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Analytical aspects in doping control: Challenges and perspectives☆

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ABSTRACT

Since the first anti-doping tests in the 1960s, the analytical aspects of the testing remain challenging. The evolution of the analytical process in doping control is discussed in this paper with a particular emphasis on separation techniques, such as gas chromatography and liquid chromatography. These approaches are improving in parallel with the requirements of increasing sensitivity and selectivity for detecting prohibited substances in biological samples from athletes. Moreover, fast analyses are mandatory to deal with the growing number of doping control samples and the short response time required during particular sport events. Recent developments in mass spectrometry and the expansion of accurate mass determination has improved anti-doping strategies with the possibility of using elemental composition and isotope patterns for structural identification.

These techniques must be able to distinguish equivocally between negative and suspicious samples with no false-negative or false-positive results. Therefore, high degree of reliability must be reached for the identification of major metabolites corresponding to suspected analytes.

Along with current trends in pharmaceutical industry the analysis of proteins and peptides remains an important issue in doping control. Sophisticated analytical tools are still mandatory to improve their distinction from endogenous analogs. Finally, indirect approaches will be discussed in the context of anti-doping, in which recent advances are aimed to examine the biological response of a doping agent in a holistic way.

1. Introduction

1.1. History of doping control analyses

The fight against drug abuse in sports has continuously improved since doping control began in the 1960s [1]. Various analytical strategies have been used to detect doping agents in the urine samples of athletes. Anti-doping analyses, first used gas chromatography (GC) nitrogen-selective detection (nitrogen-flame ionization detection; NFID) to screen for stimulants in more than two thousand urine samples during the 1972 Olympic Games in Munich [2]. Immunoassays have also been used in some laboratories as a screening procedure, but the results have to be confirmed by chromatography due to the lack of specificity [2]. In 1983 at the IX Pan American Games higher degree of selectivity and sensitivity was obtained by the introduction of benchtop quadrupole GC coupled to mass spectrometry (GC–MS) [3]. During the previous decade, liquid chromatography coupled to mass spectrometry in single-stage or tandem mode (LC–MS(/MS)) has become an attractive alternative for doping control analysis due to the development of atmospheric pressure ionization (API) sources.

The demand for more sensitive and specific methods to detect a higher number of commonly used doping agents continues to grow [4].

1.2. Banned substances and sample matrices

The World Anti-Doping Agency (WADA) has defined on the Prohibited List more than two hundred substances that are forbidden either in- and out-of-competition or only in-competition [5]. These forbidden drugs are classified into nine categories, two groups of analytes prohibited in particular sports and three forbidden methods (*i.e.*, enhancement of oxygen transfer, physical

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and chemical manipulation and gene doping). A wide range of chemical structures is thus covered, which requires the use of particular analytical strategies.

Most of the doping agents are converted to phase I or II metabolites, to facilitate excretion in urine. Phase I metabolism consists of reactions such as oxidation, reduction or hydroxylation catalyzed by cytochrome (CYP) enzymes mainly located in the liver. Glucuronidation and sulfonation are major phase II metabolic reactions that take place in the body. Large inter-individual variability is observed for doping agent metabolism, because most of these enzymes are subject to genetic polymorphisms and variations in their metabolizing activities [6]. Therefore, the relevant sample matrix and excretion pattern of each prohibited substance must be examined to ensure the proper selection of the target compounds for doping control. For example, conjugated metabolites are highly excreted in urine, but they are retrieved in only small amounts from blood samples. Careful consideration of the matrix also guides further analysis steps, such as the necessity for conjugate hydrolysis.

1.3. Analytical strategies

The analytical strategy must have high sensitivity and selectivity in complex matrices, involving mostly urine and blood samples, compounds with wide range of physico-chemical properties and molecular weight, limited sample volumes and very fast turnaround time requirements (results within 24–48 h for important sport events). Moreover, characteristic drug metabolites are relevant to confirm the extension of the detection window, which considerably increases the number of target compounds [7].

In anti-doping laboratories, a two-step analytical strategy is performed to fulfill WADA requirements (Fig. 1). First, a screening step is conducted, which consists of detecting the highest number of relevant analytes in a complex matrix (*i.e.*, urine or blood). This step must be fast, selective and sensitive, while avoiding false-negative results with a limited false-positive proportion (less than 10%). The screening step provides an indication of the presence or absence of a doping agent. Only some substances on the Prohibited List (*e.g.*, salbutamol, morphine, cathine, ephedrine, methylephedrine, pseudoephedrine and carboxy-tetrahydrocannabinol) necessitate quantitative measurement because they are considered doping agents only above a threshold concentration. In addition, high levels of endogenous substances, such as anabolic androgenic steroids, are quantified to estimate an adverse analytical finding. For other compounds, their presence in the matrix constitutes violation of the anti-doping rules. For some compounds, neither GC– nor LC–MS(/MS) techniques are able to differentiate endogenous substances from exogenous intake. Therefore, various strategies have been explored. Statistical threshold levels or ratios have been established in doping control for *e.g.*, urinary norandrosterone and testosterone [8], and isotope ratio mass spectrometry (IRMS), based on enrichment of ^{13}C, was introduced to distinguish between endogenous and synthetic steroid analogs. Indirect approaches utilizing biomarkers of the exogenous administration of the drugs of abuse are a promising way to discriminate between endogenous and exogenous substances. The athlete's biological passport allows taking into account intra-individual variation of indirect markers and reveals modifications in the biological parameters induced by the administration of a banned substance. For example, erythropoietin (EPO) misuse is indirectly monitored by the measurement of blood parameters, such as hemoglobin, hematocrit, ferritin, soluble transferrin receptor or reticulocyte [9]. However, statistical approaches and medical experts are necessary for interpretation of the results because doping comportment should not be confounded from clinical or pathological conditions. Metabolomic approaches have emerged as promising strategies to assess biochemical phenotypes and to

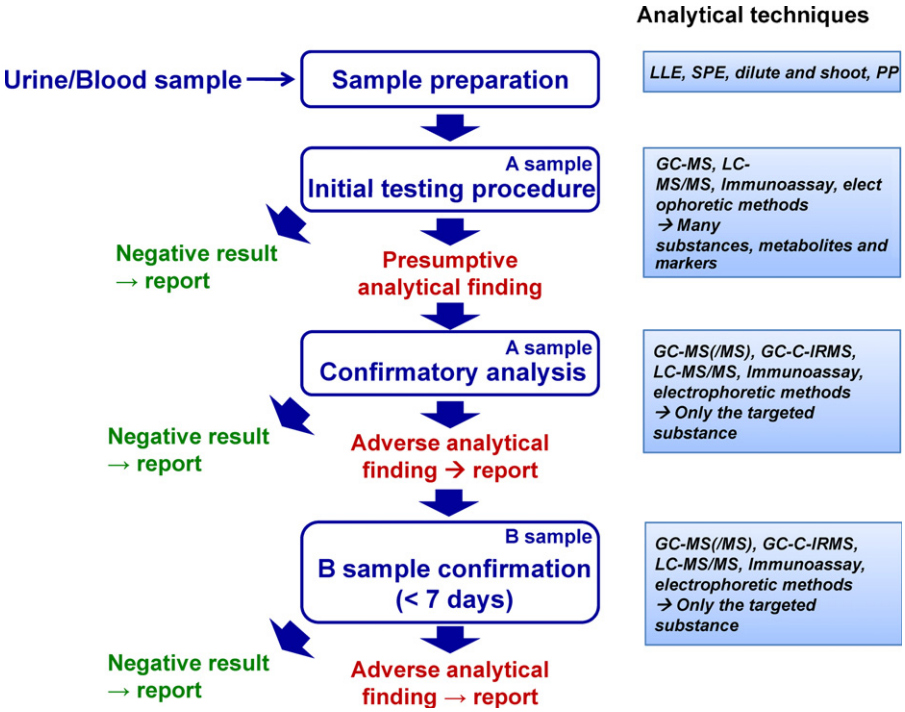

Fig. 1. Detailed anti-doping workflow for urine or blood sample analysis. In the routine doping test the urine sample is divided in two aliquot, namely A and B samples. Sample A is used for the initial testing procedure and the confirmatory analysis (fresh portions), while the B sample analysis is performed only for positive cases for cross-examination purpose.

Table 1
Overview of sample preparation and analytical techniques used for doping agents.

Substances and methods prohibited at all times	Sample preparation (adapted from reference [13])					Analytical method (adapted from references [1,60])							
In- and out-competition	LLE	SPE	Dilute and shoot	Hydrolysis	Immunoaffinity	Immunoassay	IEF	GC–MS	GC–MS/MS	GC-C-IRMS	LC–MS (/MS)	UHPLC–MS (/MS)	Other
S1. Anabolic agents													
1. Anabolic androgenic steroids (AAS)													
a. Exogenous AAS	[13]	[13]		[13]				[1]	[60]		[1]	[60]	MALDI-TOF-MS [110]
b. Endogenous AAS	[13]	[13]		[13]				[60]	[60]	[60]	[60]	[60]	Immunoaffinity chromatography [112], CE [114]
2. Other anabolic agents	[13]	[13]		[13]				[106]			[60]		
S2. Peptide hormones, growth factors and related substances													
1. Erythropoiesis-stimulating agents [*e.g.*, erythropoietin (EPO)]					[115]		[1]						CE–ESI-TOF/MS [116], MALDI-TOF [117]
2. Chorionic gonadotrophin (hCG) and luteinizing hormone (LH) in males;						[107]							
3. Insulins					[94]						[60]	[108]	nano-UHPLC-LTQ -Orbitrap [94]
4. Corticotrophins;					[96]								nano-UHPLC-LTQ -Orbitrap [96]
5. Growth hormone (GH), insulin-like growth factor-1 (IGF-1);						[109]					[111]		
S3. Beta-2 agonists	[13]	[13]	[13]	[13]				[1]			[60]	[73]	
S4. Hormone antagonists and modulators													
1. Aromatase inhibitors	[13]		[13]	[13]							[60]	[73]	
2. Selective estrogen receptor modulators (SERMs)	[13]		[13]	[13]							[60]		
3. Other anti-estrogenic substances	[13]		[13]	[13]				[1]				[73]	
4. Agents modifying myostatin function(s)													
S5. Diuretics and other masking agents													
Diuretics, desmopressin, plasma expanders (*e.g.*, glycerol; intravenous administration of albumin dextran, hydroxyethyl starch and mannitol), probenecid;	[13]	[13]	[13]					[1]			[1]	[73]	HES by MALDI-TOF [118]
Prohibited methods													
M1. Enhancement of oxygen transfer													
1. Blood doping													Flow cytometry [119]
2. Artificially enhancing the uptake, transport or delivery of oxygen (perfluorochemicals, efaproxiral (RSR13) and hemoglobin-based oxygen carriers (HBOCs))								RSR13, HES [60]				RSR13 [73]	HBOCs by CE–ESI-TOF [95]
S6. Stimulants													
a. Non-specified stimulants (*e.g.*, adrafinil, amphetamine)	[13]	[13]	[13]					[1]			[60]	[60]	
b. Specified stimulants (*e.g.*, ephedrine, strychnine)	[13]	[13]	[13]					[1]			[60]	[60]	
S7. Narcotics	[13]	[13]	[13]	[13]				[1]			[60]	[60]	
S8. Cannabinoids	[13]	[13]				[1]		[1]	[113]		[60]		
S9. Glucocorticosteroids	[13]	[13]		[13]							[1]	[60]	
Substances prohibited in particular sports													
P1. Alcohol													
Alcohol (ethanol, ethylglucuronide)						[1]		[1]					
P2. Beta-blockers	[13]	[13]	[13]	[13]				[1]			[1]	[60]	

detect variations after doping agent exposition as well as to discover and interpret new putative biomarkers.

Upon detecting a doping agent, a confirmatory step must be performed to assess the presence of the suspected prohibited substance, and a quantitative estimation may be required. Therefore, methods providing sufficient precision and accuracy have to be employed in this second step.

1.4. Standards and harmonization

In terms of sensitivity, WADA has defined the concept of minimal required performance limits (MRPL) that all accredited anti-doping laboratories should reach to ensure their results are reported homogeneously [10]. Rules are given for the detection, identification and confirmation of an investigated drug of abuse. The method must provide separation between the analyte and interfering matrix compounds, and the selected target compounds must exhibit exactly identical analytical behavior as those in reference material (positive control).

Furthermore, all laboratories must give comparable results. Therefore, inter-laboratory comparisons are continuously performed, which is particularly relevant for the profiling of an athlete for its biological passport. As the athlete is followed during sport events, the analyses are not always performed by the same laboratory. The WADA external quality assessment scheme was introduced to evaluate regularly the performance of the laboratories, and to improve the result uniformity between the testing laboratories.

Strict standards for confirmatory analysis are defined for GC or LC–MS(/MS) techniques to reach a sufficient number of identification criteria. Those criteria are described in the International Standards for Laboratories (ISL) [11], and have been discussed previously [12]. The appropriate analytical technique is selected for separation from interfering compounds and unambiguous detection and identification of the analyte. Analytical strategies, such as GC– or LC–MS(/MS), are used for screening and identification with a parallel analysis of reference compounds, which should match for both the chromatographic and MS part of the analysis. Low- or high-resolution mass spectra in the selected ion monitoring (SIM) or scan mode must include a minimum of three diagnostic ions. The accumulation of evidence is also an important parameter for unambiguous identification. Different techniques could be used in parallel on a suspect sample to ensure the analyte identity.

Techniques and procedures whose precision, accuracy, specificity and sensitivity can fulfill WADA requirements are needed, and these methods generally involve expensive technologies and specially trained staff.

In this paper, we will focus on the analytical tools for anti-doping analyses, with an emphasis on the improvement in separation techniques, new challenges and perspectives.

1.5. Routine arrangements in doping control

The analytical strategy is generally determined by the chemical structures of the substances and their biochemical behaviors, such as metabolism and excretion process [7].

Two major types of analyses, chemical (separation techniques) and biological (immunological tests) are performed in anti-doping laboratories to cover all of the prohibited substances and methods. Low-molecular weight compounds are commonly separated and analyzed by GC–MS and/or LC–MS(/MS), whereas immunological tests or isoelectric focusing (IEF) are more dedicated to the analysis of proteins and peptides. An overview of the types of sample preparation and the analytical approaches used for doping control is depicted in Table 1.

Sample preparation and chromatographic conditions may influence the final result; therefore, the analytical process comprising sample preparation, compound separation, analyte detection and data handling must be adjusted accordingly [13].

2. Sample preparation

'Adequate sample preparation is a prerequisite for successful chromatography of biosamples, which in doping control are typically urine and blood. With urine samples, various sample preparation procedures can be considered, based on the analyte physico-chemical properties and selected analytical method.

Sample clean-up extracts are, in general, directly compatible with LC, whereas hydrolysis and derivatization steps are mandatory to make samples compatible with GC. The hydrolysis is performed either enzymatically (*e.g.*, β-glucuronidase from *Escherichia coli*) or by acid treatment, and the time frame is from tens of minutes up to one day, depending on the applied incubation temperature. The derivatization step is performed with various strategies based on the targeted analytes and generally has reaction time of around 1 h.

Off-line sample preparations, such as liquid–liquid extraction (LLE), solid-phase extraction (SPE), dilute-and-shoot technique and protein precipitation (PP), are usually preferred over on-line procedures to reduce the risk of cross-contamination and carry-over. Moreover, on-line sample preparation is adapted to dedicated analysis and not to a broad range of analytes present at very low concentrations [14].

Due to the high sensitivity and selectivity of the latest generation of analytical devices, simple and fast sample treatments, such as dilute and shoot, can be applied more easily than techniques with higher selectivity. This sample treatment allows generic, high-throughput and automated analyses to be successfully used for fast screening purposes for doping control [15,16]. Direct injection of urine sample was also performed for the detection of hydroxyethyl starch (HES) and methoxyphenamine in human urine [17,18]. Despite the matrix effects, this sample treatment was also used for the quantitative determination of some threshold analytes [19].

For blood analysis, different specific sorbents, such as affinity columns and proteome partitioning kits, have recently been developed for the extraction of doping agents. Protein precipitation is the least time-consuming sample preparation for plasma and serum, and acetonitrile is the preferred and easy-to-use solvent for protein removal [20]. PP plates were developed in a 96-well format to save time when a large number of samples have to be extracted simultaneously. These plates have many advantages, such as sample volume and the possibility of automation without transfer steps (filtration is performed directly in the same well as precipitation) [21].

Despite the simplicity and rapidity of dilute-and-shoot and PP approaches, more selective sample preparations (*e.g.*, LLE and SPE) are often preferred for urine and blood analyses. Historically, LLE has been widely used and presents advantages in terms of efficiency, simplicity and cost. However, LLE suffers from non-repeatable recovery, the need for relatively large sample volumes, lack of automation possibilities and mid-low throughput [21]. On the one hand, the addition of an inorganic salt, called salting-out LLE (SALLE), enhances extractions into non-polar, immiscible organic solvents with cost-effective and possibly automated methods. On the other hand, improvements in LLE techniques have focused on miniaturization and low- or no-solvent consumption. In this context, liquid-phase micro-extraction (LPME) techniques, such as single-drop microextraction (SDME), dispersive liquid–liquid micro-extraction (DLLME)

or membrane-assisted LPME (*e.g.*, hollow-fiber LPME and membrane bag) were recently developed and reviewed [22]. Hollow-fiber LPME was proposed and applied in the context of doping analysis to determine the presence of diuretics or ephedrine in human urine [23,24]. This technique has potential applications in doping analyses because it provides enrichment of analytes directly from urine specimens without sample pre-treatment.

SPE methods are commonly used in doping analyses, allowing sample clean-up together with analyte pre-concentration, and are compatible with several analytical techniques [25]. Various stationary phases and support geometries are available, such as normal phase, reversed phase, ion exchange or mixed mode and allow for several mechanisms of retention. Mixed-mode cartridges (*i.e.*, reversed phase/anion or cation exchange) present obvious advantages in terms of selectivity, because neutral, basic and acidic analytes can be eluted in different fractions for subsequent analyses. Furthermore, SPE technique in general can be easily automated, and a large number of samples can be simultaneously prepared by using 96- or 384-well plates. However, SPE often lacks selectivity towards matrix-interfering substances, leading to matrix effects in LC–MS(/MS) analyses. New developments have recently been made to improve extraction selectivity and to reduce matrix effects. For blood samples, a strategy called "hybrid SPE-PP" was proposed, which combines the selectivity provided by SPE (zirconia-coated particles sorbent) and PP. Phospholipids are retained on the zirconia sorbent, and this material provides no affinity towards a wide range of analytes of interest [26].

SPE techniques such as molecularly imprinted polymer SPE (MISPE), dispersive SPE (dSPE), disposable pipette extraction (DPX), micro-extraction by packed sorbent (MEPS), solid-phase micro-extraction (SPME) or stir bar sorptive extraction (SBSE), were recently reviewed by Kole at al. and are interesting strategies for doping control analysis due to the improvements in selectivity, sensitivity and reduced matrix effects [21]. A clinical application using MEPS sample preparation showed a reduction in the matrix effects of the plasma by diminishing the phospholipid concentration [27]. Moreover, lower volumes of sample and solvent are consumed, which is an important feature, especially for blood samples. Although those techniques were successfully described for some doping agents (*e.g.*, MISPE for acetazolamide and furosemide; MEPS for methadone and amphetamine; SPME for amphetamines and β-blockers; and SBSE for steroid hormones, including testosterone and epitestosterone), few methods have been developed especially for anti-doping purposes. Recently, Claude et al. developed an MISPE method for tamoxifen and its main metabolite in urine [28]. The determination of anabolic steroids or carphedon in human urine was also proposed by automated in-tube SPME [29,30].

For blood collection and analysis, a very interesting approach consisting of the collection of whole blood spotted on absorbent paper was recently introduced [31,32]. This technique, called dried blood spot (DBS), offers the advantage of very low sample consumption (μL) [33] and appears promising for doping control blood analysis in terms of transportation and storage possibilities. Indeed, DBS has the potential to reduce costs, to simplify sample processing after collection, storage and shipping and to reduce animal usage for clinical trials, by decreasing the required sample volume. Moreover this sample collection approach is easily coupled to LC–MS(/MS) and was successfully applied in doping control, bioanalysis and forensic analysis for the detection of pharmaceutical drugs [34–36]. This technique may allow for the analysis of exogenous compounds in blood, in which sensitivity is not the bottleneck.

3. Analytical considerations

3.1. GC–MS(/MS) analyses

3.1.1. History of GC–MS in doping analysis

In the fight against doping, GC-NFID [7] and GC–MS quickly became the standard instrumentations for the detection and quantification of the majority of doping agents, including amphetamines and ephedrines [2]. GC is interfaced with MS with either electronic impact (EI) or chemical ionization (CI) sources. EI in full-scan mode is commonly used for anti-doping purpose in the screening step because spectral information of the analytes in the unknown sample can be compared with extensive databases [37]. However, EI is a high-energy ionization technique, and soft ionization is sometimes required for molecules that fragment readily. Therefore CI in positive or negative mode could be used to retain the (de)protonated molecular ion information in GC–MS spectra or to obtain alternative fragmentation behavior [38]. GC–MS is the most widely used technique in anti-doping laboratories, either in full-scan mode or in SIM mode.

The applicability of GC–MS for toxicological laboratories was demonstrated in 1972 with the development of mass spectra libraries including EI and CI ionization [4]. In GC–MS, the ionization energy is constant (*i.e.*, 70 eV in EI), and the mass spectra are reproducible for a molecule without inter-instrument variability, allowing for the use of routine library searches. GC–MS libraries contain many prohibited substances and their metabolites, which increase the probability to detect an unknown compound [39].

The quantitative capabilities of GC–MS have been proven by the instrument stability and the good sensitivity for determination of threshold substances. Quantitative analysis is preferably performed with stable isotope labeled analogs as internal standards, and by using the SIM mode to ensure high sensitivity of the method for analytes at low concentration. In parallel to that, full-scan mode is used as a comprehensive approach [40].

Volatile and thermostable molecules on the WADA Prohibited List are directly compatible with GC–MS, while non-volatile compounds (*e.g.*, anabolic steroids, benzoylecgonine, opiates and Δ9-THC-COOH) can be effectively analyzed by GC if the targeted molecules are derivatized first [3]. However, some derivatives are thermolabile and complex to analyze. Many metabolites are excreted in urine as conjugated glucuronides or sulfates, and they are amenable to GC–MS analysis by following rather extensive sample preparations [6]. Additional care must be taken to ensure complete deconjugation to exclude undesirable secondary activities by the enzymes and to ensure total sample dryness before the complex derivatization step.

The analytical challenge in doping control analysis is still in the detection of anabolic steroids, which are excreted in various amounts in urine (ranging from low ng/mL to high μg/mL) with a large number of endogenous isomers and metabolites [3]. GC–MS is the primary technique for the analysis of anabolic androgenic steroids, as reported in Table 1. Due to the complexity of the biological matrix, improving the resolution of GC is necessary. Improvements in separation power can be obtained by comprehensive bidimensional GC (GC × GC). Multidimensional separation methods are used for increasing the resolution of specific analytes and removing interfering compounds. GC × GC is generally designed with a non-polar or low polarity first GC column of conventional length. The second orthogonal dimension is performed in a shorter column that contains a more polar stationary phase, in which the separation is mainly driven by the analyte polarity [41]. This technique shows improvements in the peak capacity and the signal-to-noise ratio [42]. GC × GC is often hyphenated to a high resolution mass analyzer (*e.g.*, time-of-flight, TOF), which provides a fast acquisition rate and a new dimension in

selectivity as a result of accurate mass determination. This hyphenated approach could be well applicable to doping control, particularly for confirmatory purposes. However, a major drawback is the quantitative data assessment because each peak corresponds to a spot that is difficult to integrate precisely.

Higher peak capacity can also be alternatively obtained by reducing the GC column diameter down to 100 or 50 μm and enhancing the column length *e.g.*, up to 80 m [43].

Fast GC was also developed to reduce the screening time from 25 to 30 min to less than 10 min by replacing helium with hydrogen as the carrier gas [44]. The analysis time could also be reduced by using a very fast temperature gradient with the dedicated instrumentation (up to 1200 °C/min) and/or by reducing simultaneously the column diameter and length. These are interesting strategies that could be applied for doping control when the sample turnaround time is of prime importance.

Selectivity can also be improved by GC coupled to high-resolution mass spectrometry (HRMS) [45] or by using tandem mass spectrometry (MS/MS) [46,47]. These two techniques have been widely used for doping control. One area of particular interest is the coupling with IRMS [2]. Neither GC–MS nor LC–MS(/MS) are able to distinguish between endogenous analytes (*e.g.*, testosterone) and corresponding synthetic analog. To detect the misuse of testosterone or its precursors, the first indication is obtained by conventional GC–MS screening after measuring the ratio of testosterone to epitestosterone (T/E). When T/E is higher than 4 or when the steroid profile is abnormal, a confirmatory procedure must be performed, and GC combustion IRMS (GC-C-IRMS) is the technique of choice to assess the intake of exogenous steroids by measuring the $^{13}C/^{12}C$ ratio. In the nature, ^{13}C is a stable isotope with an abundance of 1.1% [1], but hemisynthetic testosterone displays a different ^{13}C content than its human counterpart [48]. This technique can detect the misuse of testosterone and several precursors and metabolites, and it was successfully applied to detect boldenone [49] or 19-norandrosterone [50]. However, this approach requires highly pure extracts and the sample preparation time is quite long.

3.2. LC–MS(/MS) analyses

Until the beginning of the 21st century, LC–MS was considered to be a complementary approach to GC–MS instead of an alternative analysis. LC–MS was not suitable for the screening of unknown drugs and metabolites because neither its separation power nor the mass spectral information were comparable to GC–MS. Anti-doping analyses were first performed by reversed-phase liquid chromatography (RPLC) coupled to UV and then by thermospray to MS [7]. Electrospray ionization (ESI) interfaces were developed in the 1980s [51] and different instruments were commercialized in the beginning of the 1990s. The initial studies examined substances not amenable to GC–MS (*e.g.*, diuretics) [52]. Today, LC is preferentially coupled to MS/MS because higher spectral information can be obtained. LC is hyphenated with mass spectrometers by using API sources, such as ESI, atmospheric pressure chemical ionization (APCI) or atmospheric pressure photoionization (APPI). ESI is the most widely used source for doping control because it is dedicated to polar compounds and offers high sensitivities, whereas APCI is more dedicated to less polar compounds. The latter has greater signal stability and is less sensitive to matrix effects [53]. APPI is used in a lesser extent in the doping control field because it often necessitates dopants, but it can be used alternatively to APCI, as reported elsewhere [54].

LC–MS suffers from an important drawback because the fragmentation is not reproducible between instruments. A generic value of fragmentation voltage cannot be set for a large panel of analytes and the obtained spectra could differ between instruments [55], which is detrimental to building a library of reference spectra [12]. Despite this drawback, LC–MS has opened new possibilities for the determination of toxicologically relevant substances and searchable libraries of LC–MS/MS reference mass spectra have been reported [56–58].

Using LC–MS(/MS), sample preparation can be simple, fast and directly compatible with both polar and non-volatile analytes without requiring the removal of water or salts, deconjugation and derivatization. For doping control, LC–MS was initially focused on the analysis of thermolabile and non-volatile analytes that were not covered by the gold standard GC–MS method [59]. During the last decades, LC–MS/MS has been routinely implemented for all groups of doping agents because of its obvious advantages. Today, a large variety of compounds can be screened using a single system. For this purpose, numerous column chemistries are available, including reversed-phase C_{18} or C_8 (RPLC), normal phase (NPLC) and, recently, hydrophilic interaction chromatography (HILIC) materials. This technique is increasingly used for diuretics, some anabolic steroids and corticosteroids [60], as reported in Table 1, but also for quantitative purposes [61]. LC–MS/MS has become a standard for pharmacokinetics, metabolism studies and phase II conjugated metabolites, especially [62]. The instrumental analysis run-time is generally shorter in comparison with GC–MS procedures and necessitates less tedious sample preparation procedures due to the high analyte selectivity of LC–MS/MS.

3.3. From LC–MS(/MS) to UHPLC–MS(/MS)

The development of techniques combining high throughput and chromatographic performance are of primary importance for routine doping control analysis. Different approaches have been recently introduced [63–66] and involve either the use of a high-temperature mobile phase (decrease in mobile phase viscosity and increase in diffusion coefficients), monolithic columns (bimodal structure made of macropores and mesopores) or columns packed with sub-2 μm particles diameter, or sub-3 μm superficially porous particles (known as fused-core, core-shell or shell particles). Different studies have demonstrated several advantages of ultra-high-pressure liquid chromatography (UHPLC; columns packed with sub-2 μm particles operating at high pressures), high-temperature UHPLC (HT-UHPLC; at 90 °C) and shell-particles technologies, particularly in the gradient mode, to rapidly and efficiently separate drugs and related substances [63,65,66]. Shell-particle supports were applied for doping control for the development of a fast multi-analyte screening method [67]. As the generated backpressure was reduced as compared to that in UHPLC, a traditional HPLC instrument can be potentially used with shell-particle technology, which is a valuable tool in terms of chromatographic performance and saving in analysis time.

Various applications for anti-doping purposes have been performed by UHPLC. A conventional HPLC method can be directly transferred to UHPLC conditions, either to increase the resolution with improvements in peak shape while maintaining a constant analysis time or to reduce the analysis time while maintaining constant resolution. Numerous column chemistries, including reversed-phase and HILIC materials for polar analytes, are commercially available [68]. Due to the reduced analysis time, the peaks become narrow (approximately a few seconds), and high acquisition mass analyzers with fast acquisition and detector responses, such as triple quadrupole (QqQ), TOF or hybrid quadrupole TOF (QTOF) mass spectrometers, are mandatory. Several studies reported multicomponent high-throughput screenings for doping control using UHPLC [15,16,69–74]. The hyphenation of UHPLC with QTOF-MS has been found particularly useful for the fast separation of hundreds of doping agents with high selectivity, due to the resolution of UHPLC and the accurate

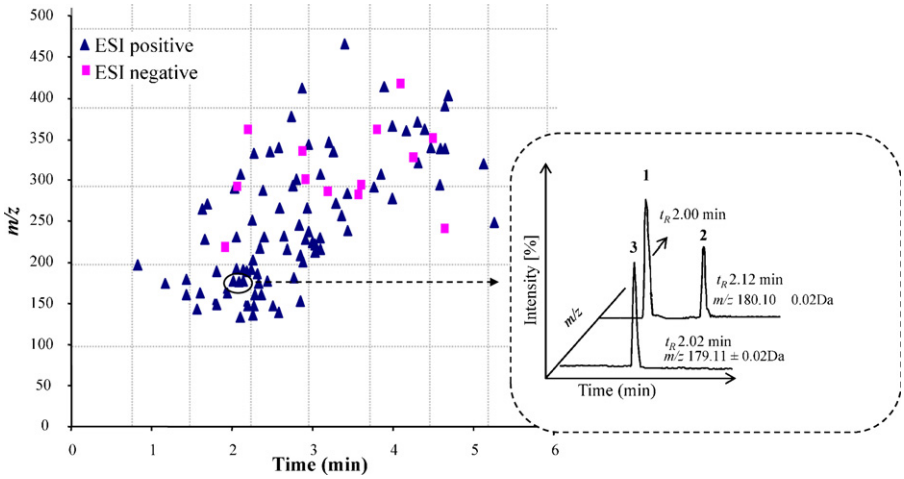

Fig. 2. Separation of hundred analytes of m/z in function of retention time (t_R). Data from ESI positive and negative modes are plotted together. The three chromatographic peaks (1) methylephedrine, (2) methylendioxyamphetamine and (3) nikethamide are separated despite closed t_R and m/z.
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mass measurement of QTOF (Fig. 2). UHPLC offers a gain in time compared to conventional LC, which is very important for sport events, in which the results are required within 24–48 h. The high resolving power of UHPLC has been used for some anti-doping applications, implying the separation of positional isomers, such as dexamethasone and betamethasone [70], ephedrine and pseudo-ephedrine [75] or conjugated steroid isomers [76]. By working with a 150-mm UHPLC column, the resolution was sufficient to achieve the baseline separation of steroid isomers as well as steroids and the matrix constituents (Fig. 3). To further improve the chromatographic resolution, 2D-LC could be considered, but this approach is still in its infancy and is not applicable to doping control.

3.4. Metabolite analysis

The increased interest in the metabolites contained in biological samples has considerably advanced the number of potential analytes that can be detected and identified [7]. The presence of major metabolites often assists the result interpretation, highlighting better the administered substance. Therefore LC–MS(/MS) is an essential tool for metabolite studies, as reported for the determination of a phase II metabolite of nandrolone. This metabolite was detected as its sulfated form (19-norandrosterone sulfate) in urine for a long period of time, thus expanding the detection window of nandrolone doping [77]. Phase II metabolites, which are very polar compounds, are not easily analyzed by RPLC except in the presence of ion-pairing reagents, which may interfere with the efficiency of ESI process. Applications based on normal phase LC (NPLC), on the other hand, are based on non-aqueous solvents, which present additional problems with analyte solubility. Alternatively, numerous providers have developed HILIC materials, which allow sufficient retention of these compounds using a polar stationary phase (bare silica or polar-bonded silica) and a hydro-organic mobile phase. Another benefit of HILIC, is the possibility to significantly increase the MS sensitivity (a factor of 10 has been reported) [78] and decrease the matrix effects in comparison with RPLC conditions. For these reasons, even if HILIC has not yet been widely employed for doping control, with the exception for some applications on threshold substances or for evaluating the prevalence of nicotine consumption in professional sport [61,79,80], it could become a valuable strategy for metabolites and/or polar compounds analysis.

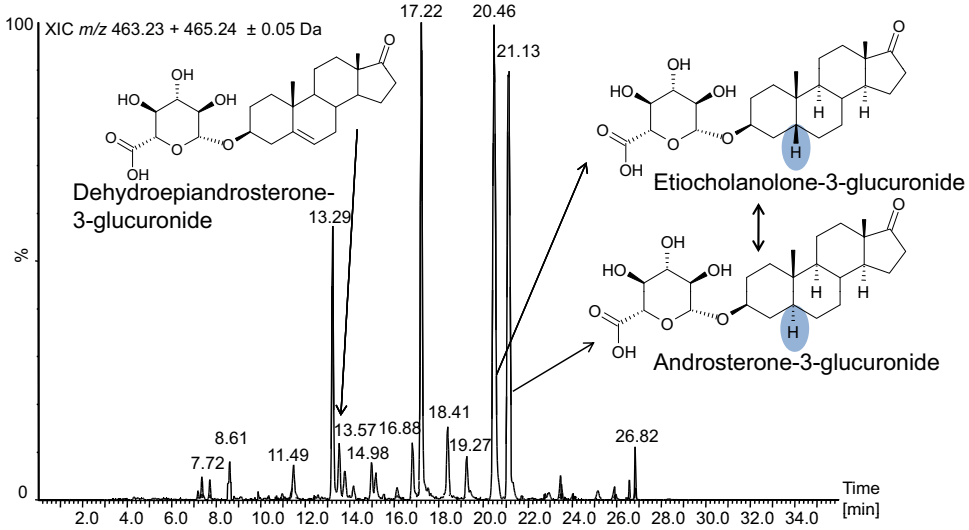

Fig. 3. Extracted-ion chromatogram (XIC) from UHPLC-QTOF-MS acquisition of glucuronidated endogenous steroids in urine matrix. Dehydroepiandrosterone-3-glucuronide can be resolved from endogenous interfering compounds, while positional isomers etiocholanolone-3-glucuronide and androsterone-3-glucuronide were baseline separated.

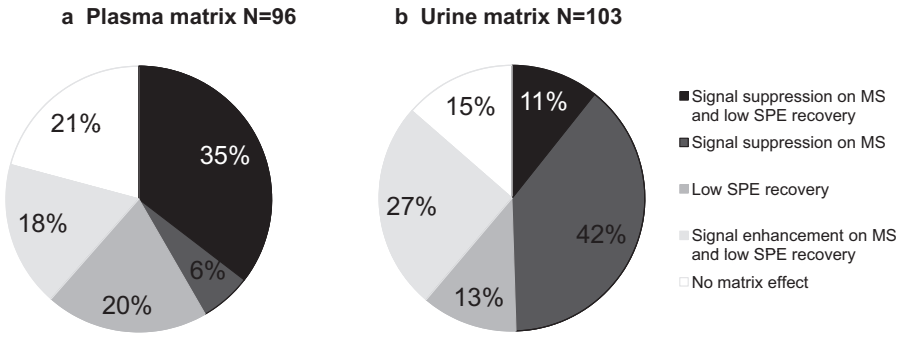

Fig. 4. Classification of matrix effects in (a) plasma and (b) urine specimen after solid-phase extraction. Different cases were reported by considering (i) signal suppression on MS as the loss of MS response due to ionization competition in ESI source; (ii) low SPE recovery as the loss of analyte during the extraction step; (iii) signal enhancement on MS as the improvement of ionization in ESI source due to the presence of the matrix; (iv) no matrix effect, when neither signal enhancement or suppression, nor loss of analyte during the extraction step was observed.

3.5. Matrix effects

LC–MS(/MS) suffers from an important drawback, which is its susceptibility to matrix constituents, namely matrix effects. Matrix effects, mostly related to ESI sources, can be estimated by either post-column infusion for qualitative estimation [81] or by the method described by Matuszewski et al. for quantitative estimation [82], after selective sample preparation. The latter approach includes the estimation of analytes loss during the extraction step and the signal alteration (ion enhancement or suppression) due to the interfering compounds from the matrix. Additional care must be taken with this phenomenon, especially in quantitative analysis. A description of the study of the matrix effects is now required in official regulatory documents. The Food and Drug Administration (FDA) recommends rapidly identifying any matrix effects during the validation process [83]. A systematic classification approach was proposed to describe these occurrences on approximately 200 analytes in urine and plasma matrices [84] (Fig. 4). Interestingly, analytes are more easily lost from plasma than urine during the extraction step. However, no particular signal suppression is encountered in plasma samples, while urine endogenous compounds lead to important signal suppression, even after SPE sample clean-up. Therefore, a strict evaluation of the matrix effects should be performed during the development of LC–MS(/MS) methods. A recent review proposed possible strategies to avoid or diminish matrix effects, including selective and sensitive sample preparation and the use of stable isotope-labeled internal standards [85].

4. Mass spectrometry

MS has emerged as the most powerful detection device for analyte identification due to its high sensitivity and selectivity. Fig. 5 shows an overview of low- and high-resolution mass spectrometers. Generally, low-resolution instruments are preferred for quantitative analysis, while high-resolution instruments are selected for substance identification or for the analysis of proteins and peptides.

4.1. Low-resolution mass analyzers

Single-quadrupole (Q), QqQ, linear ion trap (LIT) and quadrupole ion trap (QTrap) mass spectrometers are the most widely used instruments in routine doping laboratories. Further improvements in MS-based techniques, such as faster scan speeds, higher sensitivity and rapid inter-scan polarity switching, are still mandatory to deal with the ever-increasing demand of anti-doping analyses.

LIT and QqQ instruments can work in various modes, depending on the analytical strategy. Indeed, LIT was considered to be a valuable instrument for identification purposes with the help of multistage MS experiments (MS^n) and may be used for quantitative measurements. A large number of diagnostic ions could then be obtained with the mass selection of one of the product ions formed from the precursor ion and a second-generation product [86]. For QqQ instruments, multiple scan functions are available, and they can work in the product ion scan, precursor ion scan, neutral loss scan or selected reaction monitoring (SRM) mode. For doping control, SRM or multiple reactions monitoring (MRM), in which multiple reactions are followed, are the preferred modes for quantitative analysis.

Product ion scanning is commonly dedicated to the structure elucidation of a particular analyte and is mostly used for confirmatory purposes. The precursor ion scan mode is generally used to identify a class of compounds in biological samples. For example, a method was successfully developed for the screening of known and unknown corticosteroids using precursor ion scanning. This mode can follow common mass spectral fragments formed from a particular portion of the molecular structure that is common to all synthetic glucocorticosteroids [87]. Neutral loss can also be used to detect a particular class of compounds or, as an example, to highlight fluoxymesterone metabolites, a synthetic anabolic androgenic steroid, with a neutral loss scan of 20 Da, corresponding to the loss of HF [88].

4.2. High-resolution mass analyzers

By using low-resolution (500–1000 FWHM for compounds of 556 Da) instruments, such as Q, QqQ and LIT, compounds could be separated with a 1-Th difference in mass (Fig. 5). No information on the elemental formula can be obtained, although the fragmentation spectra could be used for structural determination. High-resolution instruments, such as TOF and QTOF, are sensitive instruments that are able to discriminate compounds of the same mass unit by accurately measuring the mass and determining mass defects, which allows for the assessment of the elemental formula of an ion. TOF, QTOF, linear ion trap Orbitrap (LTQ Orbitrap), single-stage Orbitrap mass spectrometer (Exactive) and Fourier-transform ion cyclotron resonance (FT-ICR) mass spectrometers are more often employed in bioanalysis for accurate mass measurements, allowing the determination of the elemental composition [16,75,89,90]. With a mass accuracy of 1–5 ppm, the identity of the metabolite can be suggested. For doping analysis, full identification of analytes requires direct comparison with authentic reference compounds. To fulfill these requirements, chemical or biochemical synthesis of new putative doping agents and their metabolites has

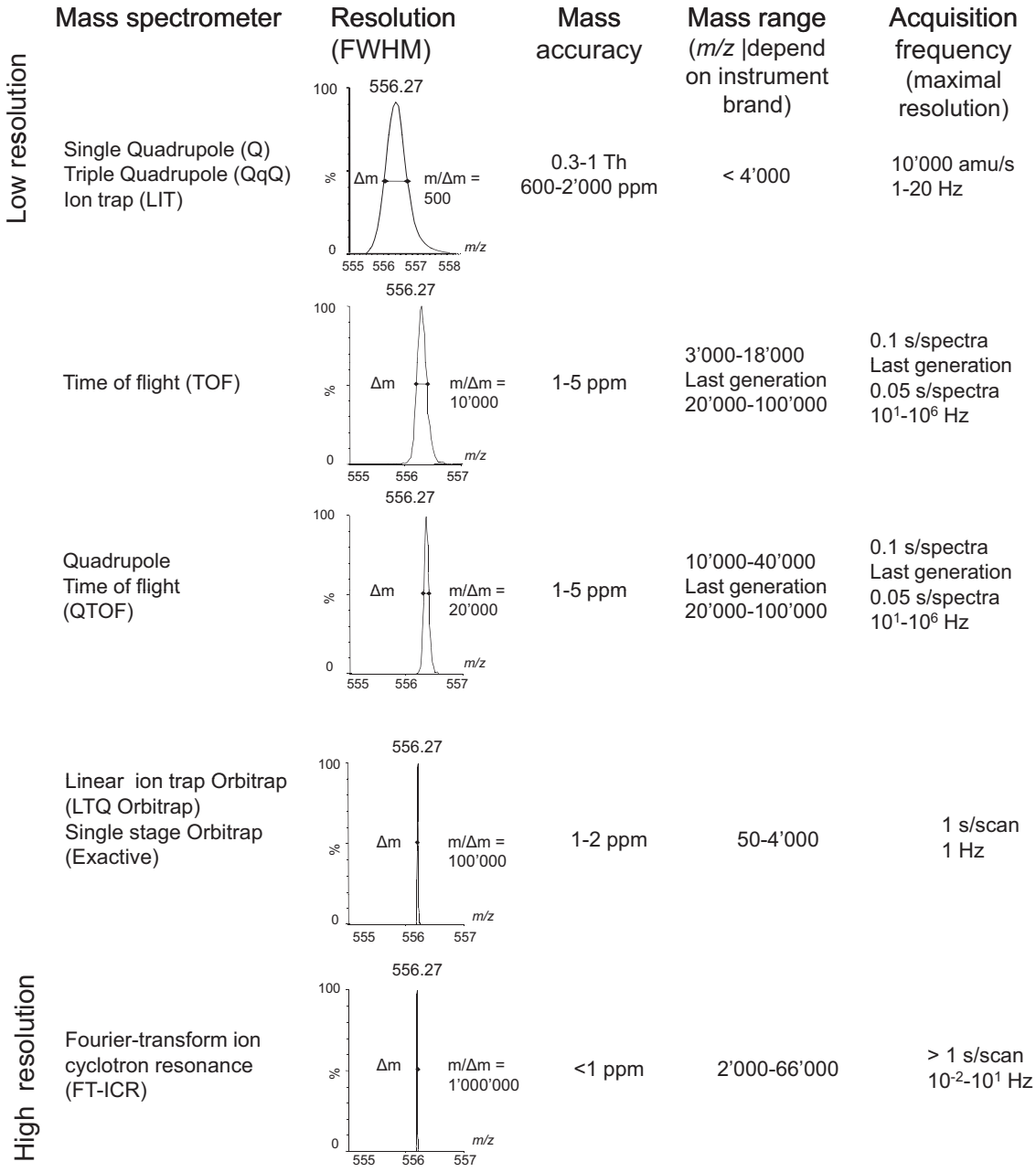

Fig. 5. Comparison of mass spectrometers in terms of resolution, mass accuracy, mass range and acquisition frequency.

to be performed [39]. The WADA technical document reports that accurate mass is considered complementary information to the required identification criteria [11].

There has been strong interest in coupling these high-resolution instruments with fast chromatographic approaches, such as UHPLC. UHPLC is coupled to TOF or QTOF mass spectrometers because they possess fast acquisition capability, as reported in Fig. 5. Due to the sharpness of the UHPLC peaks, the acquisition must be sufficiently fast to obtain enough points-per-peak and an adequate number of scans for an accurate mass measurement.

TOF, QTOF or Orbitrap mass analyzers possess an additional advantage over traditional mass spectrometers because they acquire full mass spectra over the selected mass range, which allows for a retrospective analysis of doping control samples. Data reprocessing can be performed without reanalysis [89,91], which is also in accordance with the WADA Code. QTOF allows for acquiring holistic data in the MS^E mode, which involves the parallel acquisition of a function at low and high collision energies. This method has been described for the study and extensive discovery of phase II metabolites of endogenous steroids [76]. This approach allows for the detection and confirmation of the identity of an analyte without sample re-injection, and it is promising for doping control. Moreover, full mass spectra and high sensitivity are important features of LC-QTOF-MS for database building [89,92].

Finally, an important application for high-resolution instruments involves protein analyses. In this case, the highest resolution is mandatory to clearly resolve multicharged ions.

5. Proteins and peptides analysis

Since the introduction of glycoprotein and peptide hormones as a doping class, such as EPO or human growth hormone (hGH), their detection and differentiation from endogenous analogs has been a great challenge in doping control. This particular class of doping

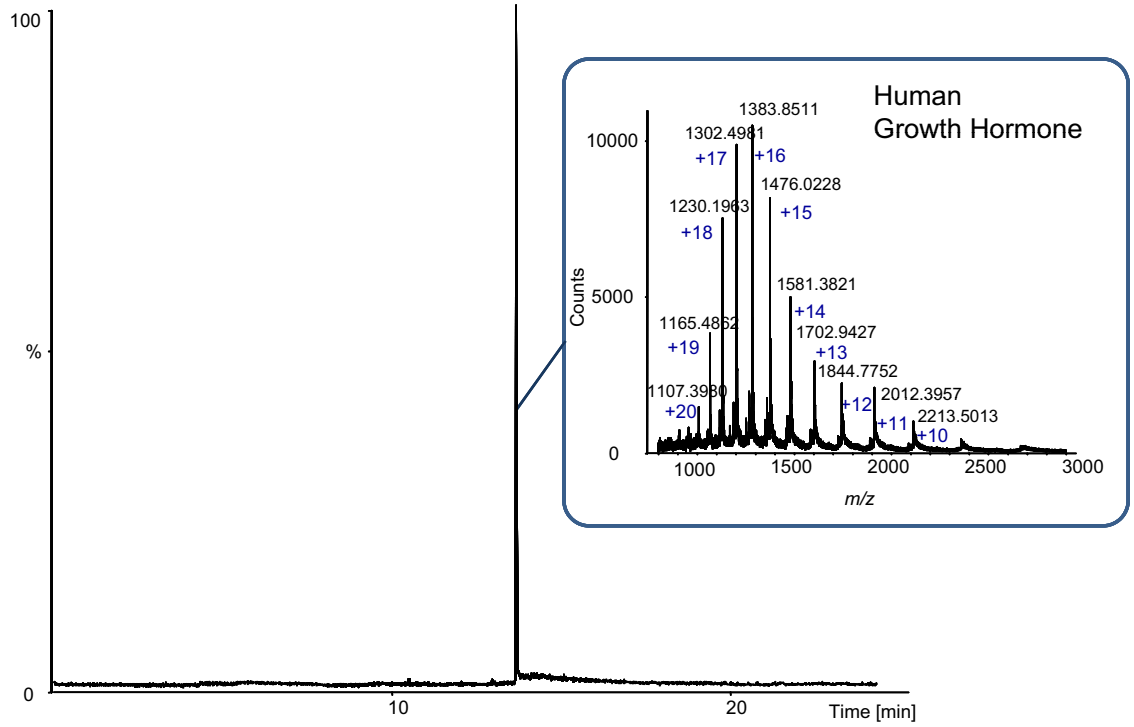

Fig. 6. CZE-ESI-TOF-MS electropherogram and mass spectrum of human growth hormone (hGH) at 200 μg/mL with 20% acetonitrile in 75 mM ammonium formate buffer set at pH 2.5.

agents requires dedicated methods that are sufficiently sensitive and selective for the detection of low physiological levels in urine and plasma (pg/mL). Traditionally, LC (size-exclusion and ion exchange modes in particular) and electrophoresis (capillary isoelectric focusing and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)) have been the reference separation techniques.

RPLC and capillary zone electrophoresis (CZE) are now employed for the analysis of these macromolecules in biological matrices because of the importance of MS for doping control (diagnostic ions) and the inherent incompatibility of the above described techniques with MS.

Proteins are commonly analyzed by MS in two main modes, either as an intact entity (top-down strategy) or as peptides after trypsin digestion (bottom-up approach).

LC–MS(/MS) presents intrinsic advantages for the analysis of macromolecules, such as the possibility to analyze intact proteins with high sensitivity and reproducibility. Furthermore, the coupling with soft ionization sources as well as high-resolution MS devices can be assessed.

Nano- and capillary-LC are particularly useful for large molecules because sensitivity often plays the main role in protein bioanalysis. As reported in the literature, improvements in sensitivity can be obtained by reducing the column diameters. For example, if considering a conventional HPLC column of 4.6 mm inner diameter (I.D.), then the sensitivity can theoretically be increased by a factor of approximately 8000 with a 50-μm I.D. column. To attain such a level of sensitivity, the injected volume should remain constant between both columns, which is usually not realistic. Therefore, a gain factor of approximately 100 should be expected because the sample volume is generally restricted and expensive for protein analysis. In addition, due to the very small column volume, all of the sources of the extra column band broadening should be reduced as much as possible, which is often a challenging task especially on a miniaturized chromatographic system. Furthermore, nano-LC system should work at a mobile-phase flow rate of approximately 100 nL/min for a 50-μm I.D. column, which is often difficult. To overcome this problem, several providers now propose all-integrated devices that are based on microfluidic technology including an injection loop, an enrichment column, a nano-LC column and a sprayer tip [93], which are extremely promising for the bioanalysis of macromolecules in which sensitivity remains a major issue.

Intact protein analysis has been performed for doping control for hemoglobin-based oxygen carriers (HBOC), gonadotrophin-releasing hormone (GnRH), insulin and corticotrophin by CZE-TOF-MS or nano-UHPLC–MS/MS (LTQ Orbitrap) [94–97]. Multicharged ions were produced by ESI and the determination of the molecular weight of the protonated or deprotonated molecules was performed after peak deconvolution [98]. This approach leads to the identification and characterization of the protein, with information on various alterations, such as post-translational modifications or from genetic or biotechnological mutation, which is particularly interesting for doping control purpose.

The bottom-up approach has also been used in anti-doping analyses. For example, peptides generated after trypsin digestion of erythropoiesis-stimulating agents or unknown proteins were analyzed by LC–MS(/MS) [99].

As previously discussed, CZE can represent a valuable alternative to nano-LC because there is no requirement for complex instrumentation and the consumption of sample (10 nL injected) is rather low. In addition, the peak widths in CZE are inversely proportional to the diffusion coefficients of the molecules, which are often very small for such large biomolecules [98]. In contrast to RPLC, very thin peaks can be obtained, even with large molecules (Fig. 6). CZE can be easily coupled to MS, and relevant protein structural information can be obtained by hyphenating it with a high-resolution mass analyzer. In this context, CZE-TOF-MS was recently used to analyze HBOC in plasma [95]. However, selective sample preparation is a fundamental prerequisite for the analyte pre-concentration to

reach the detection level of major proteins in the biological matrix using MS.

6. Untargeted approaches

Anti-doping strategies have been based on targeted approaches for the detection and quantification of selected compounds of biological relevance or related to a specific biological pathway. Recent advances in the development of analytical platforms have enabled the parallel monitoring of a large number of analytes in a single experiment with high sensitivity and specificity. These advances have opened the way to "-omic" approaches that examine biological systems in a holistic manner [100]. Such extensive data collection engenders novel perspectives for the development of anti-doping strategies and new challenges related to the extraction of the relevant biological information because doping substances induce changes in the biological response, including the metabolic cascade [101]. Metabolomic methodologies involve an interdisciplinary knowledge, from sample preparation and collection to analytical procedures, raw data processing and data modeling [102]. The methods selected for handling and analyzing such complex datasets have a great impact on the biological interpretation. Therefore, appropriate procedures should be cautiously undertaken because their choice depends heavily on: (i) the chemical characteristics of the biological compartment under study, (ii) the analytical platform employed to produce the data, (iii) the inherent properties of the data structure (*e.g.*, high dimensionality or correlations) and (iv) the subsequent data analysis methods.

Finding valuable but hidden information in large-scale data is of growing importance. Untargeted profiling has been found to be an efficient screening tool that is complementary to common testing methods to detect the illegal use of anabolic steroids in cattle [103], but it is still in its infancy for the human urinary signature [104]. Data mining and statistical tools have a fundamental role in data interpretation, by taking into account the different sources of variation and assessing the relevant biological signatures after the administration of a doping substance. However, the appropriate solutions will not come from computer programs but from separation scientists working at the bench and understanding the data-related characteristics and issues with input from a specialist in the investigated biology. Moreover, biological validation is mandatory to ensure the relevance of the results obtained from "-omic" level studies. The hypotheses derived from these approaches have to be verified by complementary studies to distinguish between false positives and proper biomarkers [105].

7. Conclusion

Doping control is currently maintained by analysis of urine and blood samples of the athletes. When a targeted substance or class of compounds is analyzed, chromatographic tools, such as GC–MS or LC–MS/MS, are preferably selected for screening and for the confirmation of low molecular weight substances. Immunoassays or electrophoretic methods are usually employed for macromolecules. The evolution of these techniques has helped to improve the detection of low levels of doping agents in a complex matrix. The development of analytical techniques for substances that are either newly marketed entities or that cannot be detected or identified with sufficient sensitivity is more challenging. Because of their ability to detect known and unknown compounds in complex matrices with high sensitivity and selectivity, advances in analytical devices and strategies constitute powerful tools towards drug-free sports. Indeed, fast or high-resolution chromatographic techniques, such as fast GC and UHPLC reduce the response time, separate positional isomers and allow for high selectivity towards the complex matrix. The identification of analytes and unknown structure elucidation can be facilitated by high-resolution mass spectrometers. In the future, the untargeted approach may become a promising tool because it allows for the detection of changes in an entire biological system (global information), instead of targeted levels and concentration ratios of a selected doping agent.

Strong efforts should be focused on sample preparation, which is still in its initial phase for doping control and remains the bottleneck in the analytical procedure. Promising possibilities are available for the reduction of sample and solvent consumption, automation and miniaturization. For blood analysis, the dried blood spot seems to be a valuable sample collection technique for the detection of exogenous compounds in the future.

Finally, the most appropriate analytical combination must be utilized, however this often involves expensive devices and specially trained staff.

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