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Review

Analytical strategies based on mass spectrometric techniques for the study of steroid metabolism

Cristina Gomez [a], Andreu Fabregat [a], Óscar J. Pozo [a,*], Josep Marcos [a,b], Jordi Segura [a,b], Rosa Ventura [a,b]

[a] Bioanalysis Research Group, IMIM, Hospital del Mar, Doctor Aiguader 88, 08003 Barcelona, Spain
[b] Department of Experimental and Health Sciences, Universitat Pompeu Fabra, Doctor Aiguader 88, 08003 Barcelona, Spain

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A B S T R A C T

Anabolic androgenic steroids (AASs) are synthetic substances derived from testosterone, whose use in sports is prohibited. This review underlines the advantages and the drawbacks of different coupled mass spectrometry-based approaches to the identification of AAS metabolites. We discuss diverse aspects of the applications in the doping-control field, including sample preparation and instrumental analysis, with special emphasis on the potential scope of each strategy.

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* Corresponding author. Tel.: +34 93 31 60 472; Fax: +34 93 31 60 499.
E-mail address: opozo@imim.es (Ó.J. Pozo).

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1. Introduction

Anabolic androgenic steroids (AAS) are included in the list of forbidden substances in sports due to their performance-enhancing and adverse health effects. They are the most prominent group of forbidden substances detected in doping controls, reflecting the wide misuse of these compounds as performance-enhancing drugs by athletes [1]. The general structure and some examples of AAS structures are depicted in Fig. 1A.

Antidoping laboratories have to develop analytical tools to detect the use of AASs in sports. AASs suffer extensive metabolism, so their misuse is normally monitored through the analysis of the metabolites excreted in urine [2,3]. It has to be kept in mind that the best marker is not always the most abundant metabolite but the metabolite excreted for longer times after the administration of the drug. For this reason, antidoping laboratories need to perform comprehensive and in-depth metabolic studies in order to identify as many metabolites as possible and to select the best markers. These studies have to include:

- detection of potential metabolites;
- identification of potential metabolites based on the structural information provided by mass spectrometry (MS) data;
- the excretion profile of each metabolite to evaluate the excretion time; and,
- confirmation of the postulated structure of each metabolite by synthesis of the authentic material [4].

Metabolic studies of AASs have been traditionally performed using gas chromatography coupled to MS (GC-MS), and the detection of the misuse of AASs by antidoping laboratories has relied for many years on the detection of the metabolites identified in the 1980s by GC-MS methods [2]. GC-MS has some limitations for the execution of metabolic studies, among which the most significant are:

- the need for derivatization of polar compounds; and,
- the impossibility of detecting conjugated metabolites directly.

In recent years, liquid chromatography coupled to tandem MS (LC-MS/MS) has shown several novel possibilities for the detection and the identification of new phase I and phase II metabolites. In particular, LC-MS/MS has the capacity to detect conjugated metabolites directly and the possibility to apply open methods [neutral loss (NL) and precursor ion (PI) scan methods] for the detection of metabolites having a common chemical structure [5].

The detection of AASs and their metabolites based on MS techniques has been reviewed [6–9]. These reviews deal mainly with the potential of MS for the target detection of several doping agents (including AASs and their known metabolites) in different biological matrices. However, they are not focused on the analytical strategies for the detection and the elucidation of these metabolites.

In this article, we review the potential of MS for the study of AAS metabolism in human urine samples. First, we briefly summarize the metabolism for AASs. Second, we present different approaches to the detection and the characterization of phase I metabolites. Then, we discuss the study of phase II metabolism, considering two approaches: indirect detection of conjugates after hydrolysis, and direct detection of the phase II metabolites. Finally, we suggest an integrated approach based on different methods for the comprehensive study of AAS metabolism.

2. Metabolism of anabolic androgenic steroids

Consideration of general metabolic pathways is fundamental for the detection and the characterization of the main metabolites of new steroids, but also for the discovery of previously unreported metabolites of known steroids.

In general, synthetic AASs follow the metabolic pathways observed for testosterone and have been comprehensively reviewed [2,3]. Metabolic reactions are grouped into two types – phase I and phase II – and they convert the steroid into more polar compounds in order usually to inactivate the drug and to facilitate its elimination from the body. Phase I reactions are enzymatically-catalyzed reactions, normally oxidations and reductions occurring in several positions of the steroid ring (Fig. 1A). Double-bond reduction towards 5α- and 5β-saturated structures, 3-keto or 17-keto reduction, 1,2-hydrogenation, 6-, 12- or 16-hydroxylations, 6,7-dehydrogenation, and 17-hydroxy oxidation are considered the main phase I metabolic reactions for AASs. Some examples of these metabolic pathways are shown in Fig. 1B.

Phase II reactions conjugate the AASs or their phase I metabolites with polar molecules, mostly with glucuronic acid or sulfate. Both conjugation reactions are enzymatically controlled. Glucuronidation is catalyzed by uridine diphosphoglucuronosyl-transferases, and formation of sulfates is catalyzed by sulfotransferase enzymes [2]. Minor conjugation reactions {e.g., conjugation with cysteine or N-acetylcysteine [10,11]} and the existence of bis-conjugates [2,12] have also been described.

Ther metabolism of AASs has been studied using classical models, *in-vitro* and, mainly, *in-vivo* studies in healthy volunteers. In recent years, *in-vivo* animal models based on mice with functional human hepatocytes have been successfully employed to study the human metabolism of some AASs [13]. The usefulness of this model has been demonstrated in the study of some AASs {e.g., methandienone [14], methyltestosterone [15], stanozolol [16] and promagnon, methylclostebol and methasterone [17]}.

3. Evaluation of phase I metabolism

Most of the metabolic studies published for AASs focus on the detection of phase I metabolites. Several parameters in sample treatment and instrumental analysis have to be considered, since the results dramatically depend on the suitability of these parameters.

3.1. Sample treatment

The study of phase I metabolites is generally performed after hydrolyzing the urinary phase II metabolites. Hydrolysis procedures are discussed in Section 4 (below). Following hydrolysis, phase I steroids have to be isolated from the rest of the complex matrix. In metabolic studies, this extraction step is crucial due to the wide range of polarities of phase I metabolites [2]. Ideally, all metabolites should be extracted while minimizing the co-extraction of interfering substances.

The most frequently employed strategy is a liquid-liquid extraction (LLE) with ether or n-pentane. Since interferences are reduced at a high pH (9–10), for most AASs, extraction in alkaline conditions is recommended. The extraction yield using *tert*-butylmethyl ether is greater than 85% for most AAS metabolites [18].

Quantitative recoveries can also be obtained for most AASs by solid-phase extraction (SPE) with C18 cartridges [19]. However, often large amounts of interferences are also concentrated in the cartridge, so we advise the use of SPE with caution. However, the wide variety of solid-phase packing materials available provides additional benefits for the study of specific AASs. For example, the basic

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