Validation and Applications of Gas Chromatography-Combustion/isotope Ratio Mass Spectrometric Method to Control Misuse of Androgens in Human

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Abstract: The misuse of anabolic androgenic steroids is of particular concern in sports and society. Thus, it is of great importance to discriminate endogenous steroids such as testosterone or testosterone prohormones from their chemically identical synthetic copies. In this study, gas chromatography-combustion/isotope ratio mass spectrometric (GC–C/IRMS) method has been developed and validated for discriminating the origin of anabolic androgenic steroids. The method involves the solid-phase extraction, enzymatic hydrolysis with β-glucuronidase, HPLC-fractionation for the cleanup and analysis by GC–C/IRMS. The difference (Δ^{13}C) of urinary δ^{13}C values between synthetic analogues and endogenous reference compounds (ERC) by GC–C/IRMS was used to elucidate the origin of steroids, and intra- and inter-day precision, specificity and isotope fractionation were evaluated. The present GC–C/IRMS method combined with HPLC cleanup was accurate and reproducible enough to be successfully applied to the test of urine sample from suspected anabolic steroid abusers.

Key words: Steroids, Endogenous, Validation, GC-C/IRMS

Introduction

The misuse of synthetic endogenous steroid copies is one of the most important issues in sports. Athletes may abuse steroids or manipulate metabolic pathways in an attempt to increase concentrations of biologically active steroids with the intent of enhancing athletic performance through increased muscle mass and more rapid recovery from injury or intense training.[1] Therefore, the administration of steroids has been strictly prohibited by World Anti-Doping Agency (WADA) and it is necessary to discriminate endogenous steroids from their chemically identical synthetic copies for elucidating the origin of steroids.[2]

Traditionally, the GC-MS method has been used for the detection of steroids.[3] However, this method has limitation on distinguishing endogenous steroids such as testosterone or testosterone prohormones from the chemically identical endogenous synthetic copies. In steroids, differences in ^{13}C content arise because synthetic steroids are derived from plant sterols such as stigmasterol and sitosterol obtained from C-3 plants that consistute about 90% of all plants[4]. They usually exhibit depleted ^{13}C/^{12}C ratio (δ^{13}C) values in range −25.9‰ to −35.6‰ in comparison with the values for endogenous steroids.[5] Based on ^{13}C isotopic differences, the measurement of δ^{13}C of steroids is highly regarded to elucidate the origin of steroids and the use of gas chromatography-combustion isotope ratio mass spectrometry (GC–C/IRMS) has proven to be the unique analytical technique of choice in this field[6-10]. Thus, the analysis and difference (Δ^{13}C)of urinary δ^{13}C values between synthetic analogues and endogenous reference compounds (ERC) such as 11-keto-etiocholanolone, 11β-OH-androsterone and pregnanediol allows endogenous steroids to be distinguished from their synthetic analogues in the urine and provides significant information that they have not administrated synthetic analogues of endogenous steroids.[11-13]

In the present study, we validated a comprehensive GC–C/IRMS method combined with HPLC cleanup for the discrimination of endogenous steroids and successfully applied to the urine samples of endogenous synthetic steroids abusers.

Experimental

Reagents

11-keto-etiocholanolone (11-keto-Et) was obtained from German laboratory and dihydrotestosterone (DHT) was purchased from Wako (Japan). 11β-OH-androsterone (11β-OH-A), 19-norandrosterone (19-NA) and pregnanediol (PD) were purchased from Steraloids (USA). Testosterone (T), 5β-androstane-3α,17β-diol (5β-diol), 5α-androstane-3α,17β-

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diol (5α-diol), dehydroepiandrosterone (DHEA), epitestosterone (EpiT), etiocholanolone (Et) and androsterone (A) were purchased from NMI. β-glucuronidase was purchased from Roche (Germany) and OASIS HLB cartridge (150 mg × 6 mL) was purchased from Waters (Milford, USA). Methanol (MeOH) (HPLC-grade) and acetonitrile (ACN) (HPLC-grade) was purchased from Burdick & Jackson (Ulsan, Korea). HPLC-grade water was prepared using a Milli-Q purification system (Millipore, Bedford, MA).

Sample preparation

OASIS HLB cartridges were conditioned with 6 mL of MeOH and 5 mL water. 10 mL of urine were loaded onto the column. After washing with 5 mL of water and the residue was eluted with 6 mL of MeOH. The eluate was evaporated under nitrogen to dryness. The dried eluate was dissolved in 1 mL of phosphate buffer (0.2 M, pH 7.2) and 50 μL of β-glucuronidase were added. Then the sample was incubated for 1 hr at 55°C. After cooling to room temperature 500 μL of potassium carbonate buffer were added. The aqueous layer was extracted with 5 mL of ethyl ether. The organic layer was transfered into a test tube and evaporated to dryness under nitrogen. The residue was redissolved in 20 μL of MeOH and injected to the HPLC.

HPLC cleanup

In order to achieve high specificity for the isolation of steroids from the urine matrix, high performance liquid chromatography (HPLC) fractionation steps were employed. The HPLC system consisted of an Agilent 1100 series (Waldbronn, Germany) with a diode-array module. The column used for the separation was a Zorbax Eclipse XDB C_{18} (4.6 mm ID × 150 mm length, 5 μm particle size, Agilent, USA). The HPLC mobile phases consisted of water (solvent A) and ACN (solvent B). A gradient program was used for the HPLC separation at a flow rate of 0.8 mL/min. The mobile phase was initially composed of 100% solvent A, linearly programmed to 20% solvent A for 18 min and changed back to the initial condition over 5 min followed by 2 min re-equilibration. The total run time was 25 min. The entire column eluates were directly introduced into a DAD detector. The different fractions were collected in test tubes and evaporated to dryness under a stream of nitrogen (Table 1). Then the dried samples were redissolved in 35 μL of ethyl acetate.

Table 1. Collection program for urinary steroids purified by HPLC.

Fraction	Time (min)	Steroid
1	13.8–14.8	11-keto-Et; 11β-OH-An
2	14.8–15.8	T
3	15.8–16.8	5β-diol, 5α-diol, DHEA, EpiT
4	16.8–17.8	Et, DHT, 19-NA
5	17.8–18.8	A
6	18.8–19.8	PD

GC–C/IRMS system

All samples were measured on an Agilent 6890N gas chromatograph (Agilent, CA, USA) coupled with a Isoprime gas chromatography-combustion/isotope ratio mass spectrometer (GV Instrument, Manchester, UK). The GC system equipped with an DB 17 column (0.25 mm ID × 30 m length, 0.25 μm film thickness, Agilent, USA). The injection was started in the split mode (1:5) at 50°C, and the temperature was ramped to to 250°C at 50°C/min. The temperature 250°C was held for 4 min, followed by a ramp at 2°C/min to the final temperature of 280°C and held for 6min. Helium was used as the carrier gas at a flow rate of 1.0 mL/min. The interface temperature was 300°C and furnace temperature was 850°C.

Results and Discussion

The aim of this study is to develop and validate the GC-C/IRMS method for the discrimination of endogenous steroids and demonstrate that the present method is useful for the identification of the anabolic androgenic steroids abusers.

An initial effort for optimizing the sample preparation was made to achieve more complete enzymatic hydrolysis to convert glucuronide conjugated metabolites of the target endogenous steroids into their free form and clean isolation of the hydrolyzed target analytes to minimize the endogenous interferences. For these purposes, the solid-phase extraction was employed prior to the enzymatic hydrolysis and HPLC-cleanup of the hydrolyzed sample. Six fractions were collected for the isolation of 12 endogenous steroids using the HPLC-cleanup condition described in Table 1. After dryness and reconstitution of fractions, the resulting samples were analyzed by GC–C/IRMS without any derivatization step. Figure 1(A) illustrates representative chromatograms of each fraction obtained from the urine samples spiked with standard. No significant interfering peak of urine matrix was observed at the retention time of all steroids and the retention times of target analytes were consistent with those of standards. Identification of target urinary steroids was confirmed by comparison with mass spectra of standards obtained from GC-MS analysis (data not shown).[14]

The present method for the discrimination of endogenous steroids was validated. The urinary $\delta^{13}C$ values for 12 endogenous steroids were obtained and intra- and inter-day precision and isotopic fractionation were evaluated. The validation results are summarized in Table 2 and 3. For the intra- and inter-day precision, quality control (QC) urine samples at concentration of 200 ng/mL were prepared from the steroids-free urine and the QC samples were processed and analyzed 3 times in the same run (intra-day precision) and 3 times in 3 separated runs (inter-day precision). The intra- and inter-day precisions for the target analytes were between 0.1% and 6.5% (Table 2), strongly suggesting that the present method is repeatable and reproducible in the measurement of urinary $\delta^{13}C$ values. Sample preparation steps such as the SPE and HPLC-cleanup may seriously give rise to the isotopic fractionation phenomenon

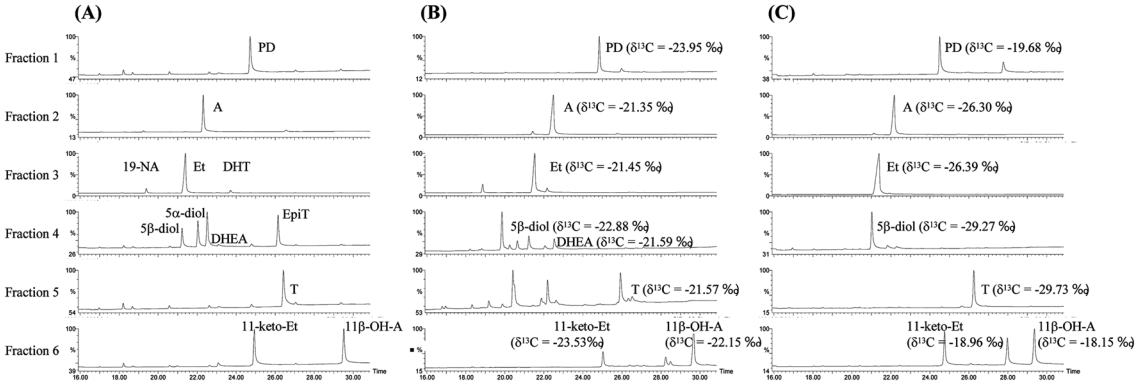

Figure 1. GC-C/IRMS chromatograms of 12 androgenic steroids after HPLC fractionation of standards-spiked urine (A), negative control urine (B) and synthetic testosterone abuser's urine (C).

Table 2. Intra- and inter-day precision. (n = 3)

Analytes	QC Concentration (ng/mL)	Intra-day Mean (δ^{13}C, ‰)	Intra-day Precision (CV, %)[a]	Inter-day Mean (δ^{13}C, ‰)	Inter-day Precision (CV, %)
11-keto-Et	200	−20.9	4.5	−20.0	5.0
11β-OH-A	200	−18.5	0.1	−18.5	4.0
T	200	−29.9	1.1	−29.5	1.9
5β-diol	200	−30.3	6.5	−30.4	4.5
5α-diol	200	−31.1	1.6	−30.6	2.0
DHEA	200	−29.8	0.6	−29.3	1.5
EpiT	200	−31.9	0.3	−31.5	1.6
19-NA	200	−29.4	0.2	−29.2	1.8
Et	200	−28.0	0.8	−27.9	0.6
DHT	200	−27.2	3.0	−26.0	4.1
A	200	−26.2	0.4	−26.2	0.3
PD	200	−32.6	1.1	−31.9	3.2

[a]CV: Coefficient of variation

Table 3. Isotopic fractionation test (n = 5)

Target compound	Steroid Standards Mean (δ^{13}C, ‰)	Steroid Standards %CV	Standard Spiked Urine Mean (δ^{13}C, ‰)	Standard Spiked Urine %CV	T test P value
11keto-Et	−19.6	3.4	−20.3	2.6	0.16
11bOH-A	−17.6	1.7	−18.1	2.6	0.12
Testosterone	−29.9	1.6	−29.5	2.4	0.32
5b-diol	−30.7	1.3	−30.9	3.1	0.65
5a-diol	−30.5	0.4	−30.8	1.5	0.25
DHEA	−29.1	0.6	−29.5	1.8	0.19
Epitestosterone	−31.7	1.3	−31.8	1.3	0.92
19-NA	−29.4	0.9	−29.2	2.7	0.52
Etiochol-anolone	−27.5	1.6	−27.9	1.2	0.24
DHT	−28.2	1.0	−27.5	4.7	0.11
Androsterone	−27.2	1.2	−26.7	2.5	0.17
Pregnanediol	−33.8	1.4	−32.8	3.1	0.11

affecting the accuracy of IRMS analyses. To determine potential ^{13}C isotopic fractionation of the steroids during the sample preparation, the isotopic fractionation test was conducted.[15] The urine samples at concentration of 200 ng/mL were prepared and analyzed (n = 5). The isotope fractionation results are summarized in Table 3. When comparing with δ^{13}C values of standards, δ^{13}C values of the analytes from urine samples were consistent with those of standards with the outcome of T test ($P > 0.05$), suggesting that no significant isotope fractionation effect was observed.

Applications

The present methods were applied to the urine samples of seven suspected synthetic testosterone abusers. Figure 1 (C) depicts the representative chromatograms and δ^{13}C values obtained from the urine sample of synthetic testosterone

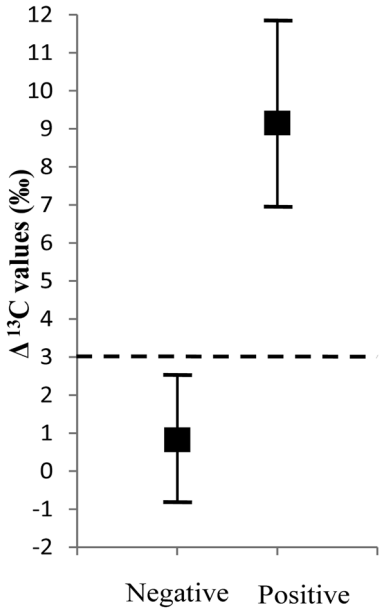

Figure 2. Δ^{13}C values of negative urine and positive urine (synthetic testosterone abusers, n = 7)

abuser. As shown in Figure 2, the results showed that the difference ($\Delta^{13}C$) of $\delta^{13}C$ values between testosterone and ERC is higher than the 3.0‰ limit currently imposed by the WADA technical document. Therefore, these results revealed that the present method is discriminative enough to exclude false positive.

Conclusions

GC-C/IRMS method combined with HPLC-cleanup has been developed and validated for the discrimination of endogenous steroids in human urine. This method was successfully applied to analysis of the urine samples from the suspected synthetic testosterone abusers. As results, the present method seems to be useful to elucidate the origin of the endogenous steroids.

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