scan electron ionization mass spectra of 17β-hydroxyexemestane and ethacrynic acid artifacts.

Key term

Athlete's biological passport: Program and methods of gathering and collating data as described in the International Standard for Testing and Investigations and the International Standard for Laboratories.

thermore, a conjugated 1,6-addition of MSTFA can explain artifacts of exemestane and its 17-hydroxy metabolite where a whole MSTFA molecule is incorporated in their structure with the same derivatization reagent. Full scan EI mass spectra of these artifacts are presented in Figure 3.

Toward the target of including the maximum possible number of analytes within a single screening procedure, attempts have been made to modify the above-mentioned derivatization procedure. Although foreign to AAS analytes were always targeted in AAS dedicated methods, comprehensive GC–MS screening methods that systematically detect other groups of prohibited substances appear only in the mid 2000.

Initially, a GC–MS screening method with MSTFA reported for stimulants and narcotics [31], replaced the frequently used combined derivatization procedure with MSTFA followed by *N*-methyl-bis-trifluoroacetamide (MBTFA). The combined detection of anabolic steroids with β_2-agonists, β-receptor blocking agents, narcotics and stimulants in human urine was reported by the same group following a single derivatization step with a diluted derivatization mixture of MSTFA/NH_4I/ethanethiol (640/1/2, v/w/v) after heating for 1 h at 80°C [27,32]. Authors concluded that some nitrogen containing analytes showed poor chromatographic behavior using the traditional AAS derivatization mixtures. This poor chromatographic behavior was largely improved after the adoption of derivatization mixture dilution.

Similar results with the application of a diluted derivatization mixture have been reported by Kiousi *et al.* [33] where a two-step 20 min derivatization process ended with the detection of 190 drugs with all classes of prohibited substances. According to this work, the application of the first step with MSTFA was necessary for some compounds such as ephedrine, in order to improve peak shape and sensitivity. Enhanced derivatization yields were also reported for some substances after combination of acetonitrile with MSTFA in the first step. These reported unified derivatization processes bring added value in doping control laboratories when reporting time is critical, especially in major international events.

More recent developments concerning the screening of prohibited substances include microwave- or ultrasonic-assisted derivatization procedures [34]. Microwave procedures benefit from a very efficient in core dielectric heating mechanism to reduce derivatization times and enhance derivatization yields where poor derivatization was observed by classical heating procedures. On the other hand, the general application of microwave-accelerated derivatization (MAD) in doping control laboratories requires the use of special scientific devices, suitable to control temperature and pressure, as it has been shown that the use of domestic kitchen microwave ovens can lead to nonreproducible results [33,34]. This is of major importance for the detection of doping with endogenous steroid substances. In this dedicated method, endogenous steroids quantification results of athlete's samples may be provided by any WADA accredited laboratory worldwide and be introduced in a global database tool, as the steroid module of athlete's biological passport [35] that creates an individual 'normal' range for every athlete for the target analytes. Abnormal values obtained by this tool trigger additional tests like GC-c-IRMS for the unambiguous decision of doping rules violation. The harmonization of the results obtained by different laboratories is critical and the impact of using different derivatization techniques between different laboratories may cause variations in quantification results and therefore must be carefully controlled.

Several publications have already demonstrated the potential of MAD in various derivatization procedures including silylation of different classes of prohibited substances (steroids, corticosteroids) included in the WADA prohibited list [7]. Bowden *et al.* [36] performed a detailed study on parameters that may affect MAD derivatizations of a subclass of steroids including reaction temperature, time and microwave power for several silyl reagents and concluded that MSTFA was found to derivatize better under microwave conditions. Derivatization time can be reduced down to 1 min at microwave power even at 100 W in comparison with traditional heating derivatization in which the same results could be obtained in over 30 min. Galesio *et al.* [37] have reported increased derivatization yields (up to 29%) for some steroids that derivatization leads to tri-TMS derivatives, in comparison with conventional heating derivatization, in only 3 min at 1200 W. Interestingly, the same authors have shown improved derivatization results upon ultrasonic derivatization of steroids as well. MAD usefulness for the derivatization of endogenous steroids with hindered hydroxyl-groups in particular has been recently tested by Casals *et al.* [38]. Authors used a different derivatization protocol compared with the one applied to doping control analysis, involving mixed methoxime (MOX)/1-(trimethylsilyl) imidazole (TSIM) derivatization. Comparison of the steroid profiles from 20 healthy volunteers showed similar results 8.

Hence, authors were able to replace prolonged heating derivatization protocol (>16 h) with MAD derivatization for 3 min at 600 W. Azzouz *et al.* [39] have also presented a method that comprised SPE extraction followed by MAD derivatization for the GC–MS analysis of pharmacologically active substances. Authors claim that these features enhance method sensitivity. An MAD approach published by Amendola *et al.* [40] allowed the efficient silylation of hindered exogenous and endogenous groups of corticosteroids by a two-step procedure; an MAD followed by a traditional heat transfer derivatization.

Substance-specific derivatization for confirmation & screening purposes by GC–MS

The suspicious screening results pertaining to prohibited substances are submitted to confirmatory analysis. Confirmation is considered the analysis of a separate aliquot treated either in the same way as the screening aliquot or usually with a more specific sample preparation procedure analyzed under verified chromatographic and mass spectrometric parameters in order to meet WADA confirmation and quantification criteria [8]. AAFs comprise qualitative and/or quantitative determinations. Numerous quantification and confirmation procedures have been used by doping control laboratories and despite the increasing importance of LC–MS(/MS), where derivatization in most cases is omitted, the use of GC–MS systems with their enhanced separation abilities and the reproducible mass spectra are still predominant for certain classes of compounds [41]. GC–MS confirmatory analysis is usually based on the use of the same derivatization procedure as in the screening analysis, but in many cases where specific problems are encountered, confirmation methods benefit from the advantages of different derivatization procedures. Key factors in the selection of the appropriate derivatization procedure for the confirmatory analysis are the chromatographic behavior and the specificity of the derivatized analytes. Numerous options are available, the most prominent of which are reviewed as follows.

Derivatization for GC-c-IRMS analysis

GC-c-IRMS is a mandatory confirmation technique in doping control field aiming at the uniquivocal proof for the exogenous administration of endogenous compounds of doping interest [42]. AAS such as testosterone, its precursors and metabolites, epitestosterone, 19-Norandrosterone, boldenone and its main metabolite, formestane and AICAR constitute the target list for GC-c-IRMS in doping control laboratories. The mentioned steroids may be subject of abuse by athletes; nevertheless, they can either be present in the normal urinary steroidome or as by-product of androgen and estrogen biosynthesis or even can be produced through the transformation of androgens by existent intestinal bacteria [43–45].

Typical identification of these compounds in athlete's specimens is not sufficient to prove doping offence and hence, samples that fulfill concentration levels specifications according to WADA guidelines or show interindividual abnormal steroids values pointed out by the steroid module of athlete's biological passport [35] have to be analyzed further by GC-c-IRMS [42]. GC-c-IRMS analysis involves the derivatization of the compounds of interest by the incorporation of exogenous carbon atoms that induce major changes on the isotopic composition of the analytes.

This phenomenon is taken into account by mass balance (Equation 1), which corrects for the isotopic interference of derivatization group [46]:

$$n_{cd}\delta^{13}C_{cd} = n_c\delta^{13}C_c + n_d\delta^{13}C_d$$

Equation 1, version 1

with n = number of moles of carbon, c = compound of interest, d = derivative group and cd = derivatized compound.

For practical purposes, a correction factor for the derivative group can be calculated according to a rearranged version of Equation 1:

$$\delta^{13}C_d = (n_{cd}\delta^{13}C_{cd} - n_c\delta^{13}C_c) / n_d$$

Equation 1, version 2

Derivatization reactions may influence the results of analysis in different ways [47]. Kinetic isotope effects (KIEs) can cause fractionation of analyte, carbon incorporation of large molar ratio of derivatization group into analyte may cause dilution of tracer and lastly incomplete derivatization and different chromatographic behavior of derivatized target compounds are issues of major concern. The number of derivatization reactions that can be implemented in GC-c-IRMS doping control analysis, where mainly hydroxyl groups have to be derivatized in conjunction with above requirements, is rather limited.

Derivatization reagents containing fluorine, such as trifluoroacetic anhydride and heptafluorobutiric anhydride can irreversibly poison the oxidation reactor resulting in incomplete combustion because of the formation of stable copper, platinum and nickel fluorides [48]. Silylation reactions are also considered 'incompatible' with GC-c-IRMS because silicon dioxide is formed upon the oxidation of silyl derivatives, and the depositions of silicon dioxide affect the functionality of the oxidation reactor with time [49].

However, silylation reactions are widespread in GC-c-IRMS community and with proper caution can improve the LODs for the GC-c-IRMS methods because of their excellent chromatographic behavior. Prevost *et al.* [50] have presented a silylation procedure that gave precise and accurate results for the control of androgens misuse in breeding animals, a field very close to doping control. Recently, Piper *et al.* [51] used a selective silylation procedure for the determination of $^{13}C/^{12}C$ ratios of endogenous AICAR. This procedure retained AICAR amine groups unaffected and derivatized ribose hydroxyl-groups as TMS. This procedure was chosen in order to reduce the derivatization reagent carbon load to the analyte.

Acetylation using acetic anhydride in the presence of pyridin is the golden standard for the derivatization of steroids in GC-c-IRMS [46]. According to WADA specifications, data evaluation demands the comparison $\delta^{13}C$ of testosterone, its precursors or its metabolites analyzed as free, mono-, and diacetylated steroids with an endogenous reference compound out of the biosynthetic pathway of androgens such as pregnanediol [42]. Comparison of the endogenous $\delta^{13}C$ requires application of corrections, typically through straightforward use of the mass balance Equation 1. Variability in KIE due to steroid structures can cause fractionation of endogenous steroid carbon isotopic ratio, resulting in inaccurate results. Correction factor is global for a certain acetic anhydride and does not consider possible variations in KIEs due to different steroid structures. This is apparently correct as differences in KIEs are found marginal [52]. Special care has to be taken regarding reaction conditions as KIEs and correction factors can vary with different reaction temperatures.

For GC-c-IRMS analysis, derivatization is considered as an additional step during the sample preparation that shall be controlled carefully in order to avoid any problems in the results evaluation. For this reason, recent methods have been reported [53,54] dealing with the GC-c-IRMS analysis of steroids without the use of any derivatization step. However, the control of chromatography is of paramount importance in these cases.

Derivatization for the confirmation of AAS

Although several attempts have been made for the screening and confirmation of AAS by LC–MS(/MS) with good results for certain classes of these compounds [55], GC–MS is still the method of choice for their analysis. TMS-enol-TMS derivatives of AAS are usually employed but when this is problematic other options are examined. Several analytical problems may lead an analyst to choose a different derivatization procedure including, but not limited to: the production of artifacts, the low number of diagnostic ions for the target analytes, the presence of interferences from the matrix and/or derivatization reagents and the separation of closely eluted peaks that share common ions. Guddat *et al.* [28] proposed the use of MSTFA/TSIM for the derivatization of oxandrolone and its metabolites in order to avoid the formation of ethanethiol adducts. Furthermore, the use of mixed MOX-TMS derivatives for the orthogonal confirmation of trenbolone type steroids have already been described previously [24]. An interesting but seldom used acetylation for the detection and quantification of urinary steroids has been presented by Zhang *et al.* using a GC x GC separation system with either chemical ionization [56] or a fast-scanning quadrupole–MS detector [57]. An online derivatization procedure was reported by Zhong *et al.* [58] for the sensitive detection of four parent steroids after on-coating with molecular imprinted polymer filaments using a mixture of BSTFA:TMCS (1%) (1:1, v/v) with acetonitrile.

Derivatization for the confirmation of stimulants

Stimulants are prohibited by WADA only in competition [7], as these substances influence the process of neurotransmission and can benefit athletes by increasing endurance and performance for the action time of these drugs [59]. This class of substances contain molecules with heterogeneous carbon skeleton backbone, however all of them contain nitrogen at various positions and thus, they show excellent ionization capabilities in ESI ion sources. Hence, traditional GC-based methodologies for screening and confirmation have been replaced by LC–MS(/MS) [60]. Despite the constantly increasing importance of LC–MS(/MS) concerning the analysis of stimulants in doping control samples, polar and low molecular weight molecules often obtained as a mixture of isomers may interfere with matrix ingredients, making their detection difficult. Complementary GC–MS-based approaches using different derivatization procedures especially for confirmation purposes are still important. The orthogonal use of GC–MS systems to confirm screening suspicious signals from LC–MS(/MS) increase the accuracy of the results. As an example, Sardela *et al.* [61] presented the confirmation of sympathomimetic alkylamine agents by GC–MS, after initial screening results from LC–MS(/MS), using an extraction acylation procedure with Mosher acid chloride as derivatization agent. A rapid and facile analytical approach to identify tuaminoheptane was reported [62] using imine formation with benzaldehyde and GC–MS analysis. Under almost identical conditions, 2-methyl hexanamine was complimentary identified after imine formation by GC–MS [62].

Ephedrines are banned only when they exceed a urinary threshold level of 150 µg/ml (pseudoephedrine), 10 µg/ml (ephedrine and methylephedrine) and 5 µg/ml (cathine). Numerous methods for the simultaneous detection of ephedrines have been reported in the literature. These methods include HPLC, LC–MS and GC (NPD or MS) detection [63].

Various LC–MS(/MS) methods have been proposed as quantification procedures by direct injection of the sample(s) [18]. Despite the fact that the proposed methods are simple and rapid, they can generate ion suppression and retention time instability because of matrix influence. These effects can be controlled only if deuterated internal standards are used in all quantitative samples and controls, as suggested recently by Deventer *et al.* [18].

Methods including detection based on the use of GC have been widely used. Among the most recent reports, a qualitative confirmation procedure was proposed by RM de Oca Porto, which allows the confirmation of ephedrines simply and easily after formation of *N*-acetyl-*O*-timethylsilyl derivatives [64]. Recently, Sardela *et al.* [65] reported the formation of 3,4-dimethyl-5-phenyl-1,3 oxazolidine artifact during GC analysis of ephedrines which can lead to false-positive results for ephedrine, false-negative results for pseudoephedrine combined also increased uncertainty in the quantitative results. To overcome this problem, the formation of *N*-TFA, *O*-TBDMS derivatives prior to GC–MS analysis was adopted allowing the unambiguous detection of ephedrines, increasing also the number of diagnostic ions required for identification.

Derivatization for the confirmation of β_2-agonists

β_2-agonists are drugs used in the treatment of bronchial asthma and their use is prohibited in sports due to claimed anabolic effects [66]. A threshold value of 40 ng/ml and 1000 ng/ml has been defined for formoterol and salbutamol, respectively. Although initial testing and confirmation procedures are mainly based on the use of LC–MS [67,68], GC–MS methods are still important for confirmatory analysis using trimethyl silylation or tert-butyldimethylsilylation as derivatization procedures. Trimethyl silylation has been reported by Di Cocsia [69] for screening and identification of 15 β_2-agonists in human urine by fast GC–MS. Single derivatives, with nearly no side products, whose mass spectra included target ions ideal for identification, were achieved using MSTFA. Theoretically, MSTFA reacts with both hydroxyl and amino-groups for the formation of TMS-derivatives. However, in practice it was revealed that TMS-ether formation was performed only for all the hydroxyl-groups for β_2-agonists. The reaction with the amine hydrogen or their β-ethanolamine substructure was limited due to steric hindrance.

Derivatization for the confirmation of β-blockers

β-blockers are included in the WADA prohibited list [7], only for in-competitions and in specific sports. Their pharmacological use concerns to the management of cardiac arrhythmias and cardio protection after myocardial infraction. They can also improve the heart's ability to relax and exhibit calming neurological effects decreasing nervousness, anxiety and stabilizing motor performance. Their calmative action may be beneficial in sports such as archery, automobile, golf, shooting as they require co-ordination, steady hands and precision.

Different techniques have been used to determine and confirm the presence of β-blockers in urine samples including methods based mainly on ELISA and chromatography [70–72]. Most of chromatographic methods are based on GC–MS requiring the derivatization of the analytes polar groups. However, various LC–MS methods have replaced GC–MS analysis, especially for the polar β-blockers. Two derivatization procedures were presented by Pujos *et al.* [70] including the formation of *O*-TMS, *N*-TFA (100 µl MSTFA at 80°C for 10 min, 30 µl MBTFA at 80°C for 10 min) and the formation of cyclic methylboronate derivatives (250 µl of methyl boronic acid at 60°C for 10 min). Results showed that the method based on TMS/TFA derivatives is more sensitive but with a much higher background compared with the boronates which also provide high selectivity. Additionally, the formation of TMS/TFA derivatives using MSTFA/MBTFA for β-blockers containing an amine functional group, substituted by a tert-butyl group (bupranolol, carteolol, nadolol, timolol), is limited as far as TFA derivative is concerned because of steric hindrance.

Miscalleaneous derivatization procedures in sport drug testing

Recent developments concerning the detection of β-blockers and morphine [73] in urine samples include in-port derivatization (or online derivatization), where the reaction occurs in the hot GC injection port. Other nonconventional procedures as syringe derivatization, on-spot derivatization, solid phase analytical derivatization, SPE with on-disk derivatization, solid phase microextraction with on-fiber derivatization, liquid phase microextraction with *in situ* derivatization and stir bar sorptive extraction with *in situ* derivatization have also been published [3].

MAD has also been successfully applied for the GC–MS analysis of hydroxyethylstarch, a polysac-

charide based plasma volume expander following a rapid sample treatment method, developed by Mazzarino *et al.* [74]. As hydroxyethylstarch detection required time-consuming pretreatment steps, the development of a derivatization process with microwave irradiation was very effective. An additional GC–MS application has been reported by de Brabanter *et al.* [75] concerning a fast quantification procedure of 11-nor-D_9-tetrahydrocannabinol-9-carboxylic acid using MAD and GC–triple quadrupole–MS.

Chiral derivatizations

The importance of enantiomeric profiling in pharmacodynamics, drug toxicology and drug disposition has been well established [76]. In doping control, the enantiomeric discrimination of drugs during metabolism and disposition is mainly ignored or considered irrelevant as drugs that may be present as pairs of enantiomers like β_2-agonists and stimulants, are prohibited including all optical isomers, for example, D- and L-, where applicable [7]. However, Jacobson *et al.* [77] have shown the higher availability of the pharmacologically active R-salbutamol in rat muscle tissues and presume a higher risk of the use of enantiomericaly pure salbutamol from athletes in order to overstep the relevant threshold. Additionally, the relevance of chiral analysis was reported recently concerning the low concentration findings of clenbuterol [78]. According to this report, the consumption of clenbuterol residues from animals treated with racemic clenbuterol is expected to be enriched to the slowly excreted enantiomer and, as a result, different enantiomeric profile is expected compared with the administration of the racemic preparation for doping purposes. Hence, enantiomeric profile may differentiate doping cases from accidental consumption of clenbuterol contaminated food.

Previously, Segura *et al.* [79] related the route of administration of salbutamol with the enantiomeric profile of salbutamol findings based on the use of chiral stationary phase columns in LC–MS(/MS) analysis. Currently, the major application of chiral analysis in doping control laboratories concerns the analysis of enantiomers of methylamphetamine, in order to distinguish the drug that had been used, as the sanctions of athletes for methylamphetamine enantiomers are different [59]. The analysis of methylamphetamines enantiomers is performed after the formation of their amides with Mosher acid chloride under an extractive acylation procedure. Recently, the same procedure was reconsidered for the confirmation of sympathomimetic alkylamine agents by Sardela *et al.* [61] based on the orthogonal use of GC–MS after initial screening results from LC–MS(/MS). Mosher derivatives proved ideally suited as they transfer early eluted analytes in much higher retention times and *m/z*, resulting in high selectivity. Interestingly, although not relevant, the enantiomeric separation of methylhexanamine, heptaminol, isometheptane, tuaminoheptane, hexan-2-amine, 5-methylhexan-2-amine and octan-2-amine were observed. *S*-(-)*N*-(trifluoroacetyl)propyl chloride proved also very efficient for the enantiomeric separation of amphetamine-like drugs [80].

Application of derivatization in metabolic studies

Many substances related to doping control do not excrete in urine as parent compounds but they are metabolized, sometimes extensively, and thus, the monitoring of their abuse is performed through the detection of their metabolites [81]. This is apparently true for AAS where in the majority of cases slowly excreted or long-term metabolites along with main excreted metabolites are preferred as markers of doping. In-depth investigations of the metabolic fate of these substances after drug administration lead to the discovery of such new metabolites that might excrete as minor metabolites but may prolong the detection of the parent substances that are nowadays one of the most important and challenging tasks for doping control scientists [82]. Since administration studies with some of these emerging steroids, nonapproved for human use, are difficult to be conducted and sometimes even impossible, metabolic studies using a chimeric mouse model [83] or using human liver microsomal preparations [84] have been alternatively proposed.

Both recently revealed, 17-ζ-hydroxymethyl 17-ξ-methyl, Δ_{13} metabolites of certain 17α-methylated steroids [85–89] and various sulfoconjugated metabolites [90–94] have shown enhanced retrospective potential for the detection of doping with AAS. Upon the discovery of a new metabolite, the assignment of certain mass spectrum profiles and retention times with exact structure is of paramount importance. Collision-induced dissociation (CID) patterns have been established for the majority of LC–MS(/MS) amenable steroids helping structure evaluation [95,96]. However, the hard ionization and the stable fragmentation patterns obtained by GC-EI/MS analysis are considered the best approach for the structure elucidation of new metabolites. The use of different derivatization procedures prior to GC–MS analysis is the most potent strategy for structure evaluation of AAS metabolites due to the fact that derivatization groups can modify mass spectra in a unique way. TMS and enol-TMS derivatives are considered the first choice with characteristic fragmentation patterns and GC retention times relationships with certain steroidal structural features to be well documented [97]. However, the mass spec-

tra of underivatized and ether-TMS, enol-TMS-ether-TMS, MOX-ether-TMS derivatives are often used in any combination in order to obtain structural important diagnostic ions [98]. Therefore, further synthesis of the proposed structures is needed for the definite characterization of a metabolite.

Role of chemical derivatization in LC–API- MS(/MS)

The development of an LC–MS(/MS) analytical method requires the analyte to fulfill the following criteria: stability, sufficient extraction recovery, high ionization efficiency in soft atmospheric pressure ionization (API) source and specific CID fragmentation patterns for MS/MS detection. One of the major advantages of LC–MS based methods is the elimination of any derivatization step. This elimination speeds up analysis, adds accuracy and precision in quantification results and makes LC–MS methods the best choice for the detection of a broad spectrum of compounds. Nowadays, several classes of doping interest compounds (anabolic steroids, diuretics, stimulants, narcotics, β_2-agonists, β-blockers, peptide hormones, proteins, etc.) are detected by LC–MS [13,99–101].

On the other hand, there are classes of compounds that cannot satisfy the above criteria and therefore, the implementation of LC–MS(/MS) in their analysis is limited.

To overcome these obstacles and widen the applicability of LC–MS(/MS), chemical derivatization has been proven to be an effective technique for improving the stability of the analyte, its extraction recovery and chromatographic separation, its ionization capability and fragmentation behavior [102–105].

Enhancement of ionization efficiency of neutral & nonpolar compounds

Although LC–API–MS is a valuable tool for the analysis of a wide range of substances, its applicability to the detection of neutral and nonpolar compounds presents some limitations. The difficulties are referred mainly to the limited ionization capability of these compounds under ESI, atmospheric pressure chemical ionization (APCI) or atmospheric pressure photoionization resulting from the lack of acidic or basic groups in their structures [106]. The derivatization strategies are focused on improving the detection sensitivity of weakly ionizable analytes by the chemical inclusion of a moiety that enhances the ionization efficiency compared with the underivatized molecule.

On one hand, the ionization process using an ESI source occurs in both the solution phase and in the gas phase and as a result the detectability is higher for analytes that are either ionic or can be easily ionized in the solution. Therefore, for ESI–MS detection mode, the introduction of permanently charged or easily protonated/deprotonated moieties may dramatically improve the ionization efficiency of the analyte. On the other hand, on the APCI or atmospheric pressure photoionization detection mode the key process comprises proton transfer, charge transfer or adduct formation and the introduction of moieties characterized by high proton or electron affinity into the analyte is being used for the ionization enhancement.

Altering fragmentation behavior

For a sensitive MS/MS detection, the analyte shall generate an intense product ion after its efficient fragmentation upon CID. Derivatization may enable the production of specific fragmentation reactions in MS/MS by the modification of the analyte with a defined structural element, which will improve the specificity of the method. Product ion formation from a derivatized molecule can be emerged from the intact analyte structure, the incorporated moiety of the derivatization agent or the derivatized molecule itself which leads to a specific and sensitive detection.

Optimization of chromatographic behavior

Derivatization can also be an effective approach of improving the chromatographic behavior of an analyte. For instance, analysis of polar compounds using reversed-phase LC columns can be optimized through the addition of a functional group that will increase the hydrophobicity of the molecule allowing for a moderate retention in the column. Furthermore, better chromatographic separation achieved due to the changes in chromatographic behavior through derivatization, or analysis of cleaner samples after the application of a clean-up step, after derivatization, that is, LLE or SPE procedure, decreases the ion suppression phenomena caused by co-eluted biomatrix components.

Substance-specific derivatization for screening & confirmation purposes by LC–API–MS/(MS)

As previously mentioned, direct analysis of poorly ionizable compounds such as AASs by LC–MS(/MS) does not provide the required sensitivity for all analytes. In such cases, chemical derivatization is the key process to improve the detectability and ensure both sensitivity and specificity.

AAS have been one major target of chemical derivatization coupled with LC–MS(/MS), as there are several members of this class of compounds including metabolites, lacking at least a keto-group conju-

gated with a double bond and hence their response in soft API sources is relatively low due to the low proton affinity of carbonyl- and hydroxyl-groups in the steroid molecule [109]. The chemical addition of the API-responsive moieties to the functional groups of steroids, that is, carbonyl- and/or hydroxyl-groups, allows for the formation of derivatives with increased sensitivity.

The introduction of permanently charged moieties or easily ionizable groups containing tertiary nitrogen into the functional group of hydroxysteroids is the main approach applied for their detection in positive ESI mode. Furthermore, a significant number of derivatization reagents have been used for the conversion of carbonyl-groups to hydrazones with quaternary ammonium and pyridinium moieties or oximes improving their ESI sensitivity.

Notably, hydroxylamine and methoxyamine convert oxosteroids into the corresponding oximes. The obtained derivatives contain a nitrogen atom, an easily ionizable moiety which improves the ESI efficiency [107–110]. The introduction of the pyridyl group, a highly proton-affinitive moiety, to the oxosteroids molecule using 2-hydrazino pyridin is also a common reaction for increasing the response of the analytes [111–113]. Furthermore, the conversion of carbonyl-groups into water-soluble hydrazones containing quaternary pyridinium and ammonium moieties is mainly applied for the derivatization of keto steroids. This specific reaction can be performed under mild, acidic conditions in either organic or aqueous solvents using Girard's reagent P (1-(carboxymethyl) pyridinium chloride hydrazide; GRP) or Girard's reagent T ((carboxymethyl) trimethylammonium chloride hydrazide; GRT) as derivatization reagents.

The ionization enhancement of hydroxysteroids can be accomplished with a wide variety of derivatization reagents. Picolinic acid, a pyridin-carboxylate acid, is one of the most widely used reagents for the conversion of the hydroxyl-groups to the corresponding picolinoyl esters [114–119]. The esterification reaction can take place either by the mixed anhydride or the acyl chloride method. The mixed anhydride method uses pyridin-carboxylate acid and 2-methyl-6-nitrobenzoic anhydride in the presence of 4-dimethylaminopyridin and triethylamine while the acyl chloride method is based on pyridin-carboxylic acid chloride hydrochloride in pyridin.

Esterification of hydroxysteroids can also be achieved by the use of sulfonyl (R-SO_2-Cl) and acyl chlorides (R-CO-Cl) which can readily react with nucleophiles such as the hydroxyl-groups. According to their chemical structure, this type of derivatization reagents can be classified into nitrobenzoyl chlorides such as 4-nitrobenzoyl chloride and 3,5-dinitrobenzoyl chloride, nitrobenzenesulfonyl chloride such as 4-nitrobenzenesulfonyl chloride, pyridin-3-sulfonyl chloride and dansyl chloride (5-dimethylamino-1-naphthalenesulfonyl chloride).

Due to the basic secondary amine moiety in its structure that helps the preformation of ions in an acidic mobile phase, dansyl chloride is a common reagent used in positive ESI mode [120–127]. Besides, the MS response in negative APCI mode is improved significantly by the use of the electron-affinitive groups of 4-nitrobenzoyl chloride, 3,5-dinitrobenzoyl chloride and 4-nitrobenzenesulfonyl chloride [128,129]. Di-hydroxysteroids such as estradiol and 5α-androstane-3α,17β-diol [130] can easily be derivatized by isonicotinoyl azide due to the presence of the high-proton affinitive pyridyl group in its structure.

Derivatization procedures applied in doping control analysis of AAS by LC–MS/(MS)

AAS are the leading group in AAF in human sports, according to WADA accredited laboratories [17]. Due to the limitations in the ionization efficiency of some AAS lacking ionizable fuctional groups, mainly saturated steroids, LC–API–MS or tandem MS techniques cannot meet the analytical specifications of sensitivity and specificity arisen by the continuous demand of the sports federations and WADA for clean sports and 'zero-tolerance' to the abuse of pharmaceutical substances and/or practices. Based on this fact, a brand new research field has been inaugurated over the past decade, by the development and application of different derivatization procedures prior to LC–API–MS or tandem MS for both screening and confirmatory doping control analysis of AAS [131].

A qualitative LC–high resolution tandem MS method using Q-TOF has been reported by Borges *et al.* for the analysis of 22 sporting federation-banned anabolic steroids or their metabolites and antiestrogens in human urine samples [132]. Sample preparation procedure involved the hydrolysis of steroids glucuronide conjugates followed by an LLE, and the use of GRP as derivatization reagent. Derivatization reaction was performed at room temperature using a 1M GRP in 50 mM ammonium acetate buffer (pH 4.2). The reversible character of the reaction required a pH adjustment in a range of 4–5 [133] for obtaining the maximum reaction yield. With the exception of androstenedione and 6α-hydroxy-androstenedione, all the analytes were converted to the corresponding mono-GRP hydrazones. Due to the *N*-alkylpyridyl group in the GRP molecule and its permanently charged character, the

$[M]^+$ ion was obtained for all the derivatives. Fragmentation of GRP steroids derivatives resulted in the loss of 79 Da corresponding to the loss of the pyridin moiety and of a pyridin fragment that preserves the charge. Detection at concentrations well below to WADA's established minimum required performance limit [134] was achieved for all the examined steroids.

Baranov *et al.* [135] developed and validated a parallel two derivatization procedures for the analysis of steroids in human urine. The method consisted of the conversion of carbonyl- and hydroxyl-groups into oximes [136] and picolinoyl esters, respectively. The picolinoyl esterification of hydroxyl-groups was performed based on the mixed anhydride method at room temperature for 30 min. A freshly prepared derivatization reagent was used containing picolinic acid, 2-methyl-6-nitrobenzoic anhydride and 4-dimethylaminopyridin along with the addition of tetrahydrofuran and triethylamine. A triple quadrupole mass spectrometer coupled with HPLC was used, operated in positive ESI–MS-SRM mode. The protonated molecular ion $[M+H]^+$ was the major ion obtained for all the tested derivatives. Characteristic fragment ions collected from the MS/MS spectra of the picolinoyl derivatives were used to establish a sensitive tandem MS screening method. ESI sensitivity was shown an enhancement of 10–15-times for 16 out of the 21 examined molecules after the implementation of the derivatization. LODs of 0.1–1 ng/ml and 0.5–2.0 ng/ml were achieved for the oxosteroids and hydroxysteroids, respectively.

The introduction of the picolinoyl moiety to the hydroxyl-groups of the steroids through pyridin-carboxylate esterification by the mixed anhydride method followed the conversion of the carbonyl-groups to Schiff bases by either GRT or 2-hydrazino pyridin was the derivatization protocol applied to a recently developed method by Athanasiadou *et al.* [137] for the screening analysis of AAS. The method included the analysis of both free and conjugated steroids after hydrolysis followed by LLE and centrifugation. The two-step derivatization procedure was applied to the dry residue of the organic layer of the step one, that is, picolinoyl esterification.

This single derivatization procedure was selected after an extended method-development and results showed that the applied derivatization procedure enables the detection of 40 out of 43 AAS, according to WADA specifications.

Though testosterone and its ester have been detected with high sensitivity without any derivatization in equine plasma [138,139], derivatization was employed complimentary for their detection in human matrices. Shackleton *et al.* [140] reported the conversion of 17β-fatty esters of testosterone into polar, water soluble GRT hydrazones in plasma as a potential method for the confirmation of testosterone misuse in sports. After plasma protein precipitation, centrifugation and evaporation under nitrogen, the dry extract was derivatized using GRT. Water-soluble hydrazones were collected by SPE. HPLC–selected-ion monitoring–MS analysis of the obtained derivatives provided simple but informative MS/MS spectra giving major ions at M-59 and M-87 for all compounds.

Another attempt for the screening analysis of nine synthetic testosterone esters using human plasma as matrix was reported by Forsdahl *et al.* [141]. Oxime derivatives of the 3-keto steroid group were formed using hydroxylamine as derivatization reagent after heating at 60°C for 10 min. Prior to derivatization step, sodium hydroxide:methanol (1:1, v/v) was added to 1 ml plasma followed by a LLE, centrifugation, addition of dimethyl sulphoxide and evaporation. Detection of the examined testosterone esters was achieved at concentrations below to 10 pg/ml with the exception of testosterone enanthate.

In addition to the use of blood as matrix for the detection of testosterone exogenous administration, the implementation of dried blood spots in doping control analysis was reported recently by Tretzel *et al.* [142]. A screening method for nandrolone and eight anabolic esters derived from nandrolone, trenbolone and testosterone was developed based on the preparation of the corresponding methyl-oxime derivatives prior to HPLC–high-resolution MS/MS analysis. Derivatization was applied to the dry residue using O-methyl-hydroxylamine (100 mM in methanol 80%). The presented assay provided a simplified approach for the qualitative analysis of eight anabolic steroid esters and nandrolone with a sensitivity in the low ng/ml range.

Another approach for the confirmation of testosterone misuse was presented by Danaceau *et al.* [143] based on the quantitation of testosterone/epitestosterone ratio in human urine after GRP derivatization and LC–QTOF–MS analysis. The same sample preparation protocol as in the case of Borges *et al.* [132] was followed, which comprised the glucuronide hydrolysis of steroids followed by a LLE, the common extraction procedure used in antidoping applications and derivatization by GRP after 1 h incubation at room temperature. Increased sensitivity and quality of MS/MS spectra along with the fast analysis speed and easy sample preparation were the main advantages of the developed method compared with the conventional GC–MS method.

Cowan *et al.* [144] investigated the elimination of the adduct formation and fragility of glucuronides after derivatization using testosterone glucuronide and epitestosterone glucuronide as model substances. The formation of hydrazone based on the addition of a GRP solution [145] and oxime obtained by the addition of a methoxyamine hydrochloride solution in pyridine [146] were the chosen derivatization procedures. The extraction of the oxime derivative from the excess of the derivatization reagent was performed using SPE as purification step. The carboxylic acid on the glucuronide moiety was derivatized by diazomethane (methylation) [147] or tris(trimethoxyphenyl)phosphonium chloride (hydrazone formation) [148]. Both the protonated steroid glucuronides were stabilized by the applied derivatization procedures allowing their MS/MS analysis.

Other applications of derivatization for the LC–API–MS/(MS) analysis of doping agents

Recently, a novel method for the extraction, derivatization and high-throughput LC–MS(/MS) screening analysis of six growth hormone releasing peptides (GHRPs) was published by Timms *et al.* [149]. The developed method was based on the acetylation of GHRPs in order to minimize their charge and hydrophilicity. Acetylation was accomplished by the use of acetic anhydride, a common reagent for the derivatization of primary amino groups in aqueous solutions forming acetyl amides. Derivatization procedure included the addition of acetylation buffer (2 ml acetic anhydride:disodium hydrogen orthophosphate 10:90, v/v) to the SPE cartridge and incubation for 5 min. All eight GHRPs and their metabolites were detected in human urine with an improved chromatography and high sensitivity.

Conclusion & future perspective

Doping control analysis requires the development of methods able to screen all substances included in the prohibited list. As prohibited list is revised annually and a lot of emerging drugs are included in it, doping control screening and confirmation methods should be able to adapt to the detection of these emerging substances. New trends in the derivatization procedures will help these methods remaining comprehensive and versatile until the final target of a single sample preparation and one injection for all the prohibited compounds. Microwave-assisted derivatizations, online derivatizations and new combinations of derivatization agents will support further the fight against doping. Especially, derivatization for the enhancement of ionization efficiency for LC–MS(/MS) is expected to decrease detection and quantification limits for target compounds.

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Executive summary

Background

Through the aim of generic sample extraction and derivatization protocols, robust and sensitive comprehensive screening methods have been established for doping control purposes.

The chemistry of TMS-enolTMS derivatives

- TMS-enolTMS derivatization has been proved ideally suited for the sensitive detection of AASs where both hydroxyl and keto functional groups are protected along with several other class of substances that contain reactive proton fuctionalities.

Derivatization in confirmation procedures

- Numerous different derivatization procedures are often used for the orthogonal confirmation of prohibited compounds in athletes' urine samples.

Chiral derivatization

- Enantiomeric separation may give information for the metabolic fate of racemic compounds of doping control interest.

Derivatization & structure elucidation

- The use of different derivatization procedures is a powerful tool for the structure elucidation of newly identified metabolites.

Derivatization for LC–MS

- The conversion of polar and poorly ionized compounds into easily detectable derivatives is considered important for their detection by LC–MS/(MS). Verification in ionization behavior, LC separation and MS fragmentation is discussed.

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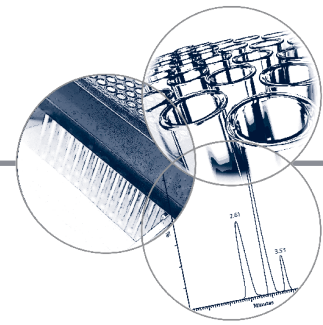

Advances in the detection of designer steroids in anti-doping

The abuse of unknown designer androgenic anabolic steroids (AAS) is considered to be an issue of significant importance, as AAS are the choice of doping preference according to World Anti-doping Agency statistics. In addition, unknown designer AAS are preferred since the World Anti-doping Agency mass spectrometric identification criteria cannot be applied to unknown molecules. Consequently, cheating athletes have a strong motive to use designer AAS in order to both achieve performance enhancement and to escape from testing positive in anti-doping tests. To face the problem, a synergy is required between the anti-doping analytical science and sports anti-doping regulations. This Review examines various aspects of the designer AAS. First, the structural modifications of the already known AAS to create new designer molecules are explained. A list of the designer synthetic and endogenous AAS is then presented. Second, we discuss progress in the detection of designer AAS using: mass spectrometry and bioassays; analytical data processing of the unknown designer AAS; metabolite synthesis; and, long-term storage of urine and blood samples. Finally, the introduction of regulations from sports authorities as preventive measures for long-term storage and reprocessing of samples, initially reported as negatives, is discussed.

The World Anti-Doping Agency (WADA) [1], which is considered to be the accepted organization by sports and governmental organizations worldwide to combat doping in sports, revises and publishes at least once per year the 'Prohibited List' as an International Standard [2]. The List identifies substances and methods that are – according to the **WADA Code** [3] – prohibited as doping, because of their potential of either enhancing performance or masking drug abuse. The substances of the List are claimed to induce pharmacological effects on the cell, the tissues and the organism. Anabolic Agents constitute Class S1 of the List and they comprise the following drug categories with anabolic action: exogenous (synthetic) and endogenous anabolic androgenic steroids (AAS), as well as other anabolic agents such as selective androgen-receptor modulators (SARMs). Examples of drugs and medicines that fall under the Class S1 are the synthetic AAS stanozolol, metandienone, oxandrolone, tetrahydrogestrinone, oral turinabol, SARMs, zeranol, and so on. However, drug interaction with cells to induce a certain pharmacological effect can be achieved by several structural features of the drug molecule, which practically creates an unlimited combination of the molecular features that could provide the particular effect. Since the List comprises prohibited pharmacological effects and respective drug categories, it is not possible to be exhaustive, hence, the following phrase has been added: *"and other substances with a similar chemical structure or similar biological effect(s)"* [2]. The meaning of the last phrase is that prohibited substances are not only those referred to as examples in the List, but also any other molecule, known, secreted or designed in the future, legally marketed or not, with or without clinical studies, having the same pharmacological effect.

The WADA accredited laboratories [4] perform the analysis mainly in urine samples, detecting small drugs contained in the List by using explicitly MS, either coupled to GC or LC. Detection and reporting of prohibited substances is based on specific criteria described in the WADA Technical Document for Identification Criteria for Qualitative Assays [5]. According to this document, in order to report for a violation of the List, laboratories must match in strict ranges chromatographic retention times and abundances of ions specific for the compounds of interest, both in the athlete's sample and in a sample originating from an excretion study or a synthetic reference material analyzed contemporaneously. Without the existence of the reference material, the reporting of a prohibited substance of the List in an anti-doping sample is impossible. As a result, there is a motive for the unethical scientists to create new molecules unknown to the anti-doping community, the designer drugs. The designer drugs are structurally modified analogs or derivatives of known substances, which were never approved

Wadha Abushareeda[1], Argyro Fragkaki[2], Ariadni Vonaparti[1], Yiannis Angelis[2], Maria Tsivou[2], Khadija Saad[1], Souheil Kraiem[1], Emmanouil Lyris[2], Mohammed Alsayrafi[1] & Costas Georgakopoulos*[1]

[1]Antidoping Lab Qatar, Sports City Road, Aspire Zone, PO Box 27775, Doha, Qatar
[2]Doping Control Laboratory of Athens, Olympic Athletic Center of Athens, Kifissias 37, 15123, Maroussi, Greece
*Author for correspondence: costas@adlqatar.com

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Key Terms

WADA Code: The Code is the fundamental and universal document upon which the World Anti-Doping Program in sport is based. The purpose of the Code is to advance the anti-doping effort through universal harmonization of core anti-doping elements. It is intended to be specific enough to achieve complete harmonization on issues where uniformity is required, yet general enough in other areas to permit flexibility on how agreed-upon anti-doping principles are implemented.

Nutritional supplements: Preparations intended to provide nutrients that may otherwise not be consumed in sufficient quantities such as aminoacids, minerals, vitamins, fatty acids, fiber. They can be contaminated with steroids either marketed or unmarketed.

for human use in the past or never made it to production by pharmaceutical companies. They are used by cheating athletes in order to avoid detection by the WADA laboratories. Designer drugs induce less, similar or better pharmacological effect and usually circulate in the market without following formal regulations (labeling, approval and clinical studies) or via the Internet as **nutritional supplements**. Another motive for illegal laboratories to produce designer drugs is to avoid legislative limitations imposed on known molecules because of public health issues.

The current Review presents several aspects of the designer AAS in sports doping. The idea of designer AAS has been around for quite a while and elements of their history, as well as the current situation, are of great importance for both the anti-doping science and public health in general. Since the financial interest to produce new designer AAS is substantial, the rationale behind the molecular changes of the already existing AAS to create new designer molecules is explained later on in the article. The problem of the production and circulation of illegal molecules is known to the sports and public authorities and certain measures have been taken against illegal laboratories. A list of the designer synthetic AAS is presented in 'The chemistry' section. The use of designer AAS does not only appear in human sports, but also in animal racing samples as well. The anti-doping laboratories have made progress for the detection of designer AAS using MS and bioassays. Anti-doping laboratories, guided by the need of elucidating the metabolism of the designer AAS, have adopted sample preparation techniques and performed synthesis of designer AAS metabolites. However, *in silico* predicted analytical data related to designer AAS have also been used. In addition, the sports authorities have introduced the element of time in the fight against cheating athletes: *"I cannot catch you now; I'll catch you later, when I know more about the designer drugs you are using"*. As a result, accredited laboratories have made relevant adaptations in their procedures such as long-term storage of samples and data reprocessing of already analyzed samples that were initially reported as negatives.

The past & present of the designer AAS

Since the 1970s, sports authorities have banned the use of AAS and other performance-enhancing drugs. Nonetheless, since 1966, in East Germany, the German Democratic Republic (DDR) government and its state security 'Stasi' coordinated the development of new synthetic AAS to enhance sports performance [6]. No further anti-doping regulations from official authorities had been established until then, thus, no doping rules' violation existed. A typical example DDR synthetic AAS is the famous oral turinabol (or dehydrochormethyltestosterone) [7].

After 1982, the DDR regime also created endogenous designer AAS to escape the anti-doping tests for testosterone abuse, which were organized by the International Olympic Committee (IOC), the International Association Athletics Federation (IAAF) and the anti-doping Laboratory of Cologne, West Germany [8]. Epitestosterone and androstenedione were also included in the synthesized endogenous steroids of that time period. The rationale behind the creation of designer endogenous AAS takes into consideration the fact that athletes trying to avoid the detection of synthetic AAS were interrupting the relevant therapy close to the competition periods, changing to taking testosterone esters. Exogenous testosterone was mixed with endogenous, making its direct urinary detection impossible, due to the fact that the mass spectra of the endogenous and the exogenous preparation are identical. Its indirect detection is based on the measurement of the ratio testosterone to epitestosterone (T/E) [8]. Epitestosterone is the inactive isomeric molecule of testosterone and its biosynthesis is inhibited after testosterone intake. The mean human population statistic for the urinary T/E is close to unity and the threshold ratio chosen to be the limit for doping purposes was set to 6:1 by both the IOC and the IAAF. To circumvent the anti-doping controls after the abuse of testosterone esters, DDR sports medicine administered athletes with testosterone and epitestosterone esters produced by the state pharmaceutical manufacturer Jenapharm [6]. Since 2005, the WADA has changed the reporting threshold for T/E from 6:1 to 4:1 in order to improve the sensitivity for the detection of T misuse [8] (see also the 'Endogenous designer AAS' section).

Nowadays, two trends for the circulation of designer AAS exist: the first trend comprises the creation of novel molecules in order to be used by cheating athletes without failing doping tests. Since the 1980s, MS detection of synthetic AAS has improved, together with improvements in anti-doping system regulations after WADA's activation in 2004. As a result, cheating athletes switched to the abuse of designer AAS. The most

striking example was the Bay Area Laboratory Co-operative (BALCO) case [9]. BALCO was a San Francisco Bay laboratory that was supplying steroids to athletes. BALCO was initially known as a vitamin and mineral shop, which was later transformed to a laboratory that illegally produced black market steroids sold to elite athletes of baseball, American football and athletics. The 'products' of BALCO comprised the designer AAS norbolethone [10], the tetrahydrogestrinone (THG) [11] and the 'cream', – a salve containing mixture of testosterone and epitestosterone. Norbolethone is a synthetic AAS that was available as a pharmaceutical in the 1960s; however, it was never marketed due to its toxicity. THG is also a designer AAS. The 'cream' was widely used by athletes because it gave normal T/E ratios following administration. Another famous synthetic AAS, seized by Canadian customs in 2004, is desoxymethyltestosterone (MADOL or DMT) [12,13] that was initially detected by the US Accredited Laboratory of University of California, Los Angeles (UCLA; CA, USA) [12]. It is worth mentioning that no Adverse Analytical Finding (AAF) for elite athletes is related to MADOL. It is probable that the UCLA and the Canadian Accredited Laboratories [12,13] timely communicated the detection data to all WADA accredited laboratories and in this way MADOL was no longer a tempting substance for cheating athletes. The cream is another illegal preparation for avoiding detection of testosterone abuse, although it is less effective than testosterone injections. In 2008 the Cologne Accredited Laboratory (Germany) revealed an important case of the abuse of the designer synthetic AAS methyltrienolone, involving 11 Greek weightlifters [14]. The origin of the methyltrienolone synthesis is unknown, but sanctioned athletes claimed the use of Chinese nutritional supplements.

The second trend for the circulation of designer AAS is the nutritional supplement market. Several countries, such as the USA, have introduced legislations to restrict the production and circulation of nutritional supplements based on AAS, such as the US Anabolic Steroids Control Act, 2004 [15]. Nutritional supplements circulate through the Internet, in shops, in gyms, and so on. Nonhormonal supplements such as vitamins and amino acids may contain designer AAS not declared on the labels of the products [16]. Unfortunately, several reports have been published relating these nutritional supplements with AAF cases in doping controls [17,18]. A thorough review was recently published by Teale *et al.* describing the phenomenon of designer drugs for the entire spectrum of the prohibited drug classes for doping control [19].

Authorities against illegal laboratories

In May 2011, WADA circulated guidelines with the title: *Coordinating Investigations and Sharing Anti-Doping Information and Evidence* [20]. In this document, WADA recognizes the crucial role of the National Anti-doping Organizations (NADOs) to expand the fight against doping, apart from their existing anti-doping programs, with further measures to be taken against illegal laboratories and illegal substances trafficking networks. As expected, the BALCO case is referred to in the document. BALCO's activities were revealed with the involvement of the United States Anti-doping Agency (USADA) [9]. Another important investigation against illegal laboratories held in the USA in 2007 – the Operation Raw Deal – is mentioned [20]. New elements of the fight against doping are described in this report [20]:

- The concept of 'non-analytical' anti-doping rule violations;
- Perpetrators falling outside sport's authorization;
- Activating the public authorities in the fight against doping in sport; and, finally;
- Strengthening relationships between NADOs and public authorities.

The Memorandum of Understanding between WADA and Interpol is also published, showing the importance of police involvement in the fight against doping [20]. Three other reports [21–23] also associate the fight against doping with the reinforcement of national legislations. The first report [21] deals with the illegal drugs trafficking in various countries. Another report studies the Italian situation of doping in sports [22]. This report, which can be considered as indicative for many other countries, examines Italy's anti-doping criminal law experience with a twofold purpose: to analyze the production and distribution of doping products; and, to give evidence of how anti-doping criminal provisions and their enforcement can contribute to improve the fight against doping, both within and outside the sports community. The multilateral use of legislation to control the production, movement, importation, distribution and supply of performance-enhancing drugs in sport (PEDS) by several countries is the subject of a report written by Houlihan and García in 2012 [23]. Furthermore,

Key Term

Designer steroids: Steroids synthesized to closely resemble existing known compounds but with sufficient chemical diversity to evade doping control tests.

The Australian Crime Commission conducted an investigation and published a report in 2013 examining the extent to which organized crime is related to illicit drug markets [24].

The aforementioned reports make several references to the role of the Chinese pharmaceuticals industries in the production of raw materials for prohibited drugs. Aligned to these references, WADA's General Director made a statement in February 2013: *"99% of the raw materials that are used through the Internet to make up in your kitchen or your backyard laboratory are emanating from China"* [25]. However, J Zhixue, the head of Chinese NADO, replied immediately [26] asking for evidence concerning the alleged "99%", albeit admitting anti-doping problems in China.

The International Federation of Pharmaceutical Manufacturers & Associations (IFPMA) and WADA have collaborated to combat the latest doping techniques [27] announcing the following declaration: *"The Joint Declaration on Cooperation in the Fight against Doping in Sports facilitates voluntary cooperation between IFPMA member companies and WADA to identify medicinal compounds with doping potential, minimize misuse of medicines still in development, improve the flow of relevant information, and facilitate development of detection methods."* The WADA report on the *Lack of (In) Effectiveness of Testing Programs* published in May 2013 [28] completes a thorough description of the problem of illegal drugs' circulation in sports.

Designer synthetic AAS: the chemistry

Designer AAS are substances with sufficient chemical diversity from known AAS, developed either in the past for clinical practice, or to evade doping control from official doping authorities. These designer AAS pose a serious health risk to consumers due to limited available pharmacological and toxicological data. The male hormone testosterone (Figure 1) is the basic steroidal structure upon which a considerable number of modifications can be applied in order to achieve the design of novel molecules with enhanced anabolic potency and reduced androgenic effect.

Androgens mediate their action through their binding to the androgen receptor (AR) [29,30]. Besides natural androgens, AR binds a variety of synthetic molecules with different affinities. AR ligands are classified as steroidal or nonsteroidal based on their structure, or as agonist or antagonist based on their ability to activate or inhibit transcription of AR target genes. The strength of the interaction between a ligand and a receptor is difficult to predict, since AAS with similar structures can possess different affinities for a given receptor, while structurally different ligands may show similar affinities [31]. Relative binding affinity (RBA) has been used as a term for the quantitative estimation of the receptor–ligand interaction. Methyltrienolone binds AR so strongly that it is used in studies as a reference substance to estimate the RBAs of other steroids, which are characterized as strong (19-nortestosterone and methenolone) or weak ligands (stanozolol and methandienone). Other compounds show RBAs that are too low to be determined (oxymetholone and ethylestrenol). A possible explanation for steroids with anabolic–androgenic activity *in vivo,* but that do not bind to AR, is the existence of an indirect mechanism of action, for example, via biotransformation to active compounds [32,33]. Structure–activity studies have revealed that the most important structural elements of a steroidal structure for effective binding to the AR are:

- The 3-keto group in the A-ring [31]. The reduction of this 3-keto group to an alcohol (either to α- or β-isomers) does not favor binding [34];
- The 17β-hydroxyl in the D-ring [31]. Any modification or elimination of the 17β-hydroxyl group reduces the AR binding affinity. A reduction in binding affinity also occurred by esterifying, for example, the 17β-hydroxyl in testosterone [34]. The 17α-hydroxyl group is not favorable to binding either;
- The 5α-steroidal framework [34];
- A small steric substitution at the 7α-position, but large substituents, reduce affinity. It has been shown that in 17β-hydroxy-4-androstenes the combined removal of the 19-methyl group and 7α-methylation can enhance binding to the AR [35].

Other studies demonstrated that key structural characteristics of a steroidal structure that affect either anabolic or androgenic activities of a given steroid are:

- The 17α-alkylation. 17α-alkylation contributes to the prolongation of the anabolic effect. The oral effectiveness of 17α-alkylated androgens is due to lower hepatic inactivation; the intracellular metabolism is limited and

transformation of this particular part of the molecule does not occur leading to liver disturbances [36,37]. 17α-alkylation also prevents aromatization of A-ring to estrogens [38];

- The 17β-hydroxyl group. Its esterification (by propionate, enanthate, cypionate, decanoate and undecanoate esters) induces enhancement of anabolic activity, and also its prolongation due to the reduction in the elimination rate as a result of the slow release of the parent non-esterified molecule. The absence of a 17-hydroxyl group induces the complete loss of androgenic activity [39], while due to the oxidation to 17-keto steroids, the androgenic activity is significantly reduced or disappears [40];

- The C-4,5 double bond. Its presence seems to cause an increase in activity;

- The 3-keto group. It is necessary for androgen activity, but has no effect on anabolic activity [40,41]. However, 3-deoxy steroids, in the presence of the C-4,5 double bond, were found to be compatible with high anabolic to androgenic activities (e.g., ethylestrenol);

- The removal of the 19-methyl group. This structural change offers, partially, dissociation of the androgenic and anabolic activities for a given molecule [42];

- The modification of ring A, either by the junction with another ring (e.g., a pyrazol ring, as in stanozolol), or by the introduction of an oxygen atom (e.g., oxandrolone), leads to a considerable increase in anabolic activity.

The structural characteristics mentioned above inspired research teams to synthesize a vast number of **designer steroids** (even for ethical purposes), retaining one or more of the above modifications while further modifying the structure of known anabolic steroids at positions where no significant reduction to AR binding or biological activity (either anabolic or androgenic) was induced. These further modifications (and/or their combinations) include:

- Alkylation at different positions in the steroidal structure, such as methylation at C-1 (e.g mesterolone), C-2 (e.g., drostanolone), C-6 (e.g., 6-methyltestosterone), C-7 (e.g., bolasterone), C-17 (also, ethylation or ethynylation, e.g., methandienone, norethandrolone and danazol, respectively) and C-18;

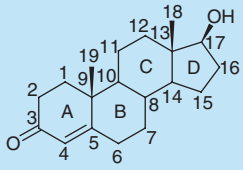

Figure 1. Testosterone, a representative steroidal structure for carbon numbering.

- Introduction of a double bond at different positions in the steroidal structure, such as at C-1,2 (e.g., 1-testosterone), C-2,3 (e.g., desoxymethyltestosterone) [12,43], C-5,6 [44] and C-5,10 (e.g., tibolone). In addition, many compounds with conjugated double bonds extending from ring A and B to C have been synthesized (e.g., methyltrienolone, methyldienolone) [41,45,46];

- Addition of heteroatoms, either to replace a carbon atom of the steroidal structure (e.g., with an oxygen atom [47,48] at C-2 as in oxandrolone, C-3, C-4, C-7, C-11 [49] or with a sulfur atom [50,51], or with a nitrogen atom [52]), or as a substituent (e.g., a chlorine at C-4 as in dehydrochlormethyltestosterone or at C-7 [53], or a fluorine at C-2, at C-6 [54], at C-7 [55] or at C-9 as in fluoxymesterone);

- Hydroxylation, such as at position C-4 (oxymesterone, oxabolone) or at C-11 (fluoxymesterone);

- Fusion of heterocyclic rings to the A-ring of the steroidal structure, such as of a pyrazole ring (stanozolol), an isoxazole ring (danazol) or a furazan ring (furazabol).

Table I summarizes literature on designer AAS circulated either on the black market or in nutritional supplements.

Endogenous designer AAS

The use of preparations containing testosterone and epitestosterone as endogenous designer AAS to escape doping tests has been described in the previous sections. Two cases of preparations have become known: the case of Jenapharm [6] and the case of BALCO [9]. In urine, a T/E ratio greater than 4.0 triggers follow-up tests to

Table 1. Designer androgenic anabolic steroids from literature.

Entry	Substance	Ref.
1	1-androstenediol	[56]
2	1-androstenedione	[56]
3	Dehydrochlormethyltestosterone	[57]
4	Desoxymethyltestosterone	[12]
5	Methasterone	[58]
6	Methylnortestosterone	[58]
7	Methyldienolone	[16]
8	Methyl-1-testosterone	[59]
9	Metribolone	[16]
10	Norboletone	[10]
11	Norclostebol	[60]
12	Prostanazol	[61]
13	1-testosterone	[62]
14	Tetrahydrogestrinone	[11]
15	Methylstenbolone	[63]
16	2α,3α epithio17α methylandrostane 17β ol	[64]
17	2β,3β epithio17α methylandrostane 17β ol	[64]
18	5β-mestanolone	[61]
19	Methylclostebol	[65]
20	Promagnon	[65]
21	17-hydroxyandrosta-3,5-diene	[66]
22	Δ6-methyltestosterone	[67]
23	17β-hydroxyandrostanol[3,2-d]isoxazole	[68]
24	17β-hydroxyandrostanol[3,2-c]isoxazole	[68]
25	6α-methylandrostenedione	[69]
26	Estra-4,9-diene-3,17-dion	[70]
27	Androsta-1,4,6-triene-3,17-dione	[71]
28	4-androstene-3,6,17 trione	[72]
29	1-adrosterone	[73]
30	Methyl drostanolone	[74]
31	7α-methyl nortestosterone	[75]
32	17α-methyl nortestosterone	[75]
33	18-methyl nortestosterone	[75]
34	Halodrol	[75]
35	4-hydroxytestosterone	[75]

investigate whether the elevated T/E ratio is of natural or exogenous origin [8]. The anti-doping analytical technology has incorporated the use of the isotope ratio MS (IRMS) to enable the differentiation between endogenously produced and exogenous testosterone. The reader is directed to a thorough review [76] for more information on this technology. Briefly, pharmaceutical preparations of testosterone are synthesized by plant extracts, whereas human endogenous testosterone originates from the endocrine system. Testosterone contains 19 carbon atoms (Figure 1). The most abundant carbon isotope is ^{12}C, approximately 99% in nature, and the less abundant carbon isotope is ^{13}C, approximately 1%. Due to the differences in the synthetic routes, endogenous testosterone contains more ^{13}C atoms among the 19 carbon atoms of the testosterone molecule, compared with the pharmaceutical preparations. This difference in ^{13}C content between endogenous and exogenous testosterone is measurable for the testosterone molecule and its urine metabolites by IRMS. Doped athletes using pharmaceutical testosterone excrete testosterone and metabolites in urine with less ^{13}C atoms compared with the endogenous testosterone, because exogenous testosterone inhibits the production of the endogenous one. Many manufacturers of reference material produce ^{13}C-labeled testosterone for the analytical and pharmaceutical industries. In these reference materials, ^{13}C atoms replace ^{12}C in the positions mainly 2, 3 and 4 (Figure 1). Unpublished data presented at the 29th Cologne Workshop on Dope Analysis (13–18 February 2011) by L Bowers and D Eichner of USADA [77], raised suspicion that athletes already use pharmaceutical testosterone preparations mixed with ^{13}C-labeled testosterone, in order to create a testosterone cocktail with a ^{13}C content similar to the endogenous, with the purpose of misleading IRMS tests.

Detection of designer AAS

Chromatographic techniques combined with MS, GC–MS or LC–MS are the first approach of the anti-doping laboratories for the detection of AAS. Commonly used instrumentation such as the mass selective detector (MSD) with a single quadrupole mass analyzer or the magnetic sector HRMS, operating in selected ion monitoring (SIM) mode, combine high sensitivity and specificity. These analytical instruments have been used for years for the detection of targeted anabolic steroids and their metabolites in the required low concentrations in urine. As an alternative to GC–MS, the combination of LC–MS instrumentation with electrospray ionization (ESI) has been introduced in the last decade for the detection of known steroids operating in multiple reaction monitoring (MRM) mode (for triple quadrupole analysers) or product ion scan mode (for ion-trap mass analysers). All of the above detection techniques allow efficient detection of known anabolic steroids that are included in the list of screened substances. Unknown designer AAS can be detected only by coincidence in cases when they share the same precursor and product ions with the targeted compounds and they are eluted in a

close chromatographic time inside the defined time window that is selected for the printout of the chromatograms. The preventive detection of unknown designer AAS requires a generic screening protocol, which combines a generic sample preparation with a sensitive high-resolution full-scan MS analysis [78–82]. Regarding sample preparation, the unification of different extraction/derivatization procedures applied for different classes of substances to a single extraction step, which will be able to isolate the unconjugated and conjugated (after enzymatic hydrolysis) low molecular weight substances, has been an important issue for the anti-doping laboratories. The analysis of this extract is performed by GC–MS (following a generic derivatization procedure) and/or LC–MS analytical systems that can acquire high-resolution, full-scan, accurate mass, spectrometric data, which allows for the detection of an unlimited number of known and unknown substances. Such analytical systems include GC–time-of-flight (TOF)-MS and GC–QTOF-MS and the combination of mass spectrometers with TOF, QTOF or Orbitrap® mass analysers with HPLC or UHPLC systems. In addition, with the use of mass analysers that can perform fast scan to scan polarity switching, such as the recently introduced benchtop Orbitrap mass spectrometer, the intact sulfoconjugated molecules of the designer steroids can also be detected as deprotonated molecules. The generic screening approach described above contributes to the enhancement of the preventive role of the anti-doping system against the use of designer drugs, especially if combined with the long-term storage of the samples. The acquisition of full-scan data enables the retrospective analysis of samples for the presence of designer drugs or new metabolites, without the need to reanalyze the samples, by simply reprocessing already acquired LC–MS and/or GC–MS data files. Important information, such as the molecular weight of the unknown and the elemental composition, can be obtained by accurate mass full-scan mode analysis, while the appearance of a combination of adduct ions can provide additional valuable information about the steroid structure.

Another approach for the detection and identification of unknown steroids, is the development of methods based on precursor ion scan and neutral loss scan using triple quadrupole or QTOF LC–MS/MS instruments, since steroids with common structural features under collision-induced dissociation (CID) or collisionally activated dissociation (CAD) can share common fragmentation patterns. The common characteristic product ions or neutral losses can be used as markers to identify unknown compounds. Published research describes protocols that can be used as complementary approaches to the existing analytical screening procedures of the laboratory [83–89], especially in cases of suppressed steroid profile as measured by GC–MS. In these protocols, product ion scan LC–MS analyses of known steroids were conducted and with the use of deuterium derivatives or modified structurally related synthetic analogues, characteristic fragmentation pathways are proposed that provide classification of the steroids by the characteristic product ions generated. For example, precursor ion scans of ions at *m/z* 97 and 109, indicate steroids with a 3-keto-4-ene structure and the detection of abundant product ions at *m/z* 241 and 199 or 227 and 199 indicate a 4,6,11-triene steroid with ethyl or methyl group at C-13. In a similar way, neutral loss scan can be used for the detection of unknown steroids with a particular structure. Some of the common losses observed in steroids are lacking in specificity (e.g., loss of water [18 amu] or acetone [58 amu], while others are considered more specific (e.g., 84 and 30 amu) and they can be used as a diagnostic tool for the detection and characterization of unknown steroids. As suggested by Pozo *et al.* [90], the integrated use of the four different types of scan modes (neutral loss and precursor ion scan followed by full scan and product ion scans) can be the most powerful tool for the detection and characterization of a designer steroid.

MS-based techniques are used as the standard highly sensitive routine screening methods for the known AAS. However, they depend on the known chemical structures. This led to the development of *in vitro* androgen bioassays, for the screening of designer AAS based on androgen-receptor activation instead of knowing the chemical structure. Androgen bioassays are not depended on the chemical structures. An approach based on the combination of LC separation – androgen bioactivity testing and QTOF-MS identification – was developed by Nielen *et al.* [91,92]. According to this protocol, urine samples after enzymatic hydrolysis and generic SPE are analysed using gradient LC and a dual 96-well fraction collector, where one plate is used for androgenic bioactivity detection by yeast-based reporter gene bioassay. If a well is found suspect, the duplicate plate is subjected to high-resolution LC–QTOF-MS

analysis, leading to elemental composition calculations of the designer steroids, search of electronic databases and structural elucidation. This approach was recently applied to detect and identify unknown androgens in herbal samples and sport supplements. Radioimmunoassays and ELISAs have been used in the past, showing good sensitivity for the screening of AAS, but with the disadvantage of limited specificity due to antibody crossreactivity profiles [93]. Recently, a multianalyte ELISA protocol based on a site-encoded ELISA microplate has been reported, which allows the simultaneous detection of up to 11 AAS in human serum samples in concentrations below minimum required performance levels (MRPL). This protocol enables the development of multiplexed immunoassays performed in a microarray format [94].

A thorough review on the androgen bioassays has recently been published [95], where the various types of this approach have been described. In the next lines, some studies on bioassays of AAS in biological matrices and nutritional supplements are presented. Nielen *et al.* had developed a simple yeast-based reporter gene bioassay for trace analysis of estrogens, characterized by direct measurement of yeast-enhanced green fluorescent protein for the detection of estrogen activity in nutritional supplements [96]. It was shown that bioassays play a valuable role in the fight against doping as compared to a LC–MS/MS screening method alone. As a test to examine its efficiency, 18 nutritional supplements were analyzed and shown negative in LC–MS/MS, while two of them screened positive by androgen yeast bioassay. The applicability of a yeast androgen and estrogen bioassay, in the detection of steroid esters in hair samples of animals treated with a hormone ester cocktail, was also shown [97]. Another approach for the advantage of a yeast androgen screening was studied by Wolf *et al.* [98]. The long-term detection of methyltestosterone abuse by a yeast transactivation system has been successfully validated. For the purpose of that study, a human volunteer was orally administered a single dose of 5 mg methyltestosterone and urine samples were collected after different time periods (0–307 h). The samples were analyzed in the yeast androgen screen and in parallel GC–MS. The results demonstrate that the yeast androgen receptor was able to detect methyltestosterone abuse for a longer period of time in comparison with classical GC–MS. It was found that bioassay was able to trace methyltestosterone in urine samples for at least 14 days while the GC–MS method was able to detect it up to the sixth day from the intake. The result of this study demonstrated that the yeast reporter gene system could detect the activity of anabolic steroids such as methyltestosterone with high sensitivity even in urine, providing further evidence for the high potential of yeast androgen screening as a prescreening tool for doping analysis. Although promising, this approach has been criticised at the following points: metabolites of many AAS may be inactive and do not show androgenic activity; the background activity from endogenous sources reduces specificity; and, its applicability is limited due to reduced sensitivity, mostly in out-of-competition collected anti-doping samples, where the AAS analytes would be more easily detected due to higher concentrations in urine.

In addition, a promising strategy of screening methods for the misuse of designer steroids by their physiological effects is the use of omics technologies [99–102]:

- Transcriptomics for finding gene expression biomarkers, with *in vivo* studies in showing alteration of gene expression in human blood cells caused by steroid hormones;

- Proteomics for investigating changes in protein expression or excretion caused by AAS, with a few publications available showing that different lipoproteins or apolipoproteins, propeptide of type III procollagen, apoptotic factors, pro- and anti-inflammatory factors can be promising biomarker candidates;

- Metabolomics for detecting perturbations in the metabolic profile after administration of AAS, with creatine, creatine kinase and plasma urea levels being potential biomarkers. Recently, Dervilly-Pinel *et al.* published two protocols based on LC–HRMS fingerprinting and multivariate data analysis, to investigate metabolome modifications upon steroid administration in calves, showing urine profiles discrimination of the treated animals from the control ones [100]; the results showed that the protocols need to be applied to a larger population of treated and control animals in order to describe generic, reliable and robust biomarkers. An untargeted steroidomic approach was proposed for the discovery of

new biomarkers for the detection of testosterone intake, by applying UHPLC–QTOF-MS urine sample analysis and chemometric tools, showing the pertinence to monitor both glucuronide and sulphate conjugates, as well as a number of promising biomarkers that can be also related to the administration of other AAS.

Recently, in 2009, WADA introduced the term 'athlete biological passport' (ABP) in the WADA Code [103] as an additional indirect tool to detect athletes manipulating their physiological steroid and hematological variables, without detecting a particular prohibited substance or method. The ABP does not replace the routine methods, but rather complements analytical methods. Although there has already been some longitudinal profiling of markers of steroid doping [8], the ABP now introduces a standardized approach to determine steroid abuse through urine sampling. The ABP regulations are based on the innovative approach developed by the Swiss WADA-accredited laboratory of Lausanne [104, 105].

Data processing

Methods based on MS produce data for known, unknown, targeted, untargeted and endogenous substances of biological samples. Specific software extracts MS information from analyzed urine samples, eliminates interferences and identifies metabolites in a series of samples from excretion studies, using data from MS libraries of known substances, spectra and accurate masses databases. A number of tools for processing MS data have been proposed in the literature and are available; for example, MetaboLynx® of Waters, Sieve® of Thermo Fischer Scientific and MetAlign® [79] of RIKILT. The MetAlign is an interface-driven tool for full-scan MS-data processing. The main purpose of this software is the automated processing of MS-based metabolomics data with baseline correction, accurate mass calculation, smoothing and noise reduction and alignment between chromatograms. By comparing data after pre-processing with MetAlign, it was noted that besides the chromatogram baseline line correction, there were better defined peaks that improved peak picking for the identification of targeted and untargeted compounds [79]. For identification of untargeted peaks, an inaccurate-mass database was constructed containing approximately 40,000 pharmacologically relevant and existing compounds extracted from the Internet-accessible database PubChem [106]. Calculation of the exact mass of each protonated and deprotonated molecules, the isotope ratio and an estimate of the retention time was also performed.

Peters *et al.* modified MetaboLynx for the determination of *in silico* predicted metabolites of glucocorticosteroids and designer modifications of anabolic steroids in human urine [79]. It was successfully used for the detection of THG [107]. Synergetic methods for the prediction of AAS metabolites, retention times and MS fragmentation have been proposed by Fragkaki *et al.* [108]. A method predicts the phase I metabolites of AAS [108]. The statistical tool principle components analysis (PCA) was used to classify the parent AAS into different classes, based on their structure's similarities. Another method [109] was used for the prediction of MS fragmentation of AAS, including designers. The results derived from the previous two studies were combined with the study of Quantitative Structure Retention Relationships (QSRR) prediction of retention times [110]. xlogP molecular descriptors have also been used [79] for retention times predictions of PubChem database compounds. Finally, an LC–MS library searching method for the identification of AAS in nutritional supplements has been developed [111].

RNA-sequencing

A recent study opened new frontiers in the detection of designer AAS, even though it was applied in meat production animals (boars and calves) [112]. Changes in the molecular level caused by the administration of AAS were quantified by a new high-throughput and sensitive technology for holistic gene expression analysis, RNA sequencing. The results demonstrated the potential of the new technology for the screening of highly regulated genes that can act as biomarker candidates for the detection of the misuse of anabolic substances in farm animals. This novel approach can be evolved as an alternative indirect detection method of designer and known AAS in human sports in the future.

Synthesis of metabolites of designer AAS

The *in vivo* production of reference substances of AAS metabolites in humans suffers from ethical, as well as practical problems associated with the implementation of clinical studies and the isolation of pure metabolites from urine. To overcome these problems, several methods of synthesis of

metabolites have been developed. The enzyme-assisted synthesis catalyzed with microsomal uridine diphosphoglucuronosyl-transferase (UGT) enzymes has been developed, offering the main advantage of the stereospecificity of the enzymes, which allows synthesis of stereospecifically pure conjugates. Moreover, enzyme-assisted synthesis is used for the rapid production of small amounts of glucuronides when needed, for example, in the build-up of an analytical method. Using the enzyme-assisted synthesis, the preparation of glucuronide conjugate standards of 11 AAS and their metabolites, which can be detected in human urine after dosing of exogenous anabolic steroids (e.g., methandienone, metenolone, methyltestosterone, nandrolone and testosterone), has been described [113]. In another study, microsomal and S9 fractions of human liver preparations were used as sources of metabolizing enzymes, and the co-substrates of the synthesis mixture were selected to favor phase I metabolic reactions and phase II conjugation reactions of relatively new AAS [114,115]. Equine liver microsomes and S9 *in vitro* fractions were also found to generate all the major phase I metabolites observed, following *in vivo* administrations of stanozolol in the equine [116].

Chemical synthesis methods have been also developed as an alternative for the synthesis and identification of AAS and/or their metabolites, as occurred in a study for 4-hydroxytestosterone [117], madol [12], tetrahydrogestrinone [11] and other AAS [13,118,119]. The approach to synthesize, characterize and certify appropriate reference materials (RMs) and certified reference materials (CRMs) from the National Analytical Reference Laboratory (NARL), which are fit-for-purpose for the current requirements of sports testing laboratories, has been described [120].

The identification of AAS metabolic pathways have been also successfully conducted through either animal experiments, as for madol [8], or using cryopreserved human hepatocytes, as for drostanolone and 17-methyldrostanolone [121] and other AAS [122,123]. The results of the *in vitro* experiments carried out using homogenized horse liver for five anabolic steroids (turinabol, methenolone acetate, androst-4–3,6,17-dione, testosterone and epitestosterone) have also been presented [124] as an alternative for AAS metabolism studies.

The chimeric uPA+/+-SCID mouse model, transplanted with human hepatocytes, has also been used to study *in vivo* human steroid metabolism, as occurred for methasterone, promagnon, methylclostebol [65], 4-androstene-3,17-dione [125] and methandienone [126].

Recently, another strategy for synthesis of the methandienone long-term metabolite, 17β-hydroxymethyl-17α-methyl-18-norandrosta-1,4,13-trien-3-one, was reported [127]. According to this, 11 recombinant strains of the fission yeast *Schizosaccharomyces pombe*, expressing different human hepatic or steroidogenic cytochrome P450 enzymes, were screened for production of this metabolite in a whole-cell biotransformation reaction. 17,17-dimethyl-18-norandrosta-1,4,13-triene-3-one, chemically derived from methandienone, was used as substrate for the biotransformation reaction, as it was converted to the final product in a single hydroxylation step. The metabolism of methyl-1-testosterone has also been studied according to this strategy [128].

Animal doping with designer AAS

In several publications related to AAS screening in animal sports, designer AAS have been introduced to the protocols, such as in [129], proving that the problem has been inherited by animal sports from the human ones. Several animal racing laboratories have conducted studies in the metabolism of designer AAS [130–132]. The *in vitro* metabolism of a designer steroid – estra-4,9-diene-3,17-dione – featuring in 2010 in a large number of marketed products on the Internet, was studied in equine, canine and human species with the major metabolites identified for target testing in sports doping control [130]. In another study the equine *in vitro* metabolism of seven steroids available for purchase on the Internet, including androsta-1,4,6-triene-3,17-dione, 4-chloro,17α-methyl-androsta-1,4-diene-3,17β-diol, estra-4,9-diene-3,17-dione, 4-hydroxyandrostenedione, 20-hydroxyecdysone, 11-keto-androstenedione and 17α-methyldrostanolone was reported [131]. Initiated by the doping scandals in human sports [11], the pharmacokinetics of THG in equine [132] and its *in vitro* metabolism [131] were also studied.

Anti-doping samples preservation: urine stabilization & blood spots

The designers AAS molecules and their metabolism are unknown to the anti-doping laboratories at the time of their first circulation. WADA Code [3] has introduced the dimension of time in the anti-doping system, allowing laboratories to organize their detection of the designers with knowledge in the structures, the metabolism

and the synthesis of reference materials. The dimension of time in the anti-doping system is practically applied with samples long-term storage. Two methodologies have been developed to facilitate the urine and blood stability over time: urine samples stabilization and the DBS.

Retrospective analysis can only be conducted provided that urine samples quality is not undermined over time due to reactions enhanced by the presence of microorganisms or proteolytic enzymes in urine. Doping control urine samples are collected and stored in doping control stations and WADA-accredited laboratories, in a way that protects their identity, integrity and security [106], which is of particular importance in case that the already analyzed samples are eventually submitted to retrospective analysis [3]. What if sample delivery to the WADA-accredited laboratories is not immediate? Hydrolysis of steroid conjugates followed by modifications of the steroids' structure by oxidoreductive reactions may take place due to the occurrence of microorganisms that can be found in the human body or the surrounding environment, especially during sample transportation in the warm periods of the year [133–138]. The best practice to ensure that samples' integrity is maintained for possible reanalysis would be to store samples frozen as well as stabilized. To date, no preservative is added to sport urine samples [139]. The implementation of a specially designed sample collection container, incorporating a generic sample stabilization mixture consisting mainly of antibiotic, antimycotic substances and protease inhibitors has been proposed [140]. The purpose of an ongoing project funded by WADA is the investigation of the efficiency of the in-house chemical stabilization mixture in spray-coated form with simultaneous minimization of analytical interferences. Preliminary results demonstrate that the cell growth of five representative microorganisms (*Escherichia coli, Nocardioides simplex, Aspergillus flavus, Candida albicans* and *Enterococcus faecalis*) is completely inhibited after a 7-day incubation period at 37°C in those urine samples that were stored in spray-coated stabilized containers. Moreover, the degradation of steroid glucuronides is prevented in the stabilized urine samples [141]. The implementation of specially designed plastic urine collection containers, spray-coated in their interior surface with the stabilization mixture is currently more realistic than it was a few years ago. If this preventive approach is applied in the doping control sampling procedure, it would be a major step towards the preservation of urine samples for long-term storage and eventual retrospective analysis.

In the context of long-term storage of samples for retrospective analysis, the DBS technique is gaining increasing importance in the doping control field. It involves collection of small volumes (10–30 µl) of whole blood obtained from heel or finger pricks, drying it on a piece of filter paper, extracting and subsequently analyzing it by LC–MS. DBS offers numerous advantages over conventional whole blood, plasma or serum analysis, such as ease of collection (even in remote control stations), minimal potential of sample manipulation, cost effectiveness, less invasiveness, simplified storage and transport of DBS samples – absence of refrigeration – enhanced stability described for many analytes on the cellulose sampling paper [142–145]. An apparent limitation of the DBS method is the small blood volume collected, thus representing a challenge for the sensitive determination of some analytes in elite sports such as anabolic steroids at sub-ng/ml levels. In addition, a new plasma screening method has been developed for the retrospective reanalysis of stored samples for new xenobiotic drugs at low ng/ml levels [146]. It is based on protein depletion, UHPLC-based LC separation and detection by means of high-resolution/high-accuracy MS. The use of either DBS or plasma samples cannot replace (at least for the time being) the conventional urine analysis procedure, however, they are both attractive alternatives and can enable the retrospective qualitative data evaluation for known and unknown xenobiotics.

Conclusion

Designer AAS represent a dark and dangerous side of drug abuse in sports. AAS remain the prevalent drug-class according to WADA statistics [147]. The borderline between the use of novel substances as new therapeutics or as potential doping agents is often a challenge for cheating athletes to overstep. Control laboratories and regulatory authorities are aware of analytical advancements and legislation improvements for successful detection and prevention of AAS. This Review presented the main issues concerning AAS, such as their scientific background, progresses in their analytical detection and the preventive anti-doping that is intended to reveal positive analytical findings in samples initially reported as negatives, as occurred in the reanalysis of stored anti-doping samples from the 2004

Olympic Games [148] and the 2005 Athletics World Championships [149].

Future perspective

The emergence of novel designer AAS constitutes a serious threat to drug-testing laboratories and sporting administrators. The successful detection of new chemical structures of AAS is mainly based on the selection and design of improved analytical protocols, from sample preparation through to advanced instrumental analysis, which will give rise to enhanced sensitivity and specificity of the methods and fulfill the stringent performance limits suggested by WADA. Alternative methods for the detection of designer AAS, such as receptor-based assays (even combined with MS) and advancements in software technology concerning MS libraries, spectra and accurate mass databases will play a major role in future detection of designer AAS.

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Executive summary

Introduction: the chemistry of androgenic anabolic steroids

- Chemically modified steroids, which are not used in clinical practice and have either been synthesized in the past or have been specifically developed to circumvent doping control, are commonly known as designer androgenic anabolic steroids (AAS) and are considered an issue of major importance in the fight against doping.

Authorities against illegal laboratories

- Anti-doping laboratories and drug-testing authorities make continuous efforts to limit the extent of the illegal circulation of designer AAS through numerous doping controls and legislation of strict analytical and regulatory guidelines.

Detection of designer AAS

- Improvements of analytical protocols, as well as advancements in preparative and instrumental techniques, are promising for successful detection of designer AAS.

Data processing: synthesis of metabolites of designer AAS

- Various different methods for synthesis or *in silico* prediction of metabolites, to overcome the problem of their *in vivo* production due to ethical and practical restrictions, contribute to the successful detection of designer AAS.

Data processing: sample preservation

- The dimension of time for future detection of designer AAS has been applied, either through reprocessing of already analyzed doping control samples with up-to-date analytical data or through facilitating samples stabilization over time.

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