Journal of Steroid Biochemistry & Molecular Biology 152 (2015) 142–154

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry & Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Metabolic study of androsta-1,4,6-triene-3,17-dione in horses using liquid chromatography/high resolution mass spectrometry

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ARTICLE INFO

Article history:
Received 19 March 2015
Received in revised form 21 May 2015
Accepted 27 May 2015
Available online 29 May 2015

Keywords:
Steroidal aromatase inhibitor
In vitro and *in vivo* metabolism
Doping control analysis
Liquid chromatography/high resolution mass spectrometry
Equine urine and blood

ABSTRACT

Androsta-1,4,6-triene-3,17-dione (ATD) is an irreversible steroidal aromatase inhibitor and is marketed as a supplement. It has been reported to effectively reduce estrogen biosynthesis and significantly increase the levels of endogenous steroids such as dihydrotestosterone and testosterone in human. ATD abuses have been reported in human sports. Its metabolism in human has been studied, and the *in vitro* metabolic study of ATD in horses has been reported, however, little is known about its biotransformation and elimination in horses. This paper describes the *in vitro* and *in vivo* metabolism studies of ATD in horses, with an objective of identifying the target metabolites with the longest detection time for controlling ATD abuse.

In vitro metabolism studies of ATD were performed using homogenized horse liver. ATD was found to be extensively metabolized, and its metabolites could not be easily characterized by gas chromatography/mass spectrometry (GC/MS) due to insufficient sensitivity. Liquid chromatography/high resolution mass spectrometry (LC/HRMS) was therefore employed for the identification of *in vitro* metabolites. The major biotransformations observed were combinations of reduction of the olefin groups and/or the keto group at either C3 or C17 position. In addition, mono-hydroxylation in the D-ring was observed along with reduction of the olefin groups and/or the keto group at C17 position. Fourteen *in vitro* metabolites, including two epimers of androsta-1,4,6-trien-17-ol-3-one (M1a, M1b), androsta-4,6-diene-3,17-dione (M2), boldione (M3), androsta-4,6-diene-17β-ol-3-one (M4), androsta-4,6-diene-3-ol-17-one (M5), boldenone and epi-boldenone (M6a, M6b), four stereoisomers of hydroxylated androsta-1,4,6-trien-17-ol-3-one (M7a to M7d), and two epimers of androsta-1,4-diene-16α,17-diol (M8a, M8b), were identified. The identities of all metabolites, except M1a, M5, M7a to M7d, were confirmed by matching with authentic reference standards using LC/HRMS.

For the *in vivo* metabolism studies, two thoroughbred geldings were each administered with 800 mg of ATD by stomach tubing. ATD, and twelve out of the fourteen *in vitro* metabolites, including M1a, M1b, M2, M4, M5, M6, M7a to M7d, M8a and M8b, were detected in post-administration urine. Two additional urinary metabolites, namely stereoisomers of hydroxylated androsta-4,6-dien-17-ol-3-one (M9a, M9b), were tentatively identified by mass spectral interpretation. Elevated level of testosterone was also observed. In post-administration blood samples, only the parent drug, M1b and M2 were identified. This study showed that the detection of ATD administration would be best achieved by either monitoring the metabolites M1b (androsta-1,4,6-trien-17β-ol-3-one) or M4 (both excreted as sulfate conjugates) in urine, which could be detected for up to a maximum of 77 h post-administration. The analyte of choice for plasma is M1b, which could be detected for up to 28 h post administration.

1. Introduction

Aromatase inhibitors are used in the treatment of breast and ovarian cancers in postmenopausal women by inhibiting the aromatase enzyme resulting in a significant reduction of estrogens [1–2]. Besides the above-mentioned clinical applications, aromatase inhibitors can also be used for building muscles by stopping the conversion of testosterone into estradiol, resulting in the increase of the testosterone level in the body [3–4]. Furthermore, aromatase inhibitors can minimise the side effects of anabolic steroids misuse [5]. Steroidal aromatase inhibitors, such as androst-4-ene-3,6,17-trione (6-OXO) [6], formestane [7] and androsta-1,4,6-triene-3,17-dione (ATD) [8], are marketed as nutritional supplements and are readily available *via* the internet.

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http://dx.doi.org/10.1016/j.jsbmb.2015.05.011
0960-0760/

They are banned by the World Anti-Doping Agency (WADA) since 2005 [9]. In 2006, three routine human sports urine samples were reported positive for a metabolite of ATD [10]. Up to 2013, a total of 29 analytical findings attributed to ATD administration have been reported by WADA laboratories. Aromatase inhibitors are also prohibited substances in equine sports. In May 2014, aromatase inhibitors have been listed as one of the classes of hormone and metabolic modulators (*Article 6E of the International Agreement of Breeding, Racing, and Wagering*) which are prohibited to be administered to racehorses at any time in their career [11]. The metabolism of ATD has been reported in human [10,12], and the *in vitro* metabolic study of ATD in horses has been reported [13], but little is known about its biotransformation and elimination in horses. This paper describes the investigation of the *in vitro* and *in vivo* metabolism of ATD in horses with an objective to identify the most appropriate targets for detecting ATD administration.

2. Materials and methods

2.1. Materials

ATD, androsta-1,4,6-trien-17β-ol-3-one, androsta-4,6-diene-3,7-dione, androsta-4,6-dien-17β-ol-3-one, boldenone, boldenone sulfate, boldione and testosterone glucuronide were obtained from Steraloids (New Port, RI, USA). A bottle of ATD capsules (Chaparral Labs, 25 mg ATD per capsule) was purchased *via* the web site: www.chaparrallabs.net, the listed amount of ATD in the capsule had been verified by comparing with the reference standard from Steraloids. 5β-Androstan-3β-ol-17-one, androsta-1,4-diene-16α,17α-diol-3-one and androsta-1,4-diene-16α,17β-diol-3-one were obtained from Alltech (Deerfield, IL, USA). Acetic anhydride, acetonitrile (Chromasolv grade), adenosine 3′-phosphate 5′-phosphosulfate (PAPS) lithium salt hydrate, uridine 5′-diphosphoglucuronic acid (UDPGA) trisodium salt, ethyl acetate, testosterone, d_3-testosterone, β-nicotine adenine dinucleotide (β-NAD), glucose-6-phosphate, glucose-6-phosphate dehydrogenase, protease (from aspergillus oryzae, ~500 units/mL), pyridine, and sodium hydroxide (pellets, analytical grade) were obtained from Sigma (St. Louis, MO, USA). Testosterone sulfate (triethylamine salt) and epi-boldenone were obtained from National Measurement Institute (Sydney, Australia). Benzyldimethylphenylammonium chloride was obtained from ACROS (Fair Lawn, NJ, USA). β-Glucuronidase (*Escherichia Coli*) was purchased from Roche (Indianapolis, IN, USA). Anhydrous methanolic hydrogen chloride used for methanolysis was prepared according to the procedures reported previously [14]. Acetic acid (100%, Suprapur), ammonia solution (25%, Emsure®), LiChrosolv grade of *t*-butyl methyl ether and methanol, potassium carbonate (Emsure®), potassium dihydrogen phosphate, sulfuric acid (96%, Suprapur), and GR grade of chloroform, citric acid, diisopropyl ether, *n*-hexane, potassium carbonate, sodium dihydrogen phosphate monohydrate and sodium chloride were obtained from Merck (Darmstadt, Germany). Magnesium chloride ($MgCl_2$) hexahydrate were obtained from the International Laboratory Limited (San Bruno, CA, USA). Sodium sulfite (GR grade) was obtained from Peking Chemical Works (Beijing, China). Ultrafree®-MC centrifugal filter (PVDF) with 0.1 μm membrane pore size was purchased from Millipore (Billerica, MA, USA). ABS Elut Nexus solid phase extraction (SPE) cartridges (60 mg, 3 mL) were purchased from Agilent Technologies (Santa Clara, CA, USA). Deionized water was generated from an in-house water purification system (Milli-Q, Molsheim, France). Homogenized horse liver was prepared from fresh horse liver supplied by the Equine Hospital of The Hong Kong Jockey Club. The liver was cut into 1 cm thick slices and stored immediately at −80 °C. When required, the frozen horse liver was further cut into 1 cm cube pieces and homogenized at 4 °C in 2 mL of the incubation reagent.

2.2. Incubation with homogenized liver

2.2.1. In vitro *study of ATD phase 1 metabolites*

The procedures for the *in vitro* metabolic study of ATD basically followed that reported previously by the authors' laboratory [15]. Homogenized liver (one piece of 1 cm cube), and 2 mL of reagent containing β-NAD (1.5 mM), glucose-6-phosphate (7.5 mM), $MgCl_2$ (5 mM), glucose-6-phosphate dehydrogenase (1 U/mL) in sodium dihydrogen phosphate solution (2 mL; 50 mM; pH 7.4) were incubated with ATD (100 μg) at 37 °C overnight with shaking. The reaction was terminated by adding acetonitrile (2 mL) to the mixture. The mixture was centrifuged at 2100 × *g* for 10 min, and the supernatant was extracted twice with ethyl acetate (5 mL). The extract was evaporated to dryness and reconstituted with methanol (1 mL), vortex, and analyzed directly by LC/HRMS. A portion of the extract (50 μL) was evaporated and derivatized by acetylation and analyzed by LC/HRMS. Control experiments in the absence of either (a) ATD or (b) homogenized liver were performed in parallel.

2.2.2. Generation of reference standards for ATD Phase II metabolites [16]

Homogenized liver (one piece of 1 cm cube), and 2 mL of reagent containing UDPGA (5 mM, for glucuronide conjugation) and/or PAPS (0.1 mM, for sulfate conjugation), and $MgCl_2$ (5 mM) in sodium dihydrogen phosphate solution (2 mL; 50 mM; pH 7.4) were incubated with 100 μg each of androsta-1,4,6-trien-17β-ol-3-one, androsta-4,6-dien-17β-ol-3-one, androsta-1,4-diene-16α,17α-diol-3-one or androsta-1,4-diene-16α,17β-diol-3-one at 37 °C overnight with shaking. The reaction was terminated by adding acetonitrile (2 mL) to the mixture. The mixture was centrifuged at 30,000 × *g* for 10 min., and the supernatant was analyzed directly by LC/HRMS.

2.3. Acetylation for LC/HRMS analysis

Acetylated derivatives were prepared by adding 50 μL acetic anhydride and 100 μL pyridine to the dry residue. The mixture was incubated at 60 °C for 30 min, evaporated to dryness and reconstituted with 40 μL acetonitrile. The resulting extract was analyzed by LC/HRMS.

2.4. Instrumentation

UHPLC–MS analyses were performed on a Thermo Scientific Q Exactive mass spectrometer (Bremen, Germany) with a heated electrospray ionisation (HESI-II) source interfaced with a Waters Acquity UHPLC system (Waters Corporation, Milford, MA, USA). The sample tray of the autosampler was kept at 15 °C. Solid phase extraction (SPE) was carried out using a RapidTrace® SPE workstation (Zymark Corporation, Hopkinton, MA, USA).

2.4.1. UHPLC conditions

A reversed-phase Acquity UHPLC® BEH C18 column (Waters Corporation; 10 cm L × 2.1 mm ID; 1.7 μm particle size) was used for the separation of the target analytes. The mobile phase for the analysis of ATD and its phase I and II metabolites were 0.1% acetic acid in deionized water as solvent A and methanol as solvent B. For the analyses of acetylated ATD metabolites, the mobile phase was composed of 0.1 % acetic acid in deionized water as solvent A and acetonitrile as solvent B. A linear gradient was run at a flow rate of 300 μL/min, with 98 % solvent A at initial condition (t = 0 min), held for 0.5 min and then decreasing to 50% solvent A from t = 0.5 min to