Journal of Steroid Biochemistry & Molecular Biology 138 (2013) 222–235

Contents lists available at SciVerse ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

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

Profiling of steroid metabolites after transdermal and oral administration of testosterone by ultra-high pressure liquid chromatography coupled to quadrupole time-of-flight mass spectrometry

F. Badoud[a], J. Boccard[b], C. Schweizer[a], F. Pralong[c], M. Saugy[a], N. Baume[a,*]

[a] Swiss Laboratory for Doping Analyses, University Center of Legal Medicine, Geneva and Lausanne, Chemin des Croisettes 22, 1066 Epalinges, Switzerland
[b] AgroParisTech, UMR 1145, 75231 Paris, France
[c] Service of Endocrinology, Diabetology and Metabolism, University Hospital and Faculty of Biology and Medicine, 1011 Lausanne, Switzerland

ARTICLE INFO

Article history:
Received 17 December 2012
Received in revised form 16 May 2013
Accepted 18 May 2013

Keywords:
Testosterone
Anabolic androgenic steroids
Phase II metabolism
Ultra-high-pressure liquid chromatography
Quadrupole time-of-flight
Quantification

ABSTRACT

The screening of testosterone (T) misuse for doping control is based on the urinary steroid profile, including T, its precursors and metabolites. Modifications of individual levels and ratio between those metabolites are indicators of T misuse. In the context of screening analysis, the most discriminant criterion known to date is based on the T glucuronide (TG) to epitestosterone glucuronide (EG) ratio (TG/EG). Following the World Anti-Doping Agency (WADA) recommendations, there is suspicion of T misuse when the ratio reaches 4 or beyond. While this marker remains very sensitive and specific, it suffers from large inter-individual variability, with important influence of enzyme polymorphisms. Moreover, use of low dose or topical administration forms makes the screening of endogenous steroids difficult while the detection window no longer suits the doping habit. As reference limits are estimated on the basis of population studies, which encompass inter-individual and inter-ethnic variability, new strategies including individual threshold monitoring and alternative biomarkers were proposed to detect T misuse.

The purpose of this study was to evaluate the potential of ultra-high pressure liquid chromatography (UHPLC) coupled with a new generation high resolution quadrupole time-of-flight mass spectrometer (QTOF-MS) to investigate the steroid metabolism after transdermal and oral T administration. An approach was developed to quantify 12 targeted urinary steroids as direct glucuro- and sulfo-conjugated metabolites, allowing the conservation of the phase II metabolism information, reflecting genetic and environmental influences. The UHPLC–QTOF-MS^E platform was applied to clinical study samples from 19 healthy male volunteers, having different genotypes for the UGT2B17 enzyme responsible for the glucuroconjugation of T. Based on reference population ranges, none of the traditional markers of T misuse could detect doping after topical administration of T, while the detection window was short after oral TU ingestion. The detection ability of the 12 targeted steroids was thus evaluated by using individual thresholds following both transdermal and oral administration. Other relevant biomarkers and minor metabolites were studied for complementary information to the steroid profile, including sulfoconjugated analytes and hydroxy forms of glucuroconjugated metabolites. While sulfoconjugated steroids may provide helpful screening information for individuals with homozygotous UGT2B17 deletion, hydroxy-glucuroconjugated analytes could enhance the detection window of oral T undecanoate (TU) doping.

1. Introduction

The screening of endogenous anabolic androgenic steroids (AAS) for doping control purpose is routinely implemented by assessing the steroid profile in urine. Monitoring ratios between precursors or biosynthetic metabolites of testosterone (T) helps to distinguish an exogenous intake from physiological levels. The most sensitive biomarker is the T glucuronide (TG) to epitestosterone glucuronide (EG) TG/EG ratio, which cut-off is set at 4 for suspicion of T or precursor administration [1]. Despite the high specificity of this parameter, the sensitivity of this test suffers from the short detection window and the high inter-individual variability due to natural elevated TG/EG ratios or genetic polymorphisms [2–5]. The majority of steroids are biotransformed into phase I and II metabolites

* Corresponding author. Tel.: +41 21 314 73 30; fax: +41 21 314 70 95.
E-mail address: Norbert.Baume@chuv.ch (N. Baume).

0960-0760/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.jsbmb.2013.05.018

Table 1
Study design.

Phases	Weeks	Day	Administration	Urine and blood samples collection
Control	1	Mo	–	C-t00 = 0 h; C-t01 = 2 h; C-t02 = 4 h; C-t03 = 8 h; C-t04 = 12 h
		Tu	–	C-t05 = 24 h
Patch	2	Mo	2 transdermal patches of 2.4 mg/24 h	P-t00 = 0 h; P-t01 = 2 h; P-t02 = 4 h; P-t03 = 8 h; P-t04 = 12 h
		Tu		P-t05 = 24 h
		We	2 transdermal patches of 2.4 mg/24 h	P-t06 = 48 h; P-t07 = 60 h {All spot urine samples produced during 48 h
		Th		P-t08 = 72 h
		Fr		P-t09 = 96 h
Wash out	3	Mo		t10 = 118 h
	4	Mo		t11 = 336 h
Oral	5	Mo	2 oral pills of 40 mg	O-t00 = 0 h; O-t01 = 2 h; O-t02 = 4 h; O-t03 = 8 h; O-t04 = 12 h
		Tu		O-t05 = 24 h
		We	2 oral pills of 40 mg	O-t06 = 48 h; O-t07 = 60 h {All spot urine samples produced during 48 h
		Th		O-t08 = 72 h
		Fr		O-t09 = 96 h

(glucuro- and sulfo-conjugates) to facilitate their excretion in urine. Among the UDP-glucuronyl transferases (UGT), the UGT2B17 is the most important isoform involved in T glucuronidation and is highly subjected to polymorphism. It was indeed reported that 40% of the individuals having a del/del genotype for the *UGT2B17* gene coding for the UGT2B17 enzyme, a polymorphism representing two third of the population of Asian ethnic origin, did not show any TG/EG elevation after T administration [6]. Moreover, today's doping habits consist in low-doses and topical route of administration, according to testimony of top-level athletes, to avoid peak concentration excretion [7]. Then, the current TG/EG ratio threshold and other steroid profile parameters might not be sufficiently sensitive to detect low doses and transdermal T route of administration [8,9]. To avoid this limitation, subject-based monitoring approach was proposed by setting individual basal threshold levels instead of population reference limit as suitable tool for steroid profiling, as proposed for the future Athlete Steroidal Passport (ASP) [10–12].

With the perspective of using multiparametric indicators to reinforce the steroid profile detection ability, the research of novel biomarkers or minor metabolites is expanding to improve current detection windows of AAS doping [5,13–15]. To date, gas chromatography (GC) coupled to mass spectrometry (MS) or tandem MS (MS/MS) remains the gold standard for steroid analysis, but necessitates hydrolysis and derivatization prior to the analysis, leading to loss of phase II metabolism information. Besides, several liquid chromatography (LC) linked to MS(/MS) developments led to the direct detection of glucuro- and sulfo-conjugated metabolites as well as alternative markers released after alkaline treatment [5,16–21].

In this study, a new generation quadrupole time-of-flight mass spectrometer coupled to ultra-high pressure liquid chromatography (UHPLC–QTOF-MS) was evaluated for its ability to assess the steroid metabolism after T administration. The procedure was adapted from a method previously reported as a relevant strategy for the quantification of AAS in urine as direct glucuro- and sulfo-conjugated metabolites [17] and applied to real-case samples issued from a clinical trial. The former method was developed with a previous generation QTOF mass analyzer allowing good mass accuracy (2–5 ppm) and resolution (12,000 FWHM), while sensitivity and dynamic range of the instrument could be further improved. Meanwhile, a new generation QTOF instrument was commercially available, promising excellent performance, particularly in terms of resolution, sensitivity, speed and dynamic range for quantitative analysis. The performance of the new generation instrument was compared with the previous approach [17], to evaluate its benefits to investigate the steroid metabolism. For that purpose, urine samples from a clinical trial protocol, collected after T transdermal and T undecanoate (TU) administration to 19 healthy male volunteers, having different UGT2B17 genotypes (del/del, ins/del and ins/ins), were analyzed. The variation of twelve targeted steroid metabolites quantified in this study in more than 500 urine samples was evaluated with respect to both route of T administration based on individual threshold levels.

2. Experimental

2.1. Study design

The cohort recruited for the clinical trial included 19 healthy young men, aged 19–28 (mean 24.3 ± 2.7 years) with a body mass index (BMI) comprised between 18.3 and 27.2 (mean 23.1 ± 2.4 kg/m^2). 4 volunteers were rejected to join the cohort, as they did not fulfill the inclusion criteria, while 2 subjects dropped out for personal reason. A complete medical history and physical exam was performed at inclusion in the study. Exclusion criteria were: regular drug, alcohol or tobacco use, anabolic or ergogenic substance intake, dyslipidemia, hypercholesterolemia, androgen-dependent tumor, hyperprolactinemia, endocrine or metabolic disturbance, use of thyroid hormone or anti-thyroid agent, cardiac, renal or hepatic deficiency. In addition, the volunteers did not practice sport at a competitive level, did not take any medication and refrained from ingesting alcohol during the study. All subjects gave signed consent form and the protocol was authorized by the Ethical Commission for the Clinical Research of the Faculty of Biology and Medicine (University of Lausanne, Lausanne, Switzerland) and Swissmedic (Protocol n°155/11). This study was conducted at the Clinical Research Center of the CHUV/UNIL. The participants received T as presented in Table 1. Control samples (C) were gathered during the first week, mainly on Monday. Kinetics urine and blood spots were collected at time C-t00 = 0 h, C-t01 = 2 h, C-t02 = 4 h, C-t03 = 8 h, C-t04 = 12 h and C-t05 = 24 h. During the second week, they received twice 2 T transdermal systems of 2.4 mg/24 h (Testopatch®, Pierre Fabre Pharma GMBH, Freiburg, Germany) on Monday and Wednesday. The patch system (P) was stuck on the shoulder and removed after 48 h. During that time urine and blood were taken at times P-t00 = 0 h, P-t01 = 2 h, P-t02 = 4 h, P-t03 = 8 h, P-t04 = 12 h, P-t05 = 24 h and P-t06 = 48 h. Time points P-t07 = 60 h, P-t08 = 72 h and P-t09 = 96 h were collected after the application of the second patch. Apart from the mentioned collection times, all the urines produced were gathered during 48 h after the application of the first patch. During the two following weeks, the first morning urine as well as a blood sample was collected on Mondays. Following a wash-out period of 2 weeks, 2 TU tablets of 40 mg (Andriol Testocaps®, Essex Chemie AG, Luzern, Switzerland) were taken orally by the volunteers on Monday and Wednesday. During the fifth week, urine and blood specimens were