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A new sulphate metabolite as a long-term marker of metandienone misuse

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ABSTRACT

Metandienone is one of the most frequently detected anabolic androgenic steroids in sports drug testing. Metandienone misuse is commonly detected by monitoring different metabolites excreted free or conjugated with glucuronic acid using gas chromatography mass spectrometry (GC–MS) and liquid chromatography tandem mass spectrometry (LC–MS/MS) after hydrolysis with β-glucuronidase and liquid–liquid extraction. It is known that several metabolites are the result of the formation of sulphate conjugates in C17, which are converted to their 17-epimers in urine. Therefore, sulphation is an important phase II metabolic pathway of metandienone that has not been comprehensively studied. The aim of this work was to evaluate the sulphate fraction of metandienone metabolism by LC–MS/MS. Seven sulphate metabolites were detected after the analysis of excretion study samples by applying different neutral loss scan, precursor ion scan and SRM methods. One of the metabolites (M1) was identified and characterised by GC–MS/MS and LC–MS/MS as 18-nor-17β-hydroxymethyl-17α-methylandrost-1,4,13-triene-3-one sulphate. M1 could be detected up to 26 days after the administration of a single dose of metandienone (5 mg), thus improving the period in which the misuse can be reported with respect to the last long-term metandienone metabolite described (18-nor-17β-hydroxymethyl-17α-methylandrost-1,4,13-triene-3-one excreted in the glucuronide fraction).

1. Introduction

Metandienone (17α-methyl-androst-1,4-dien-17β-ol-3-one) (Fig. 1) is a synthetic anabolic androgenic steroid (AAS) included in the list of prohibited substances by the World Anti-Doping Agency (WADA) which is used by athletes in order to increase muscular mass and to improve performance [1]. Metandienone is one of the most frequently detected AAS in doping analyses [2] and, therefore, there is a constant need to develop strategies to improve the detection of its misuse.

In the doping control field, the best marker for the detection of the administration of a forbidden substance is not always the most abundant metabolite but the one which can be detected for the longest period of time (the so-called long-term metabolites). Therefore, metabolic studies are very helpful in order to detect new long-term metabolites.

The use of liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) is gradually becoming more important for the detection of phase I and II metabolites of doping agents [3–7]. Regarding AAS phase II metabolites, mainly conjugates with glucuronic acid have been systematically studied by using enzymatic hydrolysis with β-glucuronidase enzymes and detecting the released phase I metabolites by using gas chromatography-mass spectrometry (GC–MS) or LC–MS/MS [4,8]. Sulphate metabolites are known to be important for some endogenous steroids and they have also been described for exogenous AAS. Recent studies by our group showed the potential of LC–MS/MS for the direct detection of sulphate metabolites of AAS like boldenone and methyltestosterone [9,10]. For methyltestosterone, minor metabolites excreted as sulphate conjugates provided longer retrospectivity than the major metabolites excreted as glucuronoconjugates [10].

The metabolism of metandienone has been extensively studied [8,11–25]. The main metabolic pathways described are depicted in Fig. 1. The metabolism of metandienone was first published by Rongone and Segaloff [11] who described 6β-hydroxymetandienone (I) and 17-epimetandienone (II) as metabolites of metandienone in humans, detected in urine in free form [12–14,18]. 17-epimerization as well as formation of 18-nor-17,17-dimethyl analogs were demonstrated to result from degradation and rearrangement of 17β-sulphate conjugates in urine [15–17,26]. 6β-Hydroxy-17-epimetandienone (III) resulting also from the formation of a 17β-sulphate, is detected in post-administration samples in free form [12,18].

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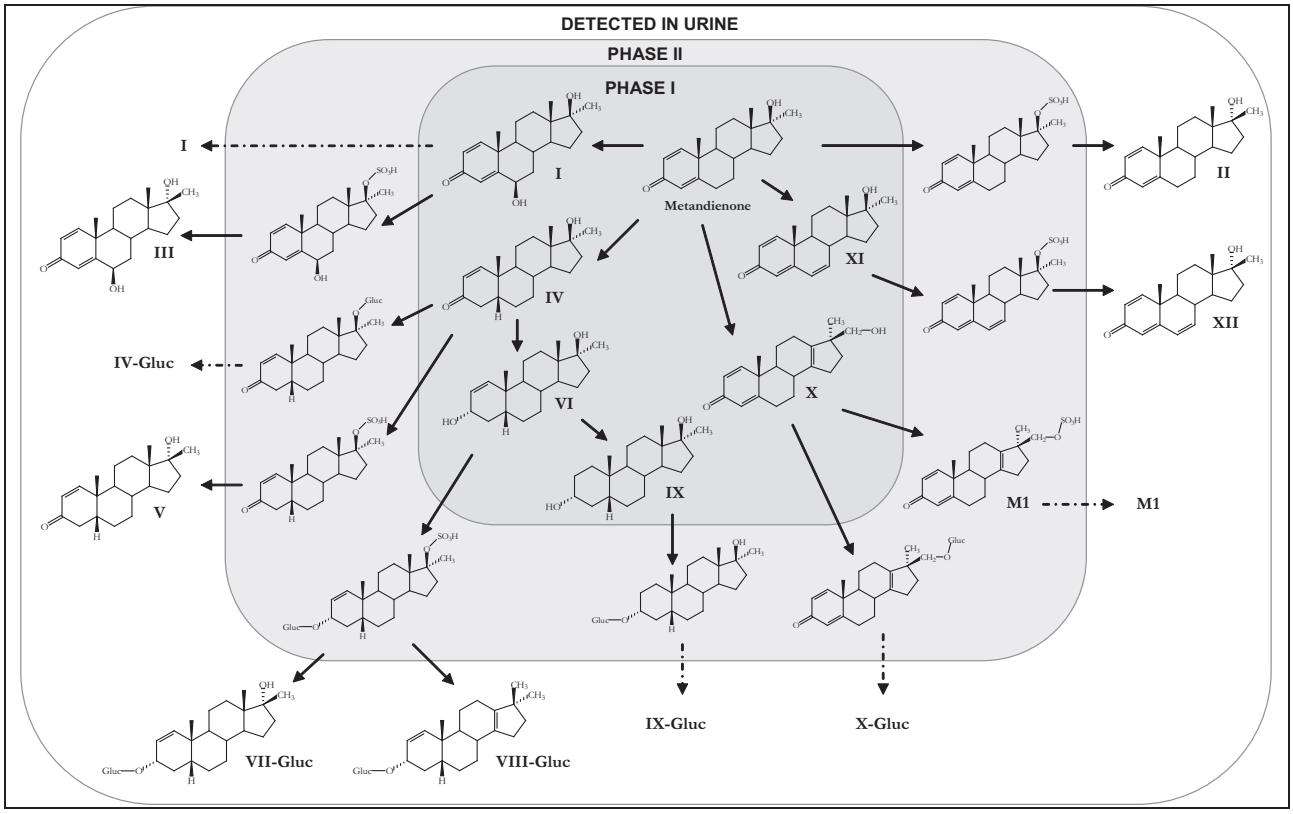

Fig. 1. Schema of metabolism of metandienone. Main metabolic pathways for metandienone based on literature [8,11–25], and location of the metabolite identified in the present work, metabolite M1.

Different phase I metabolites resulting from reduction of the A ring (metabolites IV, VI and IX) have been described [16,18]. Metabolite IV (17β-hydroxy-17α-methyl-5β-androst-1-en-3-one) is excreted in urine as a 17β-glucuronide and as a 17β-sulphate, that is decomposed in urine to the C17 epimer, 17α-hydroxy-17β-methyl-5β-androst-1-en-3-one (V), and 18-nor-17,17-dimethyl-androsta-1,4,13-triene-3-one. Further reduction of the A ring results in the formation of 17α-methyl-5β-androst-1-en-3α,17β-diol (VI) which is conjugated with glucuronic acid at the 3α position and with sulphate at the 17β position. This metabolite is decomposed in urine to form 17-epimetendiol glucuronide (VII-Gluc) and 18-nor-epimetendiol glucuronide (VIII-Gluc). Additional reduction of the A ring results in the formation of 17α-methyl-5β-androstan-3α,17β-diol (IX), which is also a metabolite of methyltestosterone, and it is excreted in urine as a glucuronoconjugate. The 17-epimer of metabolite IX, 17β-methyl-5β-androstan-3α,17α-diol and other compounds resulting from decomposition of a 17β-sulphate (18-nor-17,17-dimethyl-androsta-13-ene-3α-ol) were also detected as glucuronide conjugates in urine and, therefore, were also result of 3α-glucuronoconjugation and 17β-sulphation [13,16,18]. Moreover, dihydroxylated metabolites have been also described as 6β,16β-dihydroxy-metandienone, 6β,16α-dihydroxy-metandienone, 6β,16β-dihydroxy-epimetandienone and 6β,12α-dihydroxy-metandienone [18].

More recently, a long-term metabolite excreted in the glucuronide fraction, 18-nor-17β-hydroxymethyl-17α-methyl-5β-androsta-1,4,13-triene-3-one (X), was described [23–25], which is observed up to 19 days after metandienone administration. Finally, 6-ene-epimetandienone (XII) resulting from 6,7-dehydrogenation and the formation of a 17β-sulphate has been reported [22].

Several of the metabolites reported (Fig. 1) have 17β-methyl-17α-hydroxy or 18-nor-17,17-dimethyl structures. As indicated above, they result from the spontaneous hydrolysis of 17β-sulphates in urine [16–18]. In the case of metandienone, some metabolites having longer clearance rate (VII and XI) arise from the formation of sulphate conjugates. This fact shows the potential usefulness of sulphate conjugate metabolites for the long-term detection of metandienone misuse.

The aim of the present work was to study the sulphate fraction of metandienone metabolism using LC–MS/MS analysis, and to evaluate their potential for the improvement of the detection of metandienone compared with previously described metabolites.

2. Experimental

2.1. Chemicals and reagents

17β-Boldenone sulphate was obtained from NMI (Pymble, Australia). Boldenone was obtained from Sigma (Steinheim, Germany).

Tert-butyl methyl ether (TBME, HPLC grade), ethyl acetate (HPLC grade), acetonitrile and methanol (LC gradient grade), formic acid (LC/MS grade), potassium carbonate, sulphuric acid, sodium hydroxide, di-sodium hydrogen phosphate, sodium hydrogen phosphate, sodium chloride, ammonia hydroxyde, ammonium chloride, ammonium iodide, and 2-mercaptoethanol (all analytical grade) were purchased from Merck (Darmstadt, Germany). *N*,*N*-Dimethylformamide and sulphur trioxide pyridine complex were obtained from Sigma–Aldrich (Steinheim, Germany). *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) was purchased from Macherey–Nagel (Düren, Germany). β-Glucuronidase from *Escherichia coli* K12 was obtained from Roche Diagnostics (Mannheim, Germany). Detectabuse™ XAD-2 extraction columns were purchased from Biochemical Diagnostics, Inc. (Edgewood, NY, USA). Milli Q water was obtained by a Milli-Q purification system (Millipore Ibérica, Barcelona, Spain).