Journal of Pharmaceutical and Biomedical Analysis 115 (2015) 236–244

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

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

Determination of mepitiostane metabolites in human urine by liquid chromatography/tandem mass spectrometry for sports drug testing

Masato Okano*, Mitsuhiko Sato, Asami Kojima, Shinji Kageyama

Anti-Doping Laboratory, LSI Medience Corporation, Tokyo, Japan

ARTICLE INFO

Article history:
Received 24 April 2015
Received in revised form 16 July 2015
Accepted 17 July 2015
Available online 23 July 2015

Keywords:
Doping
Mepitiostane
Epitiostanol sulfoxide
Epitiostanol
Mass spectrometry
Sports

ABSTRACT

Mepitiostane (2α,3α-epithio-17β-(1-methoxycyclopentyloxy)-5α-androstane), which is a prodrug of epitiostanol (2α,3α-epitio-5α-androstane-17β-ol), is an epitiosteroid having anti-estrogenic and weak androgenic anabolic activities. The World Anti-Doping Agency prohibits the misuse of mepitiostane by athletes. Detection of the urinary metabolites epitiostanol sulfoxide and epitiostanol was studied using liquid chromatography/mass spectrometry (LC–MS) for doping control purposes. The use of LC–MS provided advantages over gas chromatography/mass spectrometry for detecting heat labile steroids because epitiostanol and epitiostanol sulfoxide were primarily pyrolized to 5α-androst-2-en-17β-ol. The method consists of enzymatic hydrolysis using β-glucuronidase (*Escherichia coli*), liquid–liquid extraction, and subsequent ultra-performance liquid chromatography/electrospray-tandem mass spectrometry. Epitiostanol sulfoxide was determined at urinary concentrations of 0.5–50 ng/mL, recovery was 76.2–96.9%, and assay precision was calculated as 0.9–1.7% (intra-day) and 2.0–6.6% (inter-day). Epitiostanol was determined at urinary concentrations of 0.5–50 ng/mL, recovery was 26.1–35.6% and assay precision was calculated as 4.1–4.6% (intra-day) and 3.3–8.5% (inter-day). The limits of detection for epitiostanol sulfoxide and epitiostanol were 0.05 ng/mL and 0.10 ng/mL, respectively. Epitiostanol sulfoxide and epitiostanol, as their gluco-conjugates, were identified in human urine after oral administration of 10 mg mepitiostane. Epitiostanol sulfoxide and epitiostanol could be detected up to 48 h and 24 h after administration, respectively. The results showed that the detection window of epitiostanol is much shorter than that of epitiostanol sulfoxide. The LC–MS detection of urinary epitiostanol sulfoxide, a specific metabolite with a sulphur atom in its molecular structure, is likely to be able to identify the abuse of mepitiostane.

1. Introduction

Mepitiostane (2α,3α-epithio-17β-(1-methoxycyclopentyloxy)-5α-androstane; Fig. 1a), which is a prodrug of epitiostanol (2α,3α-epithio-5α-androstane-17β-ol; Fig. 1b), is an epithiosteroid having anti-estrogenic, weak androgenic and myotropic activities [1,2]. Epitiostanol has been used intramuscularly due to its strong first-pass metabolism in both intestinal mucosa and the liver following low biological activity when administrated orally [3]. In order to avoid this disadvantage, mepitiostane, in which a methoxycyclopentane moiety has been introduced as a substituent at position 17, was developed as an oral preparation [3,4]. Mepitostane has been commercially produced in the Japanese market, and used in the treatment of anaemia of chronic renal failure and mammary cancer.

The abuse of anabolic androgenic steroids to build muscle and enhance physical performance has been for decades a serious problem in sports, despite the fact that it has the potential to cause a number of unfavourable side effects in athletes. Steroid abusers to counter side effects such as gynecomastia mainly use anti-estrogens. Therefore, the World Anti-Doping Agency (WADA) [5] prohibits the misuse of anabolic androgenic steroids and anti-estrogens by athletes at all times, both in- and out-of-competition. Mepitiostane and epitiostanol are not expressly listed as prohibited substances on the 2015 WADA prohibited list; however, they are related to anabolic agents defined in section S1, and hormone and metabolic modulators defined in section S4. Although pharmaceutical production of injectable epitiostanol (Thiodol®) has been discontinued, mepitiostane is currently available under the trade name Thioderon® in the Japanese market. Several dietary supplements containing anabolic steroids are available on the Internet [6]. In particular, 17α-methylepithiostanol, which is a 17-methylated

* Corresponding author at: Anti-Doping Laboratory, LSI Medience Corporation, 3-30-1 Shimura, Itabashi-ku Tokyo, 174-8555, Japan. Fax: +81 3 5994 2990.
E-mail address: okano.masato@mk.medience.co.jp (M. Okano).

http://dx.doi.org/10.1016/j.jpba.2015.07.016
0731-7085/

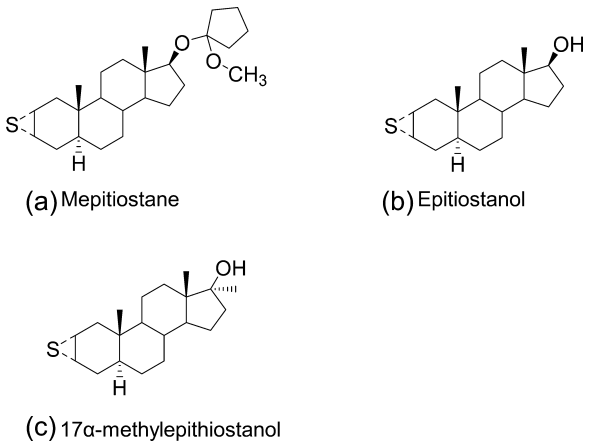

Fig. 1. Chemical structures of (a) mepitiostane, (b) epitiostanol, and (c) 17α-methylepithiostanol.

analogue of epitiostanol (Fig. 1c), has been identified in such supplements [7]. Therefore, it is of deep concern in the abuse of epitiosteroids by athletes.

Steroids are generally metabolised in the liver and kidney and then excreted in the urine, either in the free form or as conjugates of the metabolite [8]. Therefore, selecting appropriate target substances in biological matrices is important for detecting the use of steroids, and urine analysis of steroids commonly requires a detailed knowledge of the metabolic pathways. It was reported that the metabolism of epitiostanol in rat liver consists of 7α-hydroxylation, oxygenation to epitiostanol sulfoxide and dethionylation to 5α-androst-2-en-17β-ol and 5α-androst-2-en-17-one (Fig. 2) [3]. Afterwards, in in vitro study using human hepatocytes, epitiostanol sulfoxide and 5α-androst-2-en-17β-ol (as their gluco-conjugates) were identified as metabolites of epitiostanol [9].

Since the earliest doping control, analytical methods for anabolic steroids have relied on gas chromatography/mass spectrometry (GC–MS) [10]. In our preliminary study, it was suggested that the common steroid screening procedure by GC–MS is suitable for the detection of mepitiostane abuse, and that the target substance should be 5α-androst-2-en-17β-ol in urine [11]. For sports drug testing, the errorless discrimination of exogenous steroid from endogenous steroid is important. It is recognized that 5α-androst-2-en-17β-ol and 5α-androst-2-en-17-one could be identified in the urine of the Asian elephant [12]. To the best of our knowledge, the endogenous existence of two steroids in human urine is unclear; however, 5α-androst-2-en-17β-ol is an isomer of the endogenous steroid 5α-androst-16-en-3β-ol in humans. The metabolism of desoxymethyltestosterone (17α-methyl-5α-androst-2-en-17β-ol) in humans consists of the reduction of the double bond and hydroxylation, and 20 metabolites have been reported [13]. It is therefore suggested that several hydroxyl-metabolites from 5α-androst-2-en-17β-ol may exist in mepitiostane-administered urine [11]. Nevertheless, the identification of 5α-androst-2-en-17β-ol and the expected hydroxy-metabolites may not be specific evidence of mepitiostane use due to the loss of a sulphur atom from epitiostanol. For doping analysis to detect heat labile steroids such as tetrahydrogestrinone, the use of liquid chromatography/mass spectrometry (LC–MS) would provide several advantages over GC–MS [10]. Several 3-ketosteroids and their metabolites, which possess sufficient proton affinity to allow for efficient ionisation using electrospray ionisation (ESI), have been used in analytical assays employing LC–MS approaches [10,14]. However, it is reported that 5α-androst-2-en-17β-ol, 17α-methylepithiostanol, and desoxymethyltestosterone possess insufficient proton affinity for ESI [7,11].

The aim of this study was to establish a reliable LC–MS method for detecting mepitiostane abuse in sports drug testing. This paper describes the identification, by means of LC–tandem mass spectrometry (LC–MS/MS), of epitiostanol sulfoxide and epitiostanol,

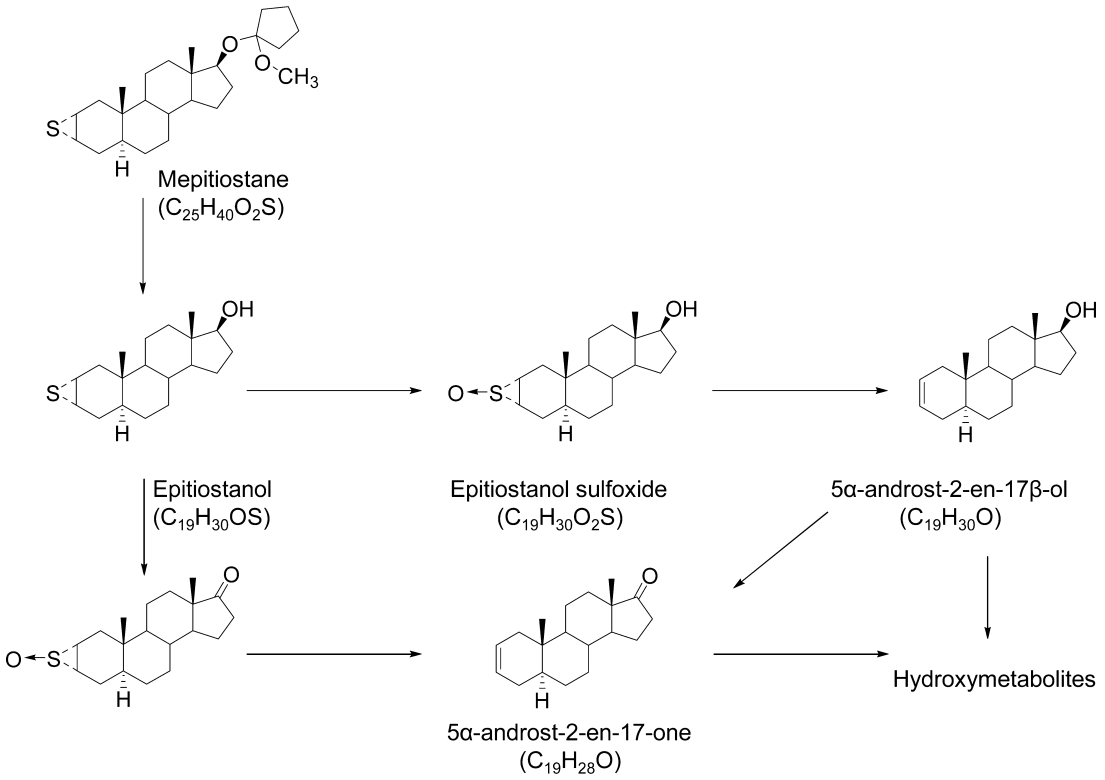

Fig. 2. Proposed metabolic pathway of mepitiostane [3,9,11,13].