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Review

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# Analytical Strategies for Doping Control Purposes: Needs, Challenges, and Perspectives

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The fight against doping in sports has been governed since 1999 by the World Anti-Doping Agency (WADA), an independent institution behind the implementation of the *World Anti-Doping Code* (Code). The intent of the Code is to protect clean athletes through the harmonization of antidoping programs at the international level with special attention to detection, deterrence, and prevention of doping.[1] A new version of the Code came into force on January 1st 2015, introducing, among other improvements, longer periods of sanctioning for athletes (up to four years) and measures to strengthen the role of antidoping investigations and intelligence. To ensure optimal harmonization, five *International Standards* covering different technical aspects of the Code are also currently in force: the *List of Prohibited Substances and Methods* (List), *Testing and Investigations*, *Laboratories*, *Therapeutic Use Exemptions* (TUE), and *Protection of Privacy and Personal Information*. Adherence to these standards is mandatory for all antidoping stakeholders to be compliant with the Code. Among these documents, the eighth version of *International Standard for Laboratories* (ISL), which also came into effect on January 1st 2015, includes regulations for WADA and ISO/IEC 17025 accreditations and their application for urine and blood sample analysis by antidoping laboratories.[2] Specific requirements are also described in several *Technical Documents* or *Guidelines* in which various topics are highlighted such as the identification criteria for gas chromatography (GC) and liquid chromatography (LC) coupled to mass spectrometry (MS) techniques, measurements and reporting of endogenous androgenic anabolic agents (EAAS), and analytical requirements for the Athlete Biological Passport (ABP).

Current doping control analysis includes the identification, and in some cases the quantification, of banned substances and methods included in the List from biological samples collected in-and out-of-competition.[3] The compounds and methods on the List are classified in ten (S0 to S9) and three (M1 to M3) different categories, respectively. Alcohol (P1) and $\beta$-blocker substances (P2) are only forbidden in few sports. A nonexhaustive description of the different classes of compounds, including examples of specific substances, is given in Table 1. Compounds are divided into nonthreshold substances, for which their simple identification could be considered an adverse analytical finding (AAF), and threshold substances (e.g., ephedrine and derivatives, salbutamol, and carboxy-THC), banned above a fixed level and for which quantitative determination in the biological sample is needed. To obtain homogeneous results between laboratories, Minimum Required Performance Levels (MRPL) for analytical methods have been established by WADA, indicating the minimum capabilities for the detection of nonthreshold substances; these values do not apply to threshold compounds, which are covered by other dedicated documents.

Urine and blood (whole blood, serum, and plasma) are considered the matrices of choice for routine antidoping analysis. The advantages of urine samples include its noninvasive collection and accessibility to large volumes of matrix, whereas blood collection is still considered invasive and with a limited volume. For these reasons, the majority of antidoping controls is still carried out on urine, even if the percentage of blood testing is continuously increasing. Therefore, determination of the presence and/or absence of a doping agent in urine is routinely carried out through a common workflow including an initial testing procedure (screening) followed by a confirmation procedure, if applicable. A schematic representation of this workflow is given in Figure 1. The screening step must be fast, selective, and sensitive to limit the risk of false-negative and false-positive results. In the case of a suspicious result, the latter should be established with a confirmation procedure targeting the potentially incriminating substance(s), including possible metabolite(s). To achieve this, and considering the important chemical diversity and wide range of physicochemical properties of forbidden substances (approximately 250 compounds), antidoping laboratories

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**Table 1. Summary of the WADA 2015 Prohibited List**

| categories | categories description | prohibition | subcategories/specific examples/observations[a] |
|---|---|---|---|
| S0 | nonapproved substances | in and out of competition | any pharmacological substance with no current approval for human therapeutic use (e.g., drugs under preclinical or clinical development or discontinued, designer drugs, substances approved only for veterinary use) |
| S1.1.a | exogenous anabolic androgenic steroids | | boldenone, boldione, calusterone, clostebol, dehydrochloromethyltestosterone, drostanolone, fluoxymesterone, mesterolone, methandienone, methasterone, methyltestosterone, oxandrolone, stanozolol, trenbolone, etc. |
| S1.1.b | endogenous anabolic androgenic steroids | | androstenediol, androstenedione, DHT, DHEA, testosterone, and their metabolites and isomers |
| S1.2 | other anabolic agents | | clenbuterol, SARMs (e.g., andarine and ostarine), tibolone, zeranol, zilpaterol |
| S2 | peptides hormones, growth factors, related substances, and mimetics | | erythropoiesis-stimulating agents, nonerythropoietic EPO-receptor agonists, HIF stabilizers and activators, hCG and LH, corticotrophins, growth hormone and their releasing factors, other growth factors, etc. |
| S3 | $\beta$2-agonists | | bambuterol, fenoterol, formoterol, salbutamol, salmeterol, terbutaline, etc. |
| S4 | hormone and metabolic modulators | | AICAR, aminoglutethimide, anastrozole, androstatriendione, clomiphene, cyclofenil, exemestane, formestane, fulvestrant, GW1516, insulins, letrozole, raloxifene, tamoxifene, testolactone, toremifene, etc. |
| S5 | diuretics and masking agents | | acetazolamide, amiloride, bendroflumethiazide, canrenone, chlortalidone, desmopressine, furosemide, glycerol, HES, hydrochlorothiazide, indapamide, spironolactone, triamterene, tolvaptan, etc. |
| S6 | stimulants | in competition | benzfetamine, cathine, cathinone, mephedrone, methedrone, ephedrine, etilefrine, heptaminol, isometheptene, MDMA, methyl ephedrine, methylphenidate, MHA, oxilofrine, pseudoephedrine, sibutramine, tuaminoheptane, etc. |
| S7 | narcotics | | buprenorphine, dextromoramide, fentanyl and derivatives, heroin, hydromorphone, methadone, morphine, oxycodone, oxymorphone, pentazocine, pethidine, etc. |
| S8 | cannabinoïds | | cannabis, hashish, marijuana, Δ9-tetrahydrocannabinol, JWH-018, JWH-073, HU-210, etc. |
| S9 | glucocorticoïds | | beclomethasone, beta/dexamethasone, budesonide, ciclesonide, deflazacort, desonide, fludrocortisone, flumethasone, flunisolide, methylprednisolone, prednisolone, prednisone, triamcinoloneacetonide, etc. |
| P1 | alcohol | in competition, in particular sports[a] | detection conducted by analysis of breath and/or blood |
| P2 | $\beta$-blockers[b] | | acebutolol, alprenolol, atenolol, betaxolol, bisoprolol, bunolol, carteolol, carvedilol, celiprolol, esmolol, labetalol, levobunolol, metipranolol, metoprolol, nadolol, oxprenolol, pindolol, propranolol, sotalol, timolol, etc. |
| M1 | manipulation of blood and blood components | in and out of competition | autologous, homologous, and heterologous blood transfusions, perfluorochemicals, RSR13, HBOCs |
| M2 | chemical and physical manipulation | | tampering or attempting to tamper, urine substitution, urine adulteration, intravenous infusions and/or injections of more than 50 mL per 6 h period |
| M3 | gene doping | | the transfer of polymers of nucleic acids or nucleic acids analogues; the use of normal or genetically modified cells |

[a]Nonexhaustive list of examples; please refer to ref 3 for the complete prohibited list. [b]$\beta$-blockers are also forbidden out of competition in archery and shooting sports.



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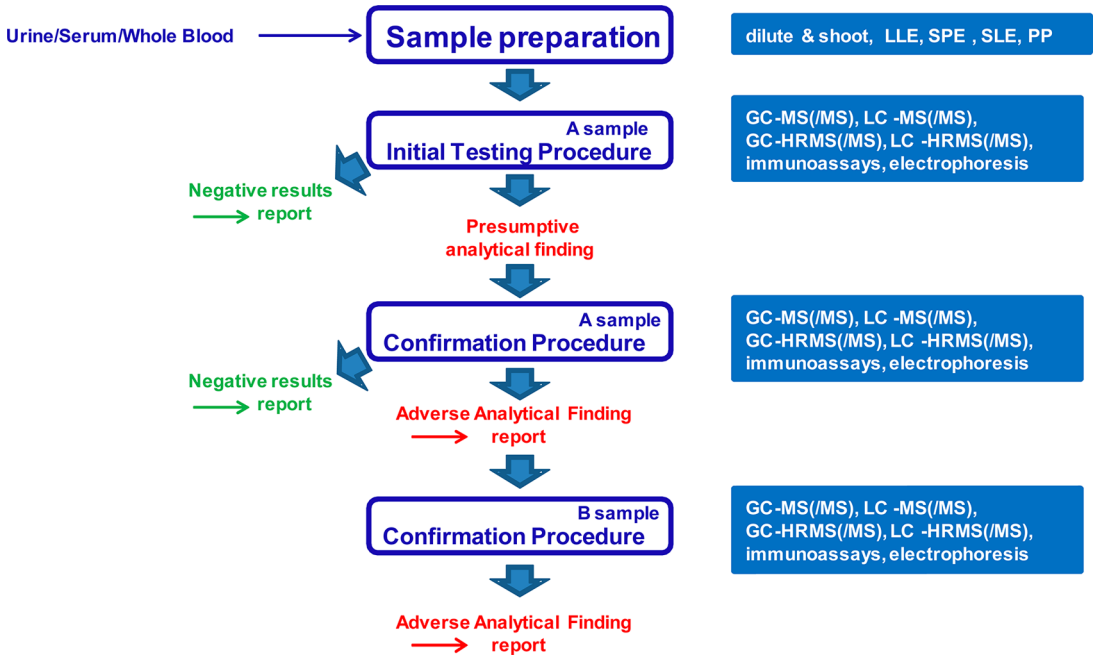


**Figure 1.** Typical workflow of doping control analysis.

should use multiple analytical techniques, including immunological, biochemical, and chromatography–mass spectrometry methods.

For the latter, both GC/MS(/MS) and LC-MS(/MS) are considered reference methods, whose effectiveness benefits from the recent excellent technical improvements in these analytical platforms in terms of sensitivity, selectivity, and rapidity. To be able to detect the current very sophisticated doping practices, these direct screening and confirmation procedures should be continuously updated, including monitoring additional substances with potential doping properties and/or new metabolites of already known compounds with increased detection windows.

The aim of this Review is to present an exhaustive and critical overview of the scientific literature related to the significant progress in antidoping analytical methodologies in the last three years. Special attention is given to the description of current advancements concerning direct detection methods of small molecules, peptides, and proteins, mostly based on GC/MS and LC-MS techniques. Benefits and limitations of each approach will be discussed in detail.

## SMALL MOLECULES ANALYSIS

In doping control analysis, the demand for modern analytical strategies with fast turnaround, high sensitivity, and selectivity in complex matrices is a major concern. The number of forbidden substances and/or metabolites on the WADA List that should be monitored has grown continuously over the last several years,[4,5] reaching approximately 250 entities. In addition, all these compounds possess very diverse physicochemical properties (e.g., polarity, molecular weight, and acido-basic properties). Considering these constraints, the analysis of small molecules remains challenging, and several methods in parallel are required to cover all the different categories and to ensure the quality of the analytical results. These methods are generally focused on the direct detection of prohibited substances as well as their major phase I and phase II metabolites because monitoring of the latter often improves detection window capabilities in urine. For this purpose, the excretion pattern of each illicit substance must be carefully examined to ensure the proper selection of the target compounds for screening purposes, favoring major metabolites or those with long-term urinary excretion profiles.[6]

**Sample Preparation Techniques.** When dealing with the bioanalysis of urine or blood by chromatographic methods, sample preparation is a critical step due to the wide heterogeneity of the analytes and the complexity of the matrices containing salts, lipids, and proteins. Sample preparation is mandatory to achieve a sufficient level of sensitivity and selectivity and also to avoid clogging the chromatographic column and contaminating the mass spectrometer, primarily used as the detector. Various sample treatments (i.e., dilute and shoot, protein precipitation) or sample preparation techniques (i.e., solid-phase extraction (SPE), liquid–liquid extraction (LLE), or supported-liquid extraction (SLE)) can be selected to obtain the best compromise between good recoveries for most of the analytes and cleanliness of the extract.[7] The choice of the best sample preparation procedure should be based on the physicochemical properties of the substances and the employed analytical instrumentation.

The sample preparation procedure for doping control analysis should be as generic as possible because many illicit substances must be monitored simultaneously during the initial screening procedure. With this in mind and thanks to the high selectivity and sensitivity offered by the latest generations of MS detectors, this step can largely be simplified, and today, the initial screening of a high number of illicit substance classes in urine is often performed after a nonselective *dilute-and-shoot* procedure with an appropriate solvent to limit matrix effects. This approach is mostly used as a screening assay of easily ionizable compounds with a limited metabolism, such as stimulants, narcotics, diuretics, $\beta_2$-agonists, and $\beta$-blockers, for which the required minimum detection levels in urine are relatively high, in the range of a few tens to hundreds of ng/mL. This sample pretreatment approach is generic, fast, and


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inexpensive and does not require any specific equipment. However, the detectability of analytes is reduced due to the dilution factor. The dilution factor varies between 1:1 and 1:25, and most of the recent applications dealing with the determination of sport drugs in urine have been carried out with a dilution factor of 1:10.[8] Because urine is a complex matrix including, but not limited to, phospholipids, proteins, salts, urea, and creatinine, as well as a wide range of organic acids and other inorganic compounds,[8] matrix effects are non-negligible in the case of a simple *dilute-and-shoot* procedure and should be evaluated case by case. To compensate for matrix effects and obtain accurate and reproducible results, the use of an isotope-labeled internal standard (IL-ISTD) remains the best choice, especially for the quantitative determination of threshold substances such as carboxy-THC, salbutamol, 19-norandrosterone, morphine, or ephedrine and its derivatives.[9]

However, in cases for which certain classes of forbidden substances should be monitored at trace levels in the biological fluid of interest (e.g., anabolic androgenic steroids), a preconcentration step is mandatory. In this context, the selective LLE procedure has been historically the most widely used preconcentration technique in doping control analysis because the sensitivity of LC-MS or GC/MS platforms was limited in the past. Sufficient elimination of major interferences is obtained with LLE, and the extraction protocol is simple and cost-efficient and provides clean extracts using solvents such as *tert*-butyl methyl ether, diethyl ether, and *n*-pentane. However, this technique is not adapted to substances with polar groups in their structure; it requires large volumes of sample and solvent and suffers from poor recovery and repeatability and lack of automation. In addition, two parallel extractions, at basic and acidic pH, respectively, are often required to simultaneously extract acidic and basic substances for screening purposes. Despite this, LLE at basic pH is still widely used as a routine protocol in antidoping analysis, mainly for compounds such as endogenous and exogenous steroids as well as glucocorticoids.

Considering the well-known drawbacks of LLE, SLE can be considered a sort of simplified and automated LLE, in which the aqueous biological sample is adsorbed on a cleaned diatomaceous earth stationary phase with high surface area loaded in a cartridge or a well plate, followed by the application of a nonmiscible solvent through the cartridge for extraction and elution of the analytes. SLE affords faster sample preparation than LLE because there is no need for phase separation, avoiding emulsion problems. In addition, SLE is available in 96-well plate format for high-throughput sample preparation. As recently reported,[7] significant improvements in recovery values with SLE were obtained compared to classical LLE. However, the suitability of this technique for the multiclass screening of illicit drugs in urine, including both acidic and basic compounds, has not been largely demonstrated thus far. For this reason, SLE in doping control is certainly better adapted for confirmation procedures of selected categories of forbidden compounds. This is, for example, the case of steroids and glucocorticoids due to their relatively low polarity, which favors SLE.

SPE is a good alternative to LLE or SLE and was recently successfully employed as a routine sample preparation strategy in doping analysis during the 2012 Olympics in London[10] for the extraction of a large number of substances including anabolic agents, $\beta_2$-agonists, hormone antagonists and modulators, diuretics, stimulants, narcotics, glucocorticoids, and $\beta$-blockers. In fact, SPE offers numerous advantages, including less solvent consumption, ease of automation (96- or 384-well plates format), and simultaneous sample clean up and preconcentration. In addition, there is a wide range of SPE sorbents for analysis in the normal phase, reversed phase, ion exchange, and mixed mode, allowing the retention of virtually any compound based on several interaction mechanisms. Polymeric C18 cartridges are particularly interesting for the screening of many illicit substances performed in doping control analysis because they allow the simultaneous extraction of neutral, acidic, and basic substances in a single elution. This feature is of prime importance due to the important diversity of the physicochemical properties of forbidden substances monitored during the initial screening. However, extracted samples can suffer from noticeable matrix effects. Mixed-mode cartridges have also emerged as a promising technique for antidoping screening and/or confirmation purposes[10,11] due to their dual-retention mechanism given by the polymeric or C18 sorbent bonded with ion exchange groups. Classical use of mixed-mode cartridges is expected to provide additional extraction selectivity by using two different elution solvents. Musenga et al. demonstrated that these cartridges allow the simultaneous extraction of approximately 180 acidic (e.g., diuretics), neutral (e.g., glucocorticoids), and basic (e.g., stimulants) compounds in only one elution after carefully selecting the washing conditions.

In a recent study,[7] seven sample preparation procedures based on SPE (with 5 different cartridges), LLE, and SLE were compared for multiclass sport drug testing in urine. In this exhaustive work, 189 illicit substances were selected as representatives from different groups of prohibited drugs, and LC-ESI/TOF-MS was employed to analyze the urine extracts. The obtained results, in terms of recoveries at a concentration of 25 $\mu$g/L, are shown in Figure 2. By comparing the

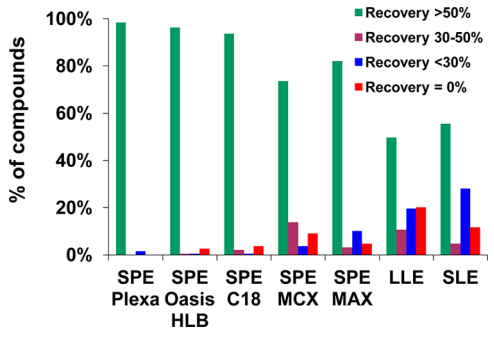


**Figure 2.** Summary of the results obtained with seven sample preparation approaches, including SPE Plexa, SPE Oasis HLB, SPE C18, SPE MCX, SPE MAX, LLE, and SLE for the large-scale sport drug testing of illicit substances at a concentration of 25 $\mu$g/L. Reprinted from *J. Chromatogr. A*, Vol. 1361, Dominguez-Romero, J. C.; Garcia-Reyes, J. F.; Molina-Diaz, A. Comparative evaluation of seven different sample treatment approaches for large-scale multiclass sport drug testing in urine by liquid chromatography−mass spectrometry, pp 34−42 (ref 7). Copyright 2014, with permission from Elsevier.

percentages of compounds with a recovery higher than 50% achieved with the seven different sample preparation approaches, this representation clearly illustrates the superiority and more generic conditions provided by SPE over SLE and above all LLE

With the advent of green chemistry, there is also a trend toward miniaturized sample preparation techniques to reduce


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the use of organic solvents or substitute organic solvents for less toxic alternatives.[12−14] In this context, many liquid-based or solid-based microextraction procedures have been proposed for a wide range of applications, including clinical and forensic toxicology. The most conventional liquid-based microextraction methods include single-drop microextraction (SDME), hollow-fiber liquid-phase microextraction (HF-LPME), or dispersive liquid−liquid microextraction (DLLME), while most well-known solid-based microextraction methods are disposable pipette extraction (DPX) or microextraction by packed-sorbents (MEPS). Until now, these approaches have not yet been applied in routine laboratories, but it is expected that the interest in these strategies will increase in the future and that some attempts will certainly be carried out soon in doping control laboratories.[11]

Finally, because blood matrix is known to provide complementary information to urine analysis, it is necessary to describe the sample preparation procedures used for this matrix. The simplest procedure is protein precipitation (PP), which is analogous to the dilute-and-shoot method for urine. Depending on sensitivity and selectivity required, LLE, SLE, or SPE procedures can also be employed in serum, plasma, and whole blood before GC/MS and LC-MS analysis, as reported above for urine. Moreover, greater attention has been paid over the past few years to the use of dried blood spots (DBS) for blood collection in doping control. Few recent applications for monitoring different categories of small prohibited compounds, peptides, anabolic steroid esters, and SIRT1 activators have been recently reported in the literature.[15−17] DBS consists of collecting a few microliters of whole blood spotted on absorbent paper and appears to be very promising for doping control blood analysis in terms of sampling (less invasive than conventional blood collection), easier shipment and storage, and improved long-term analyte stability.[16] However, although DBS is a promising sampling technique, there is still some resistance to its widespread application, mainly due to the limited preconcentration factors achieved and relatively high limits of detection. Furthermore, in a typical DBS workflow, a small disk of the spotted and dried blood spot is punched out, and these punches are therefore assumed to contain a fixed blood volume. However, the viscosity of blood depends on the hematocrit level. Then, if a uniform blood volume is placed onto a DBS card, the size of the spot formed will decrease as the hematocrit level increases. This leads to volumetric assay bias associated with the hematocrit of the blood. Various technical solutions were recently proposed to minimize this problem, and among them, Leuthold et al.[18] described the collection of a controlled volume of blood in few seconds with high reproducibility. From a commercial point of view, the Mitra microsampler from Neoteryx also allows the sampling of a precise volume of blood without hematocrit bias.[19] Finally, because plasma could represent a valuable alternative to whole blood, especially for GC/MS- and LC-MS-based testing, some technical solutions (Noviplex cards) were also recently proposed to collect volumetric plasma samples from non-volumetric applications of whole blood in only 3 min.[20]

**Chromatographic Approaches.** In doping control analysis, GC-FID and GC/MS quickly became the standard instrumentation for the detection and quantification of illicit substances. GC is generally coupled to MS through electron impact (EI) ionization, and this approach has been particularly useful for toxicological laboratories due to the low interinstrument variability and the possibility of using existing mass spectral libraries. When using GC, hydrolysis and derivatization steps are required prior to the analysis of many doping agents to make them sufficiently volatile and also to improve their sensitivity. These procedures may induce important variability and are considered expensive and time-consuming because enzymatic hydrolysis can vary from minutes to hours depending on the incubation temperature, whereas derivatization can be performed within ∼1 h. However, today, GC/MS remains the gold standard method for the screening and confirmation of anabolic steroids, which is certainly one of the most challenging classes of doping agents to analyze. Indeed, anabolic steroids are excreted in urine at very diverse concentrations, and there are also a large number of isomers and metabolites[6] that are difficult to separate in a satisfactory manner. To improve the resolving power of GC, comprehensive two-dimensional GC (GCxGC) can be used to tackle the extreme complexity of samples. In GCxGC, the resolving power is significantly increased by the use of two orthogonal columns of different polarities and lengths.[21] In addition, GCxGC is often coupled with high-resolution mass spectrometers possessing fast data acquisition rates. However, to date, GCxGC-TOF/MS has only been scarcely employed in doping control analysis likely because robust instrumentation only became commercially available very recently. For quantification purposes, GC/MS(/MS) is also considered fit-for-purpose, especially regarding the determination of endogenous steroids linked to the testosterone metabolism in urine, also called the urinary steroid profile integrated in the steroidal module of the ABP.[22] The technical document describing the sample preparation as well as the instrument settings imposed by WADA clearly states that GC/MS(/MS) is the preferred reference method. However, although this technique has been widely applied in the fight against doping, there is still a lack of standardization between the WADA accredited laboratories. Not only is the detector (single or triple quadrupole) dissimilar among laboratories but also the sample preparation (either LLE or SPE and LLE purification steps) is known to be a potential source of variation for steroid profile quantification. In addition, many other exogenous or endogenous confounding factors can influence the steroid profile.[23] Because of all these limitations, the urinary data and individual sequences generated by the steroidal module of the ABP currently suffer from more inconsistencies compared to the hematological module for which the preanalytical and analytical conditions are well established among laboratories.[24] Another application of GC in doping control analysis is the determination of xenon in urine or plasma samples. Xenon was listed by the WADA in 2014 as a banned substance categorized as a hypoxia-inducible factor activator. The presence of xenon in urine can be successfully assessed using GC/MS/MS with headspace injection down to a detection limit of approximately 0.5 nmol/mL and up to 40 h postanesthesia.[25] In addition, GC-TOF/MS and GC/MS/MS have been employed to determine xenon in human plasma or blood, also with headspace injection.[26] Depending on the type of mass spectrometer, detection limits of 0.5−50 nmol/mL have been achieved, and xenon has been detected up to 30 h after plasma and blood storage collection. However, further studies are still required to better understand the detection window for xenon under different gas mixtures and exposure time settings. Moreover, the sampling and storage of specimens should also be evaluated.[26]

Due to the great improvements in LC-MS and the need for a very fast turnaround time (results within 24−48 h from sample


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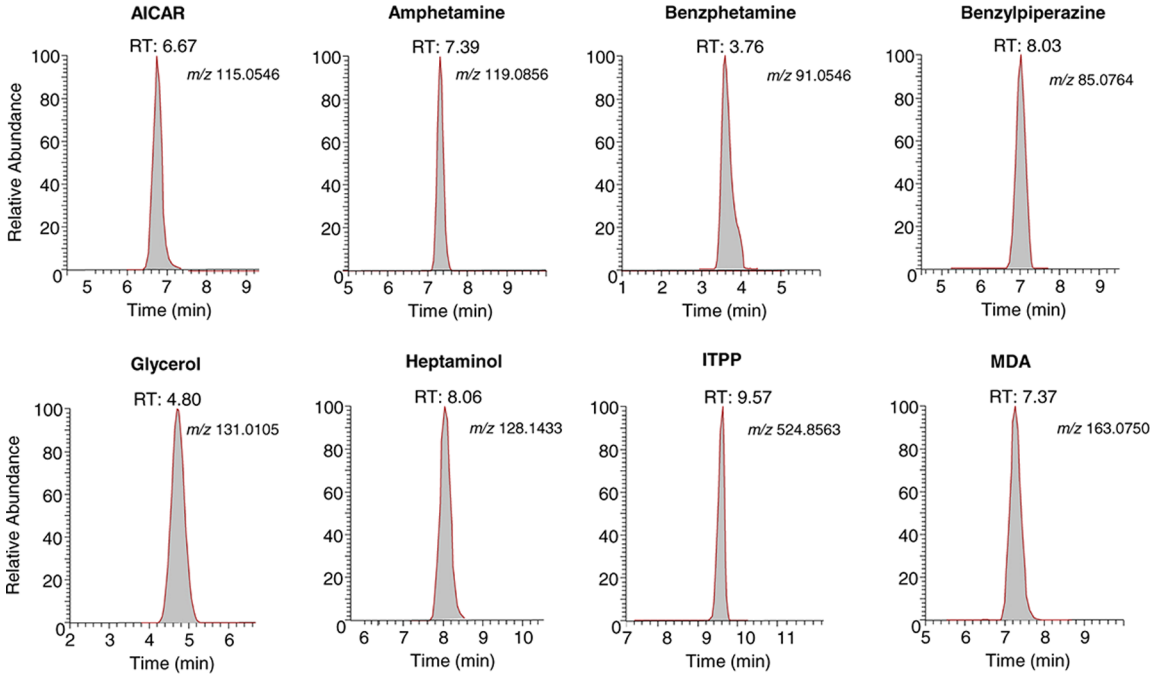


**Figure 3.** Extracted ion chromatograms of spiked urine specimens (17 500 fwhm; mass tolerance: 5 ppm) obtained from a simple dilute-and-shoot procedure on urine followed by HILIC-MS analysis. The concentration levels were as follows: 100 ng/mL for all compounds, except AICAR at 500 ng/mL. With kind permission from Springer science + business media: *Anal. Bioanal. Chem.*, "Dilute-and-inject" multitarget screening assay for highly polar doping agents using hydrophilic interaction liquid chromatography high resolution/high accuracy mass spectrometry for sports drug testing, Vol 407, 2015, 5365−5379, Gorgens, C.; Guddat, S.; Orlovius, A. K.; Sigmund, G.; Thomas, A.; Thevis, M.; Schanzer, W., Figure 2 (ref 46).

collection are required for major sporting events), LC-MS/MS tends to replace GC/MS(/MS) today for various classes of doping substances. Reversed phase liquid chromatography (RPLC) with a C18 stationary phase is clearly the most widely used approach in doping control for the analysis of biological samples. Prior to RPLC, the sample preparation is simple and rapid, and the chromatographic method is directly compatible with both polar and nonvolatile compounds without the need for derivatization. Interestingly, Miller et al. demonstrated the importance of LC data in doping control analysis.[27] They applied a predictive computing technique (neural networks procedure) for the prediction of gradient retention times in archived high-resolution urine analysis of sample data. Using this approach, they demonstrated the possibility for retrospective detection of suspected sport doping species in the postanalysis human urine samples. Their data were encouraging because the retention times of 93% of all selected doping-related substances (in total, there were 86 forbidden drugs with very diverse physicochemical properties) were within 0.5 min of their true value. The only reported constraint was the accuracy of the p$K_a$ value, which should be experimentally verified. In the end, the prediction of retention times can be considered a complementary tool to retrospective analysis for the identification of unknown compounds not included in the analytical screening. Gorynski et al.[28] also showed that the confidence in experimental results in doping control can be improved by retention time modeling. For this purpose, they used a training set of 146 drugs, metabolites, and banned substances from the WADA List.

To further improve the resolving power and/or throughput in RPLC, various approaches have been proposed during the past decade.[29] The use of columns packed with sub-2 $\mu$m fully porous particles in combination with a system possessing an extended pressure limit of 1000−1500 bar is particularly relevant, and this approach is known as UHPLC, which stands for ultra high performance (or pressure) liquid chromatography. UHPLC is particularly well suited for doping control analysis, and today, most of the accredited laboratories are equipped with UHPLC technology.[9,30−32] As was recently reported,[10,33] the screening of up to 200 multiclass sport drugs can be performed within 10−20 min using UHPLC technology. Although the analysis time reduction is an interesting attribute of the UHPLC method, the narrow peaks (only a few seconds) produced by this chromatographic technology may be critical for MS detection.[34] To mitigate this issue, new generations of faster triple quadrupole mass spectrometers possessing dwell times of only 1−3 ms and polarity switching down to 15 ms must be preferentially used. As an alternative to UHPLC, columns packed with sub-3 $\mu$m superficially porous particles (SPPs) entered the market in 2007.[35,36] This column technology is also described as core−shell or fused-core and has received considerable attention from the chromatographic community for the analysis of small compounds. Several SPPs debuted since 2007 possess a special particle design in which a 1.7 $\mu$m solid core is surrounded by a 0.5 $\mu$m thick shell of porous silica. Due to their specific morphology, the performance of sub-3 $\mu$m SPPs is almost equivalent to that of the sub-2 $\mu$m fully porous particles employed in UHPLC, while the generated pressure is 2−3 times lower due to their larger particle size. Therefore, the SPP stationary phases can be theoretically used on a conventional HPLC instrument with an upper pressure limit of 400 bar. Despite the fact that almost all column providers now offer SPP phases, this technology has surprisingly not been applied to doping control analysis yet, even though it represents a valuable alternative to UHPLC. Other SPP morphologies are also available with particle diameters varying from 1.6 to 5 $\mu$m. Recently, there has also been great interest in 2D-LC approaches[37,38] to improve the


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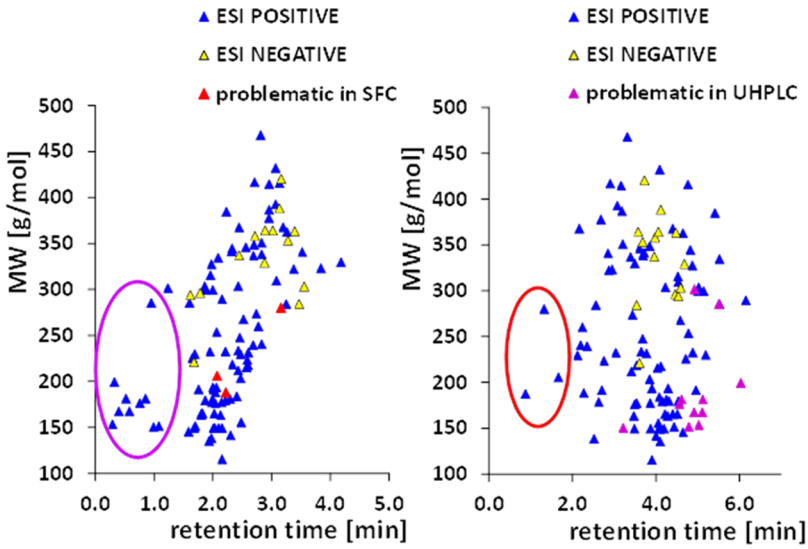


**Figure 4.** Elution distribution of 110 doping agents under RPLC−MS/MS (A) and SFC−MS/MS (B) conditions. The compounds ionized in ESI- are displayed in yellow (triangles), and those ionized in ESI+ are displayed in blue (triangles) under RPLC−MS/MS and SFC−MS/MS conditions, respectively. Reprinted from *Anal. Chim. Acta*, Vol. 853, Novakova, L.; Rentsch, M. ; Grand-Guillaume Perrenoud, A.; Nicoli, R.; Saugy, M.; Veuthey, J. L.; Guillarme, D. Ultra high performance supercritical fluid chromatography coupled with tandem mass spectrometry for screening of doping agents. II: Analysis of biological samples, pp 647−659 (ref 48). Copyright 2015, with permission from Elsevier.

resolving power of the chromatographic approach. However, the technology is not mature enough to be routinely employed in doping control laboratories because commercial instrumentation for 2D-LC operation has only recently appeared on the market.

Even if RPLC remains the most generic methodology in doping control analysis, hydrophilic interaction chromatography (HILIC) has also been evaluated by a few research groups. In HILIC, a polar stationary phase (bare silica or silica bonded with polar functional groups) is used concomitantly with a mobile phase possessing more than 60% of an aprotic solvent (acetonitrile) mixed with an aqueous buffer.[39] Under these conditions, hydrophilic partitioning of the compounds occurs between a water-enriched layer at the surface of the stationary phase and the less protic/polar mobile phase component. HILIC is thus perfectly adapted for the analysis of polar compounds but is also a viable strategy for the analysis of ionizable analytes.[40] Another important benefit of HILIC vs RPLC is the MS signal enhancement reported under HILIC conditions for ionizable compounds due to the improved desolvation and shift of $pK_a$ and pH values in the highly organic mobile phase.[41,42] Experimentally, the sensitivity improvement was comprised between 7 and 10, and a factor higher than 1000 was observed for a few basic compounds under specific pH conditions.[41] However, this gain was highly MS-dependent,[42] particularly on the ESI source geometry. In doping control, Gorgens et al.[43] developed an HILIC-MS methodology for the screening and confirmation assays of myo-inositol trispyrophosphate (ITPP), which is an illicit hydrophilic drug able to reduce the oxygen affinity of hemoglobin. The same group also developed and validated an HILIC-MS method for the determination of another hydrophilic drug, mildronate, which is still a monitored compound but will be classified as a prohibited substance in 2016.[44,45] In both cases, they demonstrated the applicability of HILIC for the determination of polar substances because these assays provided good specificity, robustness, precision, and linearity and an adequate limit of detection. Gorgens et al.[46] also established a fast and easy screening method based on the direct injection of diluted urine for the detection of 27 highly polar and charged doping agents from the WADA List using a zwitterionic HILIC stationary phase. Figure 3 shows some extracted ion chromatograms of spiked urine specimens with 8 representative hydrophilic compounds. To attain the symmetrical peak shapes and suitable sensitivity reported in Figure 3, the authors demonstrated that the dilution of urine specimens must be performed with a 100 mM aqueous ammonium acetate solution and acetonitrile in a proportion of 3:7 (v/v). In addition, to achieve robust and reproducible results, at least three blank urine samples should be injected to precondition the column before the first run of the sequence.

In addition to GC and LC, supercritical fluid chromatography (SFC) has also been investigated very recently for the analysis of banned compounds.[47−49] This old technique has made a remarkable comeback in the past few years due to improvements in instrumentation (better performance, reliability, and robustness) and column technology.[50,51] In SFC, a fluid (generally $CO_2$ mixed with MeOH) that has been pressurized and heated beyond its critical point is used. This fluid exhibits density and solvating power similar to a liquid and diffusivity and viscosity close to a gas. These properties allow good solubility and rapid transport of analytes without generating excessive pressure within the chromatographic system.[49] In addition, commercial interfaces for coupling SFC and MS are also available.[52] The role of this interface, in which a splitting device and makeup pump must be employed, is to avoid analyte precipitation and poor retention time repeatability. The applicability of SFC-MS/MS in sports drug testing was evaluated for a group of 110 doping agents belonging to different classes (i.e., narcotics, stimulants, diuretics, and $\beta$-blockers). The experiments were performed in both RPLC-MS/MS (reference method) and SFC-MS/MS using the same MS/MS device. Selectivity, sensitivity, linearity, and matrix effects were evaluated with these two analytical strategies. As illustrated in Figure 4, very diverse retentions and selectivities


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were obtained in UHPLC and UHPSFC, proving the complementarity of these analytical strategies.

Moreover, the poorly retained compounds in RPLC were sufficiently retained in SFC, and the reverse was also true. Under both chromatographic conditions, acceptable peak shapes and MS detection capabilities were obtained within 7 min of analysis time, enabling the application of these two methods for screening purposes. The sensitivity was equivalent in SFC-MS/MS vs RPLC-MS/MS for 46% of the compounds and was improved in SFC-MS/MS for 32% of the compounds. Another benefit of SFC-MS/MS over LC-MS/MS is its lower susceptibility to matrix effects in urine samples.[48] On the basis of these promising results, it would be interesting to evaluate SFC-MS/MS for the screening of other classes of illicit drugs from the WADA List such as steroids and glucocorticoids. Finally, UHPSFC-MS/MS has also been recently proposed for the enantiomeric separation of (*R*)- and (*S*)-clenbuterol to distinguish abuse from meat contamination.[53]

**Mass Spectrometry Detection.** Due to its high sensitivity and selectivity and the possibility to confirm the identification of analytes based on mass spectral information, MS has emerged as the gold standard detection mode in doping control analysis. Historically, low-resolution instruments have been preferentially used for small molecules, both for identification and quantitative determination, while high-resolution analyzers have been mainly dedicated for untargeted applications and especially the analysis and characterization of different forbidden peptides and proteins.

In routine antidoping laboratories, single quadrupole, triple quadrupole (QqQ), and quadrupole ion trap (QTrap) are the most widely used low-resolution MS devices because they generally offer suitable sensitivity, specificity, and dynamic range. For GC-based applications, single quadrupole MS remains the workhorse in routine laboratories due to its ease of use and the possibility of employing existing mass spectral libraries for analyte identification.[54] Recently, triple quadrupole mass analyzers have emerged for the detection of doping agents in targeted single-reaction monitoring (SRM) mode with GC, thereby significantly improving the detection performance from complex matrices. Due to the advantages afforded by GC/MS/MS, limits of detection of routine methods were improved compared to single-stage MS, especially for critical doping compounds such as steroids and their major urinary metabolites.[55] However, because GC peaks are extremely narrow (<1 s), ultrafast MS/MS devices with low SRM dwell times are required. Today, an SRM transition can be monitored with a dwell time of only 0.5 ms in the latest generation of GC/MS/MS instrumentation.

Despite the lack of reference libraries,[54] LC-MS is now widely employed for doping control analysis, probably more than GC/MS due to the higher versatility of LC. In LC-based applications, single quadrupole analyzers are no longer used, and QqQ and QTrap are the techniques of choice for most of the screening methods. These mass analyzers allow a rapid polarity switching between positive and negative modes, reducing the number of LC-MS injections needed for screening purposes by half. Due to the very high sensitivity and specificity afforded by these instruments, different classes of forbidden compounds (e.g., stimulants, diuretics, and $\beta_2$-agonists) can be monitored after a simple *dilute-and-shoot* procedure from urine samples. However, the electrospray ionization (ESI) source, which is by far the most commonly employed interface between LC and MS, can suffer from matrix effects produced by coeluting interferences in the sample. Ion suppression or enhancement modifies the sensitivity of the method by inducing irreproducibility. As for GC/MS, another important aspect when using LC-QqQ in doping control analysis is the need for an instrument able to provide sufficient sensitivity even at low dwell times in SRM mode. With the latest generation of LC-MS/MS instruments, the dwell time can be reduced down to only 0.8 ms, allowing the inclusion of a large number of compounds in the screening method.[34] In doping control analysis, numerous methods based on the detection of multiclass analytes have been developed with the QqQ analyzer in the past few years, with up to 100−150 banned substances screened in a single run.[56−58]

In the case of targeted MS/MS methods, the cycle time can limit the number of substances that can be screened.[8] To circumvent this drawback, a high-resolution MS (HRMS) operating in full scan mode, such as TOF or Orbitrap-based mass analyzers, can be used. Due to the current availability of benchtop HRMS platforms that are robust, easy to handle, suitable for operation by less well trained staff in daily routine work, and reasonably priced, HRMS is becoming increasingly routinely used in doping control laboratories for the screening of small molecules, especially TOF, QqTOF, and Orbitrap-based instruments. Several LC-HRMS-based methods have been recently proposed in the literature,[10,31−33,59] but antidoping methods related to the use of GC-TOF/MS remain extremely scarce; the combination of GC with Orbitrap technology has only been commercially available for a few months. The most recent generation of Orbitrap analyzers (Exactive and Q Exactive series) is remarkably fast and able to operate at acquisition rates of up to 18 Hz. Nevertheless, this speed is detrimental in terms of resolution (resolving power of only 15 000 fwhm at 18 Hz) compared to the maximal resolution that can be theoretically attained (240 000 fwhm). Regarding TOF/MS and QqTOF/MS, modern instruments provide a comparable performance to Orbitrap with faster acquisition speeds (up to 100 Hz).[33,60] However, in this case, working at very high resolution (up to 80 000 fwhm) impacts detection sensitivity, unlike with Orbitrap technology. One advantage of Orbitrap technology over TOF-based instruments is its ability to switch polarity to analyze both acidic and basic analytes within the same run; two independent runs are required with most TOF-based devices. Although the sensitivities of TOF and Orbitrap technologies are adequate for the detection of a large number of forbidden substances, they are still lower than what can be achieved with QqQ systems operating in SRM mode. This means that QqQ must be preferentially used for doping agents with critical sensitivity. For example, the recent paper from Pozo et al.[55] illustrates that the anabolic steroid, formebolone, cannot be detected with TOF/MS at a concentration of 10 ng/mL but is clearly detected with QqQ operating in SRM mode. However, despite their lower sensitivity, the quantitative performance of new HRMS platforms is now considered fit-for-purpose and similar to that of QqQ, especially in terms of dynamic range.[61] This was recently demonstrated by Rochat et al. in a recent study of the quantification of hepcidin in plasma.[62] Overall, HRMS technologies offer high resolution and excellent mass accuracy (5−20 ppm). Because these instruments operate in full-scan mode by default, any ionizable compound eluted during the run can virtually be detected. Selection of the target analytes is performed postanalysis by extracting the accurate mass of the selected doping agents within a narrow mass window. The


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importance of extracting the analytes of interest within a narrow mass window is illustrated in Figure 5, confirming that the specificity of the method can be drastically improved by decreasing the extraction mass window down to 5 ppm.

Because MS methods developed with TOF or Orbitrap-based technologies are not specific, adding an illicit drug to the method implies only the addition of the accurate mass and the corresponding retention time.[55] Therefore, TOF and Orbitrap instruments allow the retrospective analysis of previously acquired data once a new doping agent (or a new metabolite) is discovered. This feature is particularly beneficial for designer drugs, as was previously reported.[63,64] However, several key aspects of this approach have yet to be addressed or elucidated for future routine implementation of retrospective data analysis in doping control; this concept still remains a challenge primarily due to the lack of reference material for the identification of these new drugs or metabolites based on rules issued by the WADA. In this context, a promising approach for the identification of new entities based on LC-HRMS-based predictive retention time models has been recently proposed by Miller et al.,[27] as discussed in Chromatographic Approaches.

Because the initial screening of doping agents needs to be rapid and simple, there may be interest in the near future in using ambient mass spectrometry (AMS) techniques for fast, versatile, and direct analysis of samples in open air, with little or no sample preparation.[65,66] Various AMS techniques have been developed over the past few years, such as DESI (desorption electrospray ionization), DART (direct analysis in real time), and EESI (extractive electrospray ionization), but none of these is currently routinely used for doping control analysis of food supplements, pharmaceutical preparations, and/or biological fluids. However, the potential of DART hyphenated with Orbitrap-MS was evaluated for the fast identification and quantification of 21 anabolic steroid esters in oily commercial preparations.[67] Direct analysis in high-resolution scan mode was used to screen for steroid esters based on the accurate mass measurement. Steroid ester identification was further supported by collision-induced dissociation (CID) experiments through the generation of two additional ions. Moreover, the use of labeled internal standards allowed quantitative data to be recovered on the basis of isotopic dilution. DART-MS was also applied for the rapid determination of dimethylamylamine (DMAA), which is a stimulant banned by the WADA.[68] As shown in Figure 6, TOF/MS was used in this work to rapidly and unequivocally identify the presence of DMAA in pills and urine samples. However, current AMS methods suffer from drawbacks, such as poor quantitative performance, high limits of detection, lack of universality, and lack of convenience for practical applications, that should be addressed before potential widespread use in a doping control laboratory.[66]

Finally, another MS-based strategy that becomes increasingly popular is ion mobility spectrometry-mass spectrometry (IMS-MS). This analytical method separates gaseous phase ions according to their mobility under an electrical field on a millisecond time scale using IMS, followed by the detection of ions according to their mass-to-charge ratio on a microsecond time scale.[69,70] IMS could thus be considered an ultrafast replacement of chromatography prior to MS or as an additional dimension when combining liquid chromatography with IMS-MS. Much work is currently being performed on this technique to further improve the resolving power when compounds with similar collisional cross sections need to be separated, and a

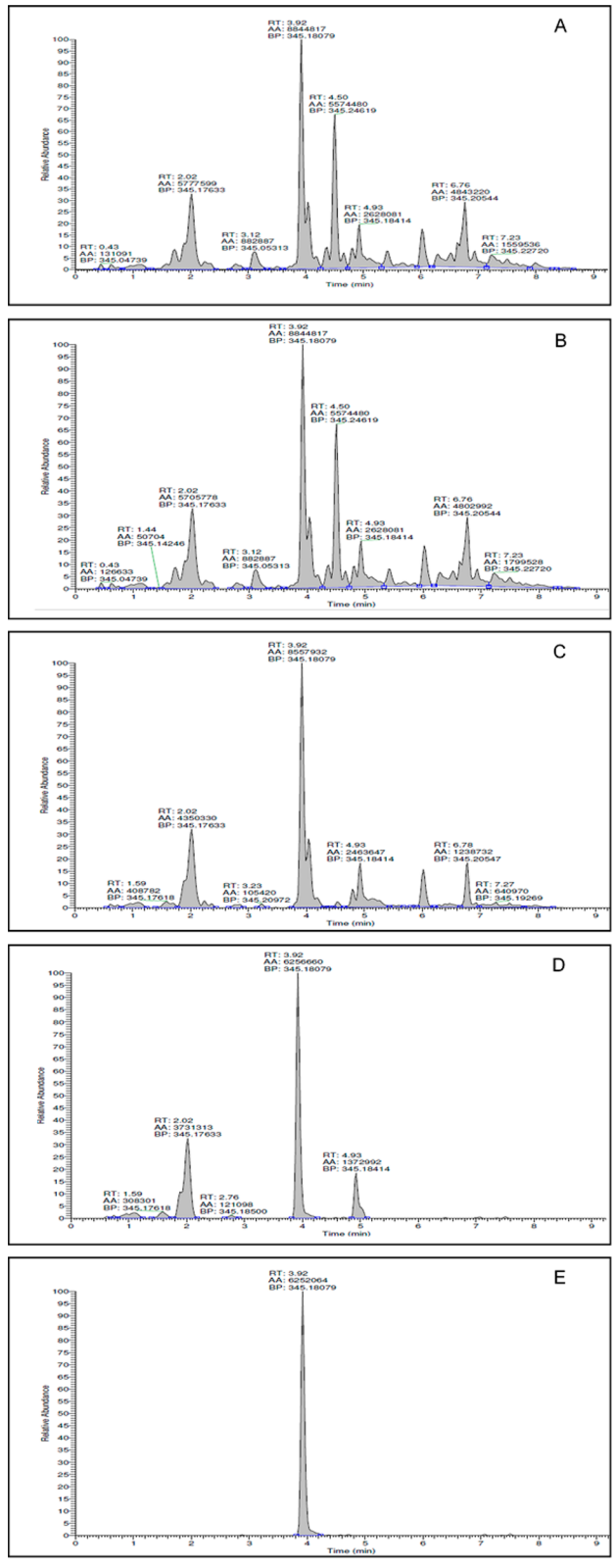


**Figure 5.** Extracted ion chromatograms of a urine sample containing 30 ng/mL formoterol ($m/z$ 345.1809) obtained with different mass range windows: (A) 1 amu (low resolution), (B) 500 ppm, (C) 100 ppm, (D) 20 ppm, and (E) 5 ppm. Reprinted from *J. Chromatogr. A*, Vol. 1288, Musenga, A.; Cowan, D. A. Use of ultrahigh pressure liquid chromatography coupled to high resolution mass spectrometry for fast screening in high throughput doping control, pp 82−95 (ref 10). Copyright 2013, with permission from Elsevier.


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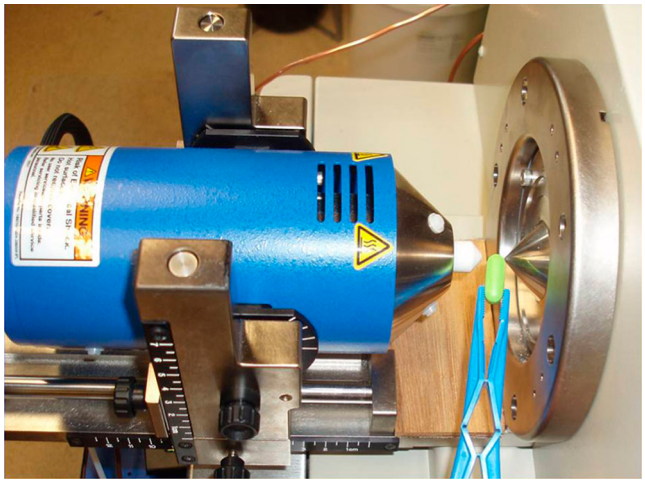

**Figure 6.** Example of a DART-MS analysis platform. Solid, liquid, or gas can be analyzed by introducing the sample between the ion source and the mass spectrometer inlet without requiring any sample preparation. Reproduced from DART-MS for rapid, preliminary screening of urine for DMAA, Lesiak, A. D.; Adams, K. J.; Domin, M. A.; Henck, C.; Shepard, J. R. E. *Drug Test. Anal.* Vol 6, Issue 7−8 (ref 68). Copyright 2014 Wiley.

promising study on the analysis of human insulin and its analogues was recently published.[71] In the near future, this approach will become valuable for antidoping control.

## PEPTIDE ANALYSIS

Peptides that stimulate growth hormone (GH) secretion have been particularly investigated in doping control analysis in the past few years due to their potential misuse as doping agents in sports. These peptides can be classified into two main groups: (i) growth hormone releasing hormones (GHRHs) such as sermorelin, tesamorelin, CJC-1288, CJC-1293, and CJC-1295, all possessing molecular masses between approximately 3 to 5 kDa; and (ii) growth hormone secretagogues (GHS) and releasing peptides (GHRPs), including, but not limited to, GHRP-1 to GRHP-6, alexamorelin, hexarelin, and ipamorelin, with smaller molecular masses of <2 kDa. In addition to these peptides, numerous methods for the analysis of human and synthetic insulins, with sizes ranging from 5 to 6 kDa as well as insulin-like growth factors (IGF) of slightly larger sizes, have been developed by doping control laboratories in recent years. Finally, there is also a range of additional peptides that need to be monitored, such as desmopressin, LHRH (GnRH) and its agonists (e.g., leuprolide, buserelin, and triptorelin), ACTH, and Synacthen and, more recently, several growth factors such as MGFs, as listed elsewhere.[72,73]

Currently, the key chromatographic method for analyzing peptidic drugs in sport drug testing is based on the use of RPLC-MS. When analyzing peptides in RPLC, it is recommended to use 0.1% TFA in the mobile phase to improve peak shapes through ion pairing to neutralize the positive charges at the surface of the peptides. However, ESI-MS sensitivity is reduced by a factor of approximately 10 compared to the use of 0.1% formic acid.[74,75] If a charge surface hybrid (CSH) C18 stationary phase is employed, the peaks observed in the presence of formic acid remain highly symmetrical and narrow, while MS sensitivity is not altered. Therefore, the CSH C18 column has been used in a few recent studies dealing with the determination of peptidic drugs in sports doping and appears as a promising stationary phase.[75,76]

Several years ago, the trend was to develop dedicated procedures for each class of peptides, but today, many laboratories want to develop a multiclass/multianalyte initial testing and confirmatory methods for peptidic substances. The most frequently reported approach for the analysis of small peptides, such as GHRPs and similar molecules, involves using mixed-mode weak cation exchange SPE from a few mL of urine samples or a few μL of protein-depleted serum/plasma specimens, followed by LC-ESI-MS/MS or LC-ESI-HRMS(/MS). A routine screening for the small GHRPs by LC-MS/MS was successfully implemented during the winter Olympic Games in 2014,[77] and positive cases were also recently identified in Montreal and Moscow using a similar methodology.[78] In addition, a screening assay was developed by Thomas et al. for the determination of 11 prohibited peptides (9 GHRPs, desmopressin, and LHRH) containing between 4 and 8 amino acids (<1.5 kDa) by combining SPE with nano-LC-HRMS.[79] A representative chromatogram showing the 11 peptides at a concentration of 25 pg/mL and four internal standards is provided in Figure 7. The method was fully validated, and the limits of detection were in the range of 2−10 pg/mL, which is much better than the most recent WADA recommendations (MRPL set at 2 ng/mL) for this class of substances. However, one of the main issues when analyzing small peptide hormones is the short half-life in plasma and their rapid elimination.[80] To further improve the current analytical approaches and improve detectability of GHRPs, the metabolism of GHRP-1, GHRP-2, GHRP-6, hexarelin, and ipamorelin in urine was investigated by Semenistaya et al.[78] In this study, a nano-LC-HRMS was employed to identify metabolites. Then, GHRPs and their major metabolites were included in a routine UHPLC-MS/MS procedure that had been fully validated. Esposito et al.[80] also highlighted the fact that *in vitro* models such as human liver microsomes and the S9 fraction could be used to detect peptidic metabolic markers in biological fluids without the need for long and tedious pharmacokinetic studies with human volunteers.

Although GHRPs and related substances are extracted and enriched through SPE alone, peptides >2 kDa (e.g., sermorelin, tesamorelin, CJC-1295, and Synacthen) should be better isolated from biological matrices using SPE or ultrafiltration followed by immunoaffinity purification. This allows one to obtain extracts of higher purity and better detection limits in biological fluids, far below the MRPL recently set by the WADA at 2 ng/mL in urine. In fact, using nano-LC coupled with HRMS detection, detection limits in urine down to 1−5 pg/mL were reported.[81,82] This procedure is relatively fast and allows the analysis of approximately 25 samples per day, which is of utmost importance for fast results reporting. However, the use of specific antibodies and magnetic beads makes this type of analysis expensive, limiting its application to targeted specimens based on previously obtained suspicious results and/or linked to high-risk sport disciplines.

Among biopharmaceuticals, insulin is one of the oldest and most well-known substances, which remains today as the primary treatment for diabetes.[83] Due to the suspected performance enhancement properties of insulin and its analogues, they have been among the first peptides implemented in routine doping controls using an MS-based strategy, and methods for their detection in human serum/plasma or urine have been continuously improved over the


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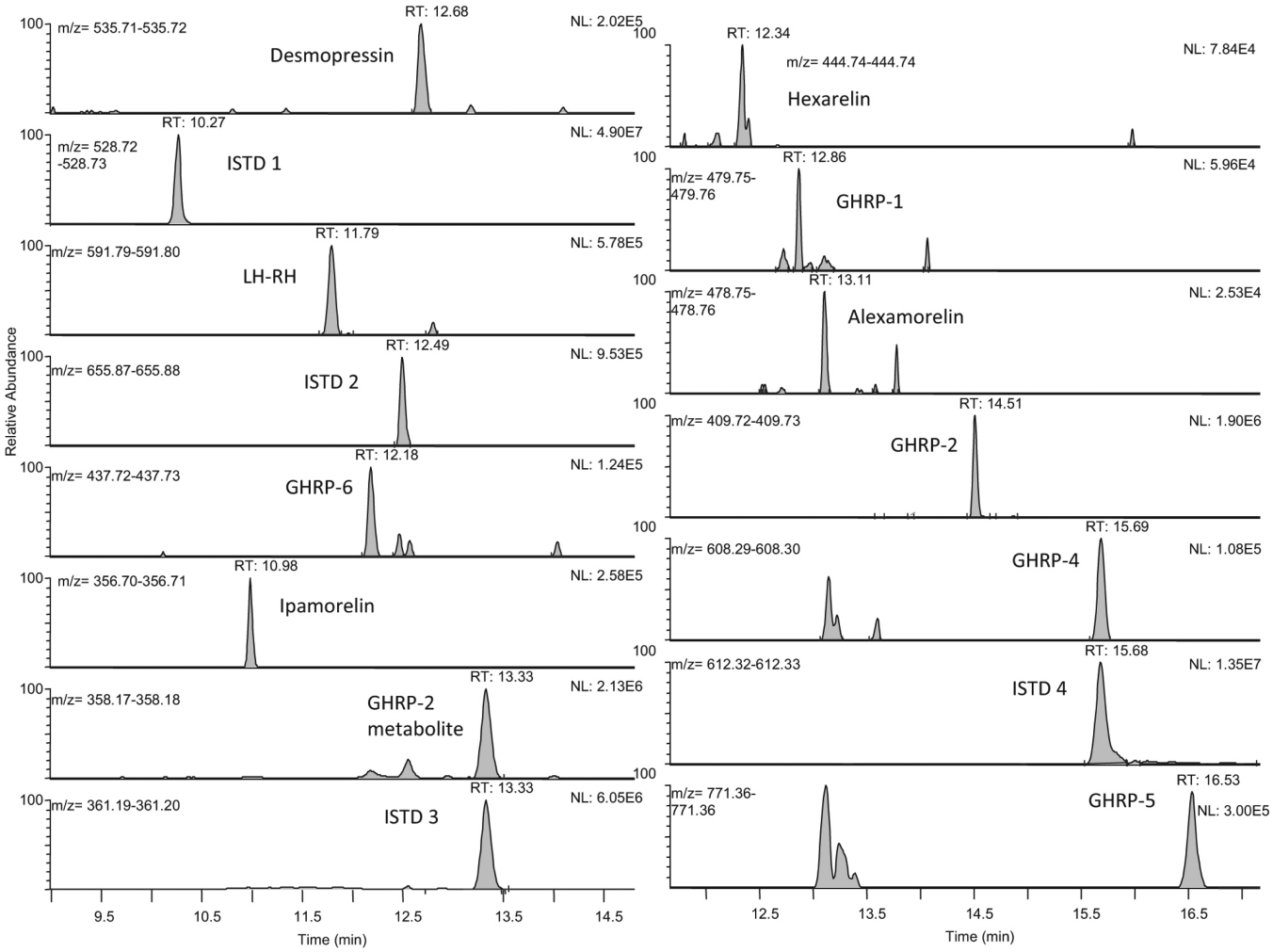


**Figure 7.** Extracted ion traces of a fortified blank sample containing 25 pg/mL of 11 illicit peptides and 4 internal standards. For desmopressin, GHRP-1, GHRP-6, and hexarelin, the extraction of the second isotope yielded better signal-to-noise in the chromatogram. Reprinted from *J. Chromatogr. A*, Vol. 1259, Thomas, A.; Walpurgis, K.; Krug, O.; Schanzer, W.; Thevis, M. Determination of prohibited, small peptides in urine for sports drug testing by means of nanoliquid chromatography/benchtop quadrupole orbitrap tandem-mass spectrometry, pp 251−257 (ref 79). Copyright 2012, with permission from Elsevier.

years. As reported in refs 73 and 84, modest modifications (i.e., the same amino acids but in a slightly different order, a truncated version of insulin, or amino acid substitutions in the sequence) were made to the primary structure of human insulin to create synthetic analogues with improved pharmacological profiles, which facilitate their unequivocal detection and identification in biological matrices (Figure 8).

To achieve the desired level of sensitivity and selectivity for the different insulins in human plasma/serum and urine, immunoaffinity purification followed by nano-LC and HRMS has been proposed. This approach allows the measurement of synthetic and endogenous human insulin down to 1−5 pg/mL.[72,73] However, although affinity purification is highly selective, it is less amenable to automation. In addition, nanoflow methods do not offer very high throughput. Therefore, Chambers et al. recently proposed a simple LC-MS/MS method for the rapid determination of four synthetic insulins in plasma with a total chromatographic analysis time of only 3.5 min.[83] A simplified and faster sample preparation procedure based on the use of the 96-well plate SPE was employed prior to LC-MS/MS, affording detection limits of 0.2−0.5 ng/mL with an overall throughput that is 6 to 10 times better than existing methods. Then, the same group of scientists[84] also designed a multidimensional platform to achieve better selectivity and sensitivity for the simultaneous analysis of human insulin and five recombinant analogues in plasma. Using a 96-well plate with mixed-mode SPE cartridges followed by a multidimensional-trapping and back dilution step prior to RPLC analysis, quantification limits down to 50−200 pg/mL were achieved for the 6 insulins, extracted from 250 $\mu$L of human plasma. Another promising strategy based on liquid chromatography coupled to ion mobility mass spectrometry was also developed[71] to achieve the orthogonal separation and fast analysis in only 8 min of isobaric insulins (human insulin and insulin lispro) in the ion mobility cell without losing specificity (Figure 9). Using this strategy, an LOQ of 0.2 ng/mL was achieved with only 200 $\mu$L of plasma or serum. Finally, when analyzing insulin, the major limitation is still the unsolved issue of distinguishing endogenous human insulin from the respective identical recombinant product. Further research is needed to support the unambiguous identification of the illicit administration of recombinant human insulin.[73]

Because IGF-1 is the primary mediator of growth hormone action and is responsible for many of its anabolic effects, this


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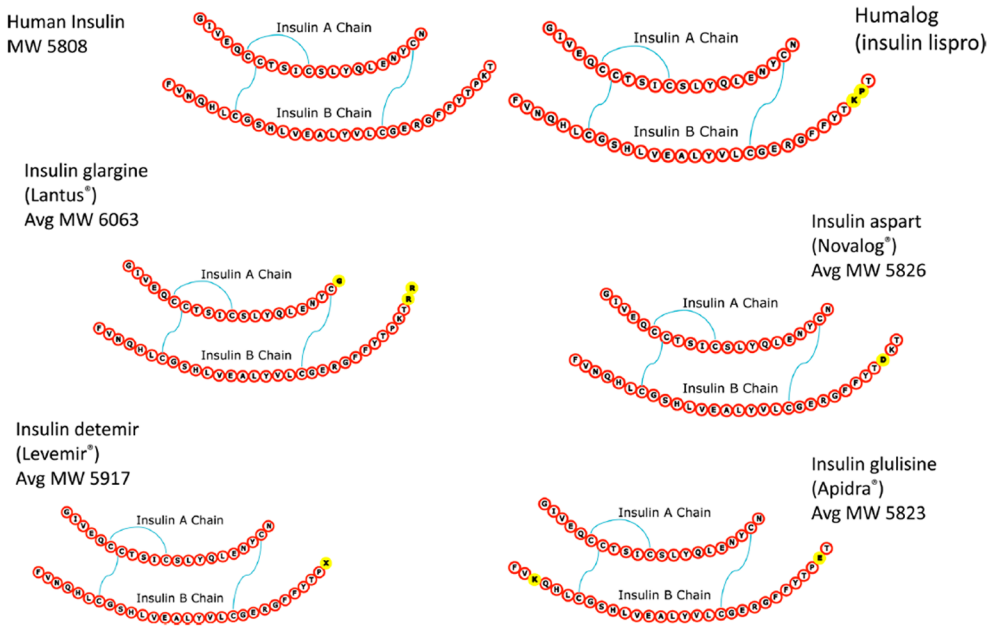


**Figure 8.** Amino acid sequences of human insulin and several analogs of importance in doping control analysis. Reproduced from Chambers, E. E.; Fountain, K. J.; Smith, N.; Ashraf, L.; Karalliedde, J.; Cowan, D.; Legido-Quigley, C. *Anal. Chem.* 2014, 86, 694−702 (ref 84). Copyright 2014 American Chemical Society.

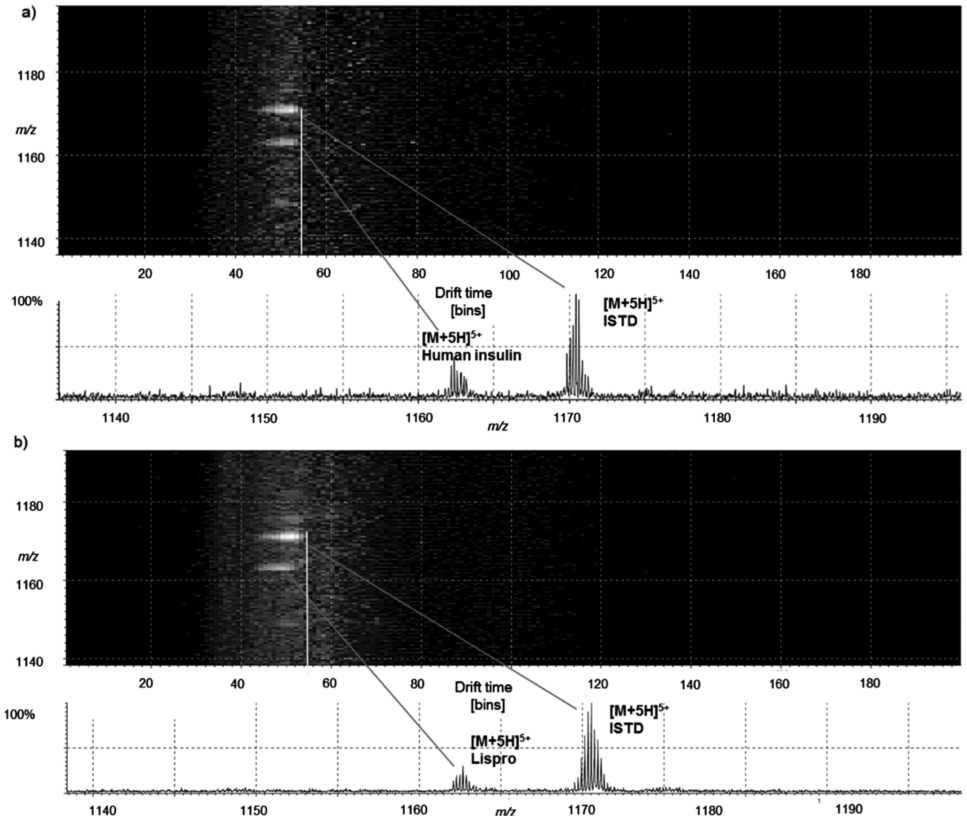


**Figure 9.** Mass vs drift time plots and mass spectra from administration samples for (a) recombinant human insulin and (b) insulin lispro. Reproduced from Determination of human insulin and its analogues in human blood using liquid chromatography coupled to ion mobility mass spectrometry (LC-IM-MS), Thomas, A.; Schanzer, W. *Drug Test. Anal.* Vol 6, Issue 11−12 (ref 71). Copyright 2014 Wiley.

peptide and its modified synthetic versions are prohibited according to antidoping regulations. Some methods have been proposed to analyze these peptide hormones of relatively high molecular masses, while addressing the issue of the natural presence of IGF-1 in humans.[72] IGF-1 can be identified in urine today, but there is no current criterion for detecting abuse, similar to human insulin. In addition, the concentration of IGF-1 in blood is used as one factor in the biomarker test for GH abuse.[85] Recently, an analytical methodology for the determination of IGF-1 as a biomarker for rhGH abuse detection was


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proposed using DBS as sample collection, rather than the traditional and invasive collection of venous blood. Using UHPLC-MS/MS, the LOQ achieved for IGF-1 in blood was estimated at 50 ng/mL, and a good agreement of concentration values was found between the venous blood and finger prick DBS collection.[86]

In addition to the misuse of IGF-1, peptide hormones categorized as mechano growth factors (MGFs) have been explicitly mentioned as prohibited substances in the WADA List since 2005.[87] Various reports have described MGFs' capability to stimulate muscle cell proliferation, increasing muscle strength, and regeneration. A method for the determination of MGF analogues was recently published[88] using IgG-coated magnetic beads in the sample preparation, followed by nano-LC and HRMS detection (Orbitrap). Even if the results in urine look promising and allow detection at 0.25 ng/mL, it remains to be clarified whether the intact agent is mainly excreted into urine in its intact form or if its metabolites could represent better targets for doping analysis.

## PROTEIN ANALYSIS

Stimulating erythropoiesis is a very efficient way to enhance performance. The resulting increase in the number of circulating red blood cells makes more hemoglobin available for oxygen transport from the lungs to the peripheral tissues, including muscles. This way of doping is of particular interest for endurance athletes performing aerobic activities. In this context, erythropoietin (EPO), a ca. 30−34 glycoprotein, is a key hormone to stimulate erythropoiesis. Secreted primarily by the kidneys, EPO acts in bone marrow. Direct detection methods of EPO should discriminate between naturally produced hormone by humans and the recombinant counterpart although their structural similarity is very high. Their protein moiety is identical as production of recombinant EPO is performed by introducing the human gene of natural EPO into mammalian cells such as Chinese hamster ovaries (CHO). However, this process results in different post-translational modifications, especially glycosylation and sulfonation. Indeed, isoelectric focusing (IEF) was chosen as the initial detection method to exploit this difference of glycosylation by separating proteins according to their isoelectric points (pIs).

Since 2007 (patent expiration), biosimilar erythropoiesis-stimulating agents have become widely available on the black market.[89] Although the latter are almost all produced in CHO cell lines, the difference of distribution of isoforms was observed between new EPO biosimilars and the originator molecule. This is attributed to the different manufacturing process such as cell medium employed or chromatographic enrichment of specific isoforms. Measurement of molecular mass distribution by sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) was therefore proposed as an additional method to detect the presence of these new biosimilars in urine and/or blood samples. Excretion studies of different biosimilars revealed that the detection window after their intake was several days. In addition, it was demonstrated as "glycofrom smear" profiles above the endogenous EPO band can be detected in urine and blood samples after microdose injections using this technique. However, compared to other epoetins, the continuous erythropoietin receptor activator (CERA) has shown lower sensitivity in SDS-PAGE, mainly due to the strong interaction between SDS and the PEG moiety of CERA. The latter is the third generation of rhEPO that is PEGylated to increase its half-life in blood up to 122 h. This problem was addressed by changing SDS to sarcosyl (SAR), a methyl glycine-based anionic detergent which interacts only with the protein (EPO) part of CERA without influencing detection of other epoetins. Because SDS-PAGE can be a more standardized method compared to the IEF procedure, many antidoping laboratories have recently replaced IEF by SDS/SAR-PAGE techniques to detect epoetins at physiological levels in urine and plasma (pg/mL). The required specificity and sensitivity were achieved by employing different monoclonal antibodies for immunoaffinity purification and Western blotting. Furthermore, SAR/SDS-PAGE could be adapted with a different protocol to improve the turnaround time of the analysis.[90]

Another erythropoietin-mimetic agent investigated in the past few years is EPO-Fc, which is a fusion protein composed of EPO and the fragment crystallizable (Fc) part of human immunoglobulin G (IgG1). A fast and efficient technique to detect EPO-Fc in serum was developed thanks to the interaction of the Fc part with protein A beads, followed by detection of the eluate with a commercial ELISA kit for the quantification of EPO. Using this approach, the LOD was determined at 5 pg of EPO-Fc independently of the serum volume.[91] For confirmation purposes, a second method was also developed consisting of immunopurification followed by SDS-PAGE or SAR-PAGE and western double-blotting with chemiluminescence detection, similar to the approach already used in routine EPO antidoping control. With this strategy, EPO-Fc can be detected in serum, together with all other recombinant erythropoietins, with an identical LOD as for the rapid screening procedure.

In addition to electrophoretic approaches, some alternative procedures based on the use of LC-MS have been employed to distinguish several recombinants from endogenous EPO. For example, Okano et al. recently demonstrated the detectability of darbepoetin alfa (a long-acting erythropoiesis-stimulating agent) using bottom-up LC-MS/MS following immunoaffinity purification from 10 mL of urine and enzymatic hydrolysis, yielding a prototypical target peptide for unambiguous identification.[92] The lower limit of detection of urinary darbepoetin alfa was 1.2 pg/mL, while the limit of detection for the confirmation analysis was estimated at 5 pg/mL. The developed method allows relatively fast confirmation analysis, including 6 h for sample preparation and an analytical run time of only 10 min per sample. Peginesatide is another type of EPO of approximately 45 kDa comprising several amino acid modifications and possessing a polyethylene glycol support. A method was developed for the determination of peginesatide in several specimens. In urine, a simple protein precipitation followed by proteolytic digestion was implemented, and purification and concentration of the resulting target peptide with SPE was used prior to LC-MS/MS. In urine, a limit of detection of 0.5 ng/mL was achieved.[93] In another study, the same erythropoietin-mimetic peptide (peginesatide) was determined in plasma using DBS collection. The same sample preparation was employed as that for urine, and a limit of detection of 10 ng/mL was attained using LC-MS/MS.[94] As reported in Figure 10, an alternative strategy was also proposed by Vogel et al. for the isolation, enrichment, and analysis of erythropoietins.[95]

This innovative approach allows the successful determination of endogenous EPO and the recombinant forms of EPOzeta, darbepoetin alfa, and CERA from human urine. It is based on regular ultrafiltration for preconcentration of the target substances, followed by specific isolation through human


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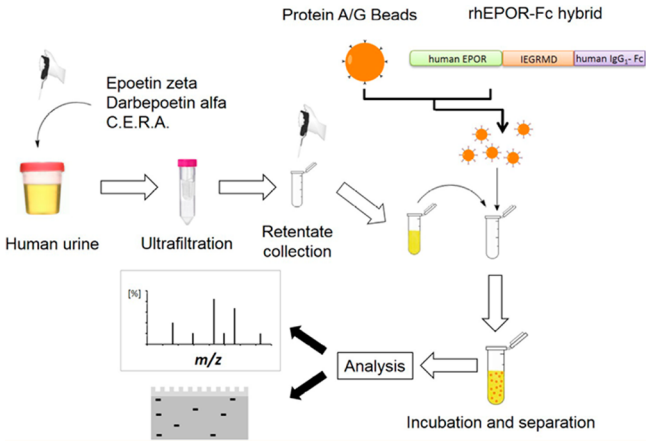


**Figure 10.** Analytical procedure for the analysis of erythropoietins at concentrations down to 20−80 pg/mL in doping control analysis. Reproduced from Vogel, M.; Blobel, M.; Thomas, A.; Walpurgis, K.; Schanzer, W.; Reichel, C.; Thevis, M. *Anal. Chem.* 2014, 86, 12014−12021 (ref 95). Copyright 2014 American Chemical Society.

erythropoietin receptor bound to magnetic beads. Then, the analytical method consists of either gel-based electrophoresis (SAR-PAGE and immunoblotting) or nanoliquid chromatography coupled with high-resolution tandem mass spectrometry (Orbitrap technology). Limits of detection achieved with the latter strategy ranged from 20 to 80 pg/mL. Even if the achieved sensitivity was found to be satisfactory for doping control analysis, approaches based on antibody-purified extracts yield superior results. However, sample preparation based on the use of human erythropoietin receptor bound to magnetic beads is much more generic and can be theoretically applied to any new recombinant EPO that will appear on the market. Finally, another interesting strategy was proposed by Reichel[96] based on the difference in sialic acid O-acetylation between human urinary and recombinant EPOs. The study demonstrated that O-glycans of human urinary EPO significantly differed in sialic acid O-acetylation compared to many recombinant EPOs. Moreover, only traces of O-acetylation were observed on two glycans of human urinary EPO.

## CONCLUSIONS

This Review highlights the analytical advances in doping control analysis achieved in the past few years, from small molecules to peptide and protein analyses. After a brief introduction describing the regulatory bodies and documents currently in force in the antidoping field, an overview of significant advancements described in the literature in chromatography−mass spectrometry-based methods for sports drug testing was provided. Because there is a need to analyze a high number of urine and blood samples, antidoping laboratories should apply diverse sample preparation techniques that allow faster and automated workflow, considering the short reporting times. Among them, dilute-and-shoot, LLE, reversed phase and mixed-mode SPE, and SLE represent the most widely used preparation techniques today. In addition to these techniques, other liquid- or solid-based microextraction procedures look very promising and could rapidly gain interest for doping control analysis in the near future to reduce the volume of samples and organic solvents needed. New analytical trends, especially in GC- and LC-MS(/MS)-based methods, for the detection of prohibited substances were also described in detail. In this context, the availability of new analytical LC columns packed with particles of diverse morphologies (i.e., fully porous sub-2 $\mu$m, core−shell sub-3 $\mu$m particles) and the use of highly sophisticated MS devices such as high-resolution-high accuracy instruments (TOF, Orbitrap) have recently emerged as important approaches for screening, confirmation, and identification purposes. Despite using cutting-edge technologies to detect doping substances, laboratories must also adapt their approaches in the future to detect new doping practices employed by athletes and their entourage to improve performance. In addition to chromatographic-based techniques, the implementation and use during recent years of indirect approaches such as ABP have equipped laboratories with very powerful and robust tools to catch doped athletes. However, due to the long validation processing times required to investigate and implement new biomarkers such as long-term metabolites of new doping compounds, antidoping laboratories are often required to possess detection methods "in due time". To address these concerns, retrospective analyses of antidoping samples could be a possible solution. Indeed, national antidoping organizations and sport federations can request that the collected urine and blood samples be stored for up to ten years in the latest version of the Code. This storage time allows reanalyses of samples collected from major sports competition events (e.g., Olympics Games, World Championships) after a few years using newly available analytical technologies.

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### Author Contributions

¶R.N. and D.G. equally contributed to this Review.

### Notes

The authors declare no competing financial interest.

### Biographies

***Dr. Raul Nicoli***, after graduating as a Pharmacist in 2003, completed his PhD thesis in the Laboratory of Pharmaceutical Analytical Chemistry of the University of Geneva. In 2010, he joined a contract research organization specialized in analytical services based on chromatography and mass spectrometry. Since April 1, 2012, Raul Nicoli has been part of the Swiss Laboratory for Doping Analyses and in charge of different research projects as well as of the supervision of the routine analyses by liquid chromatography coupled to mass spectrometry. His interests focus on new emerging sample preparation techniques as well as on the development of analytical methods for the identification/quantification of small molecules, peptides, and proteins in biological fluids.

***Dr. Davy Guillarme*** holds a PhD degree in analytical chemistry from the University of Lyon, France. He is now a senior lecturer at the University of Geneva in Switzerland. He has authored 140 journal articles related to pharmaceutical analysis. His expertise includes HPLC, UHPLC, HILIC, LC-MS, SFC, and analysis of proteins and mAbs. He is an editorial advisory board member of several journals including *Journal of Chromatography A*, *Journal of Separation Science*, *LC-GC North America*, and others.

***Dr. Nicolas Leuenberger*** obtained his biochemistry diploma at Fribourg University after working in Prof. Sandro Rusconi's group. Then, he was a visiting scientist at Melbourne University in the Biochemistry faculty. In 2005, he started his PhD thesis in the Walter Wahli laboratory at the Center of Integrative Genomics of University



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of Lausanne. Since June 2009, Nicolas Leuenberger has supervised methods linked to biology/biochemistry in the Swiss Laboratory for Doping Analysis. His interests focus on the development of new detection methods concerning recombinant proteins (EPO, growth hormones, and so on) and autologous blood transfusion in biological liquids.

***Dr. Norbert Baume*** completed his PhD thesis in the Swiss Laboratory for Doping Analyses in Lausanne. At the same time, he obtained a master's degree in Sports and Physical Education at the University of Lausanne. Norbert Baume accomplished a one year postdoctoral fellowship in Dr. Benjamin Miller's lab, part of the Department of Sport and Exercise Science, University of Auckland (New Zealand). His main expertise is anabolic steroids (especially nandrolone and testosterone) and glucocorticoids metabolism as well as nutritional supplement composition and use in sports. Dr. Baume also has skills in clinical trial management and in analytical chemistry (GC/MS(/MS) and LC-MS(/MS)).

***Dr. Neil Robinson*** received his MSc degree in Biology in 1998 from the University of Lausanne (UNIL). He then pursued his PhD at the Swiss Laboratory for Doping Analyses-Centre Hospitalier Universitaire Vaudois under the supervision of professor Martial Saugy. In 2003, he obtained his PhD degree in Sciences. A lot of the data from his thesis was used to implement the so-called Athlete Biological Passport (ABP) hematological module. Since then, he has been in charge of various routine antidoping analyses and especially responsible for the Lausanne APMU (Athlete Biological Passport Unit). His current work focuses on improving the steroidal module of the ABP passport and finding new strategies to fight against autologous blood transfusions as well as micro EPO doses.

***Prof. Martial Saugy*** studied Biology at the University of Lausanne where he received his PhD degree in 1986. After a postdoctoral fellowship at the department of Biochemistry at McGill University (Montreal, Canada), he worked as biochemist in the analytical toxicology laboratory of the Legal Medicine Institute of the University of Lausanne. In 1990, the antidoping unit, later becoming the Swiss Laboratory for Doping Analyses, was created and Martial Saugy became the deputy director. Since 2003, he has been director of the laboratory and from an academic point of view he is associate professor at the Faculty of Biology and Medicine of the University of Lausanne.

***Prof. Jean-Luc Veuthey*** is professor at the School of Pharmaceutical Sciences, University of Geneva, Switzerland. He also acted as President of the School of Pharmaceutical Sciences, Vice-Dean of the Faculty of Sciences, and finally Vice-Rector of the University of Geneva. His research domains are development of separation techniques in pharmaceutical sciences and, more precisely, study of the impact of sample preparation procedures in the analytical process; fundamental studies in liquid and supercritical chromatography; separation techniques coupled with mass spectrometry; analysis of drugs and drugs of abuse in different matrices. He has published more than 300 articles in peer-review journals.

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