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Detection and quantification of glucuro- and sulfoconjugated metabolites in human urine following oral administration of xenobiotic 19-norsteroids

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ABSTRACT

Recently, the endogenous origin of nandrolone (19-nortestosterone) and other 19-norsteroids has been a focus of research in the field of drug testing in sport. In the present study, we investigated metabolites conjugated to a glucuronic acid and to a sulfuric acid in urine following administration of four xenobiotic 19-norsteroids. Adult male volunteers administered a single oral dose (10 mg) of each of four 19-norsteroids. Urinary samples collected from 0 to 120 h were subjected to methanolysis and β-glucuronidase hydrolysis and were derivatized by *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) before gas chromatography–mass spectrometry analysis. We confirmed that 19-norandrosterone (19-NA) and 19-noretiocholanolone (19-NE) were present in both glucuronide (g) and sulfate (s) conjugates and 19-norepiandrosterone (19-NEA) was excreted exclusively as a sulfate fraction in urine of all 19-norsteroids tested. The overall levels of the three metabolites can be ranked as follows: 19-NA_{g+s} > 19-NE_{g+s} > 19-NEA_s. The concentration profiles of these three metabolites in urine peaked between 2 to 12 h post-administration and declined thereafter until approximately 72–96 h. 19-NA was most prominent throughout the first 24 h post-administration, except for a case in which an inverse relationship was found after 6 h post-administration of nandrolone. Furthermore, we found that sulfate conjugates were present in both 19-NA and 19-NE metabolites in urine of all 19-norsteroids tested. The averaged total amounts of metabolites (i.e. 19-NA_{s+g} + 19-NE_{s+g} + 19-NEA_s) excreted in urine were 38.6, 42.9, 48.3 and 21.6% for nandrolone, 19-nor-4-androsten-3,17-dione, 19-nor-4-androsten-3β,17β-diol and 19-nor-5-androstene-3β,17β-diol, respectively. Results from the excretion studies demonstrate significance of sulfate-conjugated metabolites on interpretation of misuse of the 19-norsteroids.

1. Introduction

Nandrolone has been the most frequently abused synthetic anabolic steroid for performance-enhancing purposes in sports since the late 1950s [1]. Its highly prevalent use among athletes can be reflected in adverse analytical findings from drug testing in sports during the past decade. For instance, internationally, 212 cases were found in 1995 and 232 cases in 1996 [2]. In our laboratory, between 1996 and 2004, nandrolone was ranked the highest (57%) among the violations of anabolic steroids (unpublished data). Despite several reports on the side effects of norsteroids [3,4], athletes believe that nandrolone

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may increase body weight and lean body mass to a greater extent than training alone and help to decrease recovery time between training sessions [5].

Recent studies have shown that nandrolone may be derived endogenously. A trace amount of endogenous production has been found in pregnant [6,7] and non-pregnant women [8,9], in males [10] and after exercise [11–13]. Nandrolone metabolites were found linked to oral consumption of other norsteroids and dietary supplements [14–18]. This also includes a minor metabolite of norethisterone that is used as a contraceptive in women and for the treatment of menstrual dysfunction [17]. In situ demethylation of endogenous steroids in stored urine was found to lead to the formation of 19-norsteroids, i.e. 19-norandrosterone (19-NA) and 19-noretiocholanolone (19-NE) [19].

Nandrolone and other 19-norsteroids given orally are metabolized to form 19-NA, 19-NE and 19-NEA. The first two are predominantly excreted in urine as glucuronide derivatives, while the last one is found exclusively as its sulfoconjugate (3β-sulfate) [20,21]. In 1998, a cutoff value of 19-NA at 2 ng/mL for male athletes and 5 ng/mL for female athletes was adopted by the International Olympic Committee (IOC) [22] to avoid false positives as a result of meat consumption of nandrolone-treated animals or endogenous production [2,8,11,23]. Currently, according to the World Anti-Doping Agency (WADA) Code, an adverse analytical finding with a concentration of 19-NA greater than 2 ng/mL is reported in any urine sample from either a male or a female [24]. Nevertheless, the possibility there is a risk of false positive doping tests due to endogenous metabolic complicity of 19-NA and 19-NE was raised [25].

In routine doping analysis, urinary metabolites including nandrolone are hydrolyzed by β-glucuronidase and analyzed by GC–MS. In the present study, we investigated urinary glucuroconjugate and sulfoconjugate fractions of the nandrolone metabolites using acid and enzyme hydrolyses following oral administration of xenobiotic 19-norsteroids.

2. Experimental

2.1. Urine samples

Urine samples were obtained from four Taiwanese healthy adult male volunteers (age 23–57 year; body weight 65–76 kg) who gave an informed written consent to the study approved by the Human Subjects Research Review Committee. A single dose (10 mg) of one of the four 19-norsteroids, as listed in Section 2.3 below, was administered orally to each volunteer ($n = 3$). When each volunteer ingested more than one substance, 7 days washout period was given before taking the next substance to ensure no presence of carry-over effect. Urine samples from the volunteers were collected at 0 (pre-administration), 2, 4, 6, 8, 12, 24, 48, 72, 96 and 120 h post-administration. Sample pH and specific gravity were measured and the samples were stored at −20 °C until analysis.

2.2. Chemicals, solvents, and reagents

Acetic acid, anhydrous dibasic sodium phosphate, monoacid sodium phosphate, potassium carbonate, sodium bicarbonate, sodium acetate, acetonitrile, dichloromethane, sodium dihydrogen phosphate, diethyl ether, *n*-hexane, methanol, ethyl acetate, triethylamine, *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA), trimethyliodosilane (TMIS), trimethylchlorosilane (TMCS) and β-glucuronidase from *Escherichia coli* (*E. coli*) were purchased from Sigma (St. Louis, MO, USA). Ethanethiol was obtained from Fluka (Milwaukee, WI, USA). All chemicals and solvents were of analytical grade or better. Deionized water was generated with a Millipore Milli-RO4/Milli Q water purification system.

2.3. Reference standards

19-Norandrosterone and 19-noretiocholanolone were obtained from Cerilliant (Austin, TX, USA). 19-Norepiandrosterone was purchased from Steraloids (Newport, RI, USA). 19-Norsteroids including nandrolone, 19-nor-4-androsten-3,17-dione, 19-nor-4-androsten-3β,17β-diol, 19-nor-5-androstene-3β,17β-diol and 19-norandrosterone-d_4 (internal standard; I.S.) were purchased from Sigma (St. Louis, MO, USA).

2.4. Derivatization mixture

A one-step derivatization process was used to convert steroids to the trimethylsilyl (TMS)enol–TMS ether derivatives [26,27]. Trimethyliodosilane solution (0.1 M) was first prepared by mixing trimethyliodosilane, triethylamine and dichloromethane (70:1:430). The final mixture was then prepared by adding 3.0 mL of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide, 60 μL of ethanethiol and 60 μL (0.1 M) of trimethyliodosilane solution. The mixture was stored at −20 °C before use.

2.5. Urine sample preparation

All urine samples, either for validation (unconjugated standards) or for excretion studies, were pretreated as follows: a Sep-Pak Plus C_{18} solid phase extraction (SPE) cartridge (Waters, USA) was conditioned with 5 mL (2×) methanol and 5 mL (2×) deionized water. To a volume of 2 mL urine in a glass tube, an equal volume of acetate buffer (acetic acid/sodium acetate trihydrate 0.1 M; pH 5.2) and 25 μL I.S. was mixed. After passing through the SPE cartridge, the sample was washed with 5-mL water and 3-mL hexane. The sample was collected after flushing the cartridge through with methanol (5-mL). The extract was evaporated to dryness under nitrogen gas.

One milliliter of phosphate buffer ($NaH_2PO_4{\cdot}H_2O/Na_2HPO_4$ 0.1 M; pH 6.9) and 50-μL of β-glucuronidase (1 mg/mL in phosphate buffer) were added to the tube. The enzymatic hydrolysis was performed at 50 °C for 1 h. Methanolysis was used to simultaneously deconjugate both glucuronides and sulfates of 19-NA and 19-NE metabolites in the urine as described in detail by Dehennin et al. [28]. One milliliter TMCS solution (1 M TMCS in methanol) was added and incubated at 50 °C for 1 h. The solution was evaporated to dryness under nitrogen gas. Subsequently, 1-mL phosphate buffer and 100 mg solid buffer ($NaHCO_3/Na_2CO_3$ 10:1, w/w) were added to the tube. A liquid–liquid-extraction was then performed by adding 5-mL diethyl ether to each tube followed by agitation and centrifugation (2000 rpm; 8 min). The organic layer was evaporated to dryness under nitrogen gas. The residue was resuspended