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Analysis of sulfate metabolites of the doping agents oxandrolone and danazol using high performance liquid chromatography coupled to tandem mass spectrometry

S. Rzeppa[a,*], L. Viet[a,b]

[a] Oslo University Hospital, Norwegian Doping Control Laboratory, Oslo, Norway
[b] University of Münster, Institute of Food Chemistry, Münster, Germany

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ABSTRACT

The direct detection of sulfate conjugates of anabolic androgenic steroids (AAS) can be a powerful tool in doping control analysis. By skipping the solvolysis step analysis time can be reduced, and due to long term sulfate metabolites the detection time can be significantly extended as demonstrated for some AAS. This study presents the successful identification of sulfate metabolites of the doping agents oxandrolone and danazol in excretion urines by high performance liquid chromatography coupled to tandem mass spectrometry (HPLC–MS/MS). The sulfate conjugate of 17β-hydroxymethyl-17α-methyl-18-nor-2-oxa-5α-androsta-13-en-3-one could be identified as a new metabolite of oxandrolone. Sulfate conjugates of the danazol metabolites ethisterone and 2α-hydroxymethylethisterone were identified in an excretion urine for the first time. In addition, these sulfate conjugates were synthesized successfully. For a confirmation analysis, the number of analytes can be increased by additional sulfate conjugates of danazol metabolites (2-hydroxymethyl-1,2-dehydroethisterone and 6β-hydroxy-2-hydroxymethylethisterone), which were also identified for the first time. The presented validation data underline the suitability of the identified sulfate conjugates for doping analysis with regard to the criteria given by the technical documents of the World Anti-Doping Agency (WADA).

1. Introduction

Currently, detection of AAS and their metabolites in doping control analysis relies on methods involving enzymatic hydrolysis and subsequent liquid–liquid extraction or solid phase extraction (SPE) [1]. Hence, samples are normally hydrolyzed with β-glucuronidase from *Escherichia coli* methods in doping analysis are limited to the free drugs and the glucuronides which are cleaved by this enzyme. Phase II metabolites like sulfate and cysteine conjugates are not released, and are therefore not covered by the commonly used screening methods in routine doping control analysis.

One way to overcome these limitations is through the direct detection of phase II metabolites using high performance liquid chromatography coupled to tandem mass spectrometry (HPLC–MS/MS) or high-resolution mass spectrometry (HPLC-HRMS) [2,3]. In addition to the broader spectrum of analytes the direct detection of phase II metabolites can offer improvements in sample preparation time and expansion of detection times. Especially, sulfate metabolites of metandienone, methyltestosterone, mesterolone and clostebol attract attention as so called long term metabolites in the last years [4–8]. Even though sulfate conjugates have been identified for some additional AAS used in sports doping [9,10], the knowledge concerning the excretion of sulfate conjugates is limited to only a few AAS.

The metabolism of the two commonly used doping agents oxandrolone [11,12] and danazol [13,14] is well investigated, but limited to the free fraction and the glucuronide fraction. Figs. 1 and 2 give an overview of the metabolites excreted as free analytes and as glucuronide conjugates, and is based on literature data [12,14]. Oxandrolone is excreted as drug itself and as its epimer [11]. The two metabolites 17β-hydroxymethyl-17α-methyl-18-nor-2-oxa-5α-androsta-13-en-3-one (oxandrolone metabolite 4, Fig. 1) and its epimer 17α-hydroxymethyl-17β-methyl-18-nor-2-oxa-5α-androsta-13-en-3-one (oxandrolone metabolite 5, Fig. 1) are known as long term metabolites [12]. 17,17-dimethyl-18-nor-2-oxa-5α-androsta-13-en-3-one (oxandrolone metabolite 3, Fig. 1) is another metabolite observed in urine after intake of oxandrolone. Danazol is also excreted as drug itself, and a broad

* Corresponding author.
E-mail address: Sebastian.Rzeppa@dopinganalyse.no (S. Rzeppa).

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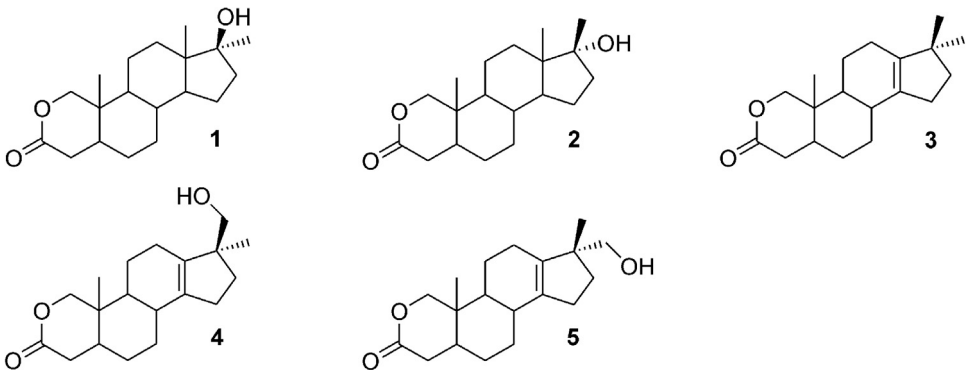

Fig. 1. Chemical structures of oxandrolone and its metabolites: (1) oxandrolone (17β-hydroxy-17α-methyl-2-oxa-5α-androstan-3-one), (2) epioxandrolone (17α-hydroxy-17β-methyl-2-oxa-5α-androstan-3-one), (3) 17,17-dimethyl-18-nor-2-oxa-5α-androsta-13-en-3-one, (4) 17β-hydroxymethyl-17α-methyl-18-nor-2-oxa-5α-androsta-13-en-3-one and (5) 17α-hydroxymethyl-17β-methyl-18-nor-2-oxa-5α-androsta-13-en-3-one [13].

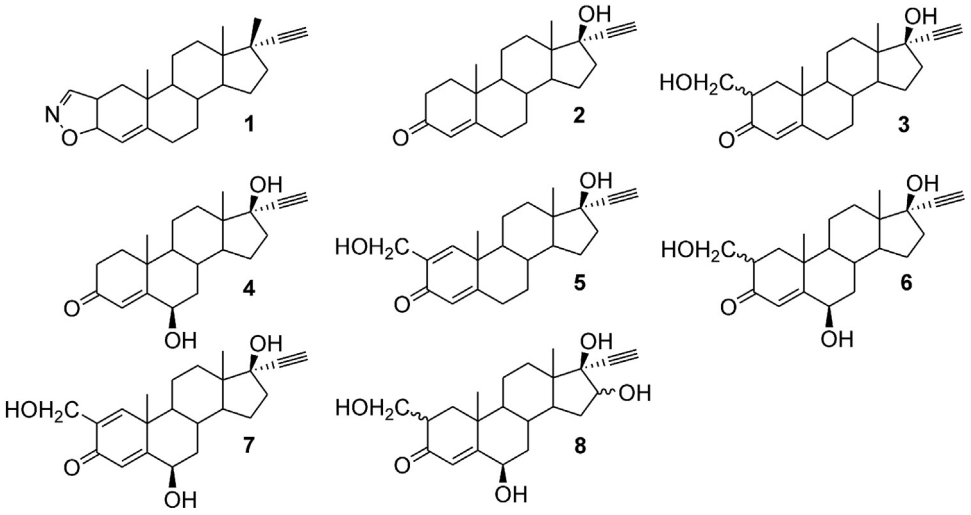

Fig. 2. Chemical structures of danazol and its metabolites: (1) danazol (17α-ethinyl-17β-hydroxyandrost-4-eno[2,3-*d*]isoxazol), (2) ethisterone (17β-hydroxy-4,17α-pregnen-20-yn-3-one), (3) 2-hydroxymethylethisterone, (4) 6β-hydroxyethisterone, (5) 2-hydroxymethyl-1,2-dehydroethisterone, (6) 6β-hydroxy-2-hydroxymethylethisterone, (7) 6β-hydroxy-2-hydroxymethyl-1,2-dehydroethisterone and (8) 6β,16-dihydroxy-2-hydroxymethylethisterone[15].

spectrum of additional metabolites is described in literature [13,14]. Main metabolites used for detection of doping are 2-hydroxymethylethisterone (danazol metabolite 3, Fig. 2), excreted as α- and β-isomer, and ethisterone (danazol metabolite 2, Fig. 2).

The aim of this study was to investigate the metabolism of oxandrolone and danazol with focus on the excretion of sulfate conjugates of the substances itself and/or their metabolites. A former published method based on SPE and detection by HPLC–MS/MS was used for identification of sulfate conjugates in excretion urines [9]. When possible, reference standards of the sulfate conjugates were synthesized in addition. Suitability of the identified sulfate conjugates as target analytes in routine doping control analysis was shown by validation and analysis of routine doping control samples.

2. Experimental

2.1. Chemicals and reagents

Acetontrile and methanol (both in LC–MS grade) were purchased from Sigma (Schnelldorf, Germany). *tert*-Butyl methyl ether (*t*BME), ethyl acetate, ethanthiol, NH_4I, HCl, Tris(hydroxymethyl)aminomethane (TRIS) and sulfuric acid (all in analytical grade) were obtained from Merck (Darmstadt, Germany). Dimethylformamide (DMF), 1,4-dioxane and sulfur trioxide pyridine complex (all in analytical grade) were purchased from Sigma. Water was purified with a Milli-Q Gradient A system (Millipore, Schwalbach, Germany). 2α-Hydroxymethylethisterone, epioxandrolone and 19-norandrosterone-d4 were obtained from National Measurement Institute (Sydney, Australia). Ethisterone and oxandrolone were purchased from Steraloids (Newport, RI, USA). Danazol, 17,17-dimethyl-18-nor-2-oxa-5α-androsta-13-en-3-one and methyltestoserone were obtained from Sigma. β-Glucuronidase was purchased from Roche (Basel, Switzerland). *N*-Methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) was supplied by Macherey-Nagel (Düren, Germany). Solid phase extraction cartridges (OASIS WAX, 3 mL, 60 mg resin) were obtained from Waters (Milford, CT, USA).

2.2. Synthesis of reference substances

Sulfate conjugates of ethisterone and 2α-hydroxymethylethisterone were synthesized at room temperature according to the method of Waller et al. [15]. In brief, the steroid metabolite was dissolved in 1,4-dioxane (1 mg/mL). 100 μL was added to a solution of the sulfur trioxide pyridine complex (10 mg) in DMF (100 μL). After 4–24 h of stirring at room temperature, 4 mL water was added. Oasis WAX cartridges were used for purification. Cartridges were preconditionated with 2 mL methanol followed by 2 mL water. Afterwards, the reaction mixture was loaded onto the column. The washing step consisted of 8 mL hydrochloric acid in