Multianalyte LC–MS-based methods in doping control: what are the implications for doping athletes?

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Over the last 50 years, the list of doping substances and methods has been progressively expanding, being regularly reviewed by the international antidoping authorities (formerly the Medical Commission of the International Olympic Committee, and afterward, following its constitution in 1999, the World Anti-Doping Agency [WADA]). New substances/classes of substances have been periodically included in the list, keeping the pace with more advanced and sophisticated doping trends. At present, and apart from the prohibited performance enhancing and masking methods (e.g., blood transfusions and tampering strategies), the list comprises several hundreds of biologically active substances, with broad differences in their physicochemical properties (i.e., molecular weight, polarity and acid-basic properties) [1]. As a consequence, the 'one class – one procedure' approach, which had been followed by nearly all accredited antidoping laboratories worldwide until the turn of the millennium, is no longer sustainable. The need to minimize the overall number of independent analytical procedures, and, in parallel, to reduce the analytical costs, stimulated the development of multitargeted methods, aimed to increase the overall ratio of 'target analytes: procedure' [2–6].

The above evolution has not always been a straight forward process. The need to comply with the WADA technical requirements (both in terms of identification criteria and of minimum required performance limits [7,8])

and with the reduction of the reporting time (a constraint that becomes even more critical during international sport events, where the daily workload also drastically increases) has imposed a thorough re-planning of the analytical procedures.

The development of an antidoping analytical method requires the appropriate knowledge not only of the biophysicochemical properties of the target analyte, but also of its PK profile. Historically, immunological methods and GC-based techniques were applied in antidoping science, as preferential screening methods for the detection of prohibited substances, which were originally limited to nonendogenous stimulants and narcotics. In the 1980s, GC–MS became the reference analytical platform for the detection and quantification of the majority of the low molecular weight doping substances [3–6]. In the following two decades, with the inclusion in the Prohibited List of new classes of low molecular weight, hydrophilic, thermolabile, nonvolatile analytes (including, but not limited to, glucocorticoids and designer steroids) and simultaneously of peptide hormones, scientists were obliged to design, develop, validate and apply techniques based on LC–MS/MS.

From single-class to multiclass approaches
Analysis of small molecules
As mentioned above, LC–ESI-MS(/MS)-based methods were initially developed to

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target those categories of prohibited drugs that would have been undetectable (e.g., peptide hormones) or barely detectable (e.g., glucocorticoids) with conventional GC-MS-based techniques, as well as for compounds that (although detectable by GC–MS) required extensive, time-consuming sample-preparation procedures (e.g., diuretics). The progressive advancement of the technical performance of bench-top mass spectrometers, in terms of ionization sources, scan speed and polarity switching capability, paralleled by a similar improvement of the chromatographic separation techniques, in terms of chromatographic hardware, stationary phases (e.g., hydrophilic interaction chromatography) and packing material characteristics and sizes (e.g., fused-core, monolithic, sub-2 μm), allowed the development of rapid and effective multitargeted screening procedures, permitting to simplify the sample preparation procedure, while at the same time reducing the volume of biological sample required for the assay, and the overall cost and time of operation per sample. A peculiar feature of the newly developed LC–MS(/MS)-based procedures was the possibility to screen for a considerable number of markedly different analytes/classes of analytes in a single chromatographic run, initially using HPLC interfaced with low-resolution mass spectrometers and, more recently, coupling UHPLC technology with either low- or high-resolution mass spectrometers [9–13].

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Analysis of macromolecules

The inclusion in the Prohibited List of peptide hormones with low molecular weight (<2000 Da) and with minimal differences in the aminoacidic sequence with respect to those produced endogenously has imposed the development of analytical strategies based on mass spectrometric identification. Different procedures were initially developed to detect insulin and its analogs, the corticotrophin synacthen (ACTH 1–24), the luteinizing hormone releasing hormone (LH-RH, gonadorelin), desmopressin and its analogs, and the entire class of GHRPs using low-resolution mass spectrometers and μLC techniques. Nano-LC coupled with high-resolution mass spectrometers was then preferred, due to sensitivity and selectivity issues [4,9,14]. Recently, multianalyte screening procedures have also been developed to detect small peptides by means of mixed-mode weak cation exchange SPE followed by LC–ESI-MS-based techniques [15–17]; while peptide

hormones with medium-molecular weight (e.g., insulins, tesamorelin, sermorelin, CJC-1293, CJC-1295, synacthen and IGF1 analogs) were proposed to be screened for by methods based on immunoaffinity purification followed by LC–ESI-MS/MS [18].

From targeted to 'omic' approaches

Analytical procedures based on targeted approaches are very specific and sensitive, however, their application is limited to preselected groups of compounds, previously characterized in sufficient details both in terms of their PK properties and chromatographic-spectrometric profile. To also detect potentially unknown targets, high-resolution mass spectrometers operating in full-scan mode can be used for the detection of a virtually unlimited range of known and unknown substances. TOF- and Orbitrap-based technologies are currently the most common high-resolution mass analyzers used in doping control analysis. The main advantage of these instrumental platforms is their capability of collecting data at very fast scan rates, which also allows the retrospective interpretation of the results originally obtained on the samples (including those that may have originally been reported as negative), to verify the possible presence of prohibited substances that were unknown at the time of the original analysis. This is possible by reprocessing the data files without the need of reanalyzing the samples [3–6]. This feature is particularly useful not only to detect new designer drugs, but also to increase the detectability of several prohibited agents and to ensure the detection of new doping practices. The possibility of monitoring a huge amount of data opens the way to 'omic' approaches, where the alteration of specific biomarkers/biomarker profiles might be used as proof of a doping offense.

Conclusion: what is next?

From the early days of sports drug testing, the 'doping community' has continuously moved toward strategies capable of increasing athletic performance, always with the parallel objective of circumventing existing antidoping regulations, testing strategies and laboratory methods. Doping analysis has always been a race against time. A race that is usually intended as the need, for the scientists of the antidoping community to quickly identify emerging substances of abuse and practices employed by athletes to improve performance, and to immediately update, improve and expand the existing panel of analytical methods. LC–MS has been one of the most effective tools in this race.

The combination of direct and indirect methods, as well as the combination of mass spectrometric-based techniques with molecular and cellular biology methodologies, seems to be the most promising detection

strategy to ensure the most complete coverage of doping substances and methods, narrowing the gap between the number of athletes who dope and the number of athletes who are caught [19,20]. In this scenario, either multitargeted (carried out on specific analytes or selected biomarkers) or untargeted methods based on LC–MS, represent the prevalent analytical tool to keep the pace with the constantly evolving doping trends. Nonetheless, although highly effective, rapid and sensitive, the multianalyte procedure find their most appropriate use as screening analyses (also defined 'initial testing analysis', e.g., by the WADA). Although modern LC–MS/MS systems ensure remarkable selectivity, in terms of chromatographic separation and mass spectrometric identification, the risk of potential interferences still exists, particularly from nonprohibited substances and/or their metabolites, which may also be present in the tested biological sample, and that are generally not considered when a new method is validated. To ensure the unambiguous identification (and where so required by the WADA rules, the quantitative determination) of all relevant analytes, the ideal analytical strategy should always be based on the combination of: multianalyte, highly sensitive, initial testing ('screening') procedures (no false-negative results, with the occurrence of false-positive kept to a minimum); and highly sensitive and, crucially, highly selective confirmation procedures, allowing virtual annulment of the risk of false-positive results.

As previously mentioned, the development of effective laboratory methods requires the knowledge of physicochemical properties, and of the PK profile of a doping substance. Information on metabolism and excretion kinetics of doping substances should also always be taken into account when a doping test session is planned. As no laboratory method, as effective as it can be, can counter balance the uselessness of an ill-timed test. In this scenario, modulation of the PK profile – either by the use of drug-delivery systems, to incorporate and transport the prohibited substances, and/or the co-administration of a doping substance with nonprohibited drug, to take advantage of drug–drug interactions altering the metabolism of the former – may be the next strategy to challenge the efficacy of laboratory methods. But WADA-accredited laboratories are already reacting, and the race continues.

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