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Urine ratio of tetrahydrocortisol to tetrahydrodeoxycortisol to screen for the systemic administration of cortisone and hydrocortisone

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ABSTRACT

We use gas chromatography–mass spectrometry (GC–MS) to determine the urine peak area ratio of tetrahydrocortisol (THF) to tetrahydrodeoxycortisol (THS) in spot urine samples of eight male volunteers after a single intramuscular injection of 100 mg hydrocortisone (HC) and after a single oral administration of 10 mg HC at six different post-treatment times over 24 h with 1 week between the two treatments. Control spot urine samples were also obtained from a group of 100 volunteers of each sex for GC–MS analysis. In addition, one female volunteer was collected for GC–MS and isotope ratio mass spectrometry (IRMS) analysis after a single oral administration of 40 mg HC and 40 mg cortisone (C) at 15 and 10 different post-treatment times over 30 h, respectively. IRMS analysis focused on the acetylated derivative of 11-keto-etiocholanolone (11KE) and 11β-hydroxy-etiocholanolone (11OHE) as target metabolites, and on androsterone (A) as an endogenous reference compound (ERC) for calculating the corresponding $\delta^{13}C$ (‰) depletion values. There was a small but significant sex-related difference for the THF/THS ratio in the control group with mean THF/THS ratio values of 10 and 13.5 for women and men, respectively. A cut-off value of 28 (mean + 2 S.D.) for the THF/THS ratio offered a narrow detection window with 39% of suspicious samples after HC-oral treatment, and a wide detection window with 94% of suspicious samples after HC-intramuscular administration in men. For the woman the same cut-off value offered a wide detection window after HC and C administration with 100% and 90% of suspicious samples, respectively. On the basis of a cut-off value of 3‰ for the $\delta^{13}C$ (‰) depletion, the exogenous origin was widely evidenced for at least one target compound in 93% and 80% of the HC and C samples, respectively. We conclude by discussing the predictive ability of the urine THF/THS ratio and its usefulness in pointing out suspicious samples resulting from the systemic administration of HC and C.

1. Introduction

Among the substances controlled for anti-doping purposes in sports, there is the prohibited class of glucocorticoids (GCs) that can be grouped into one of the following two categories: those that are exogenous synthetic GCs and those that are synthetically derived endogenous GCs, namely hydrocortisone (HC) and cortisone (C) [1]. The detection of synthetic GCs in urine is routinely performed and many robust and reliable methods are available for this purpose [2–6]. However, some difficulties remain when HC and C are involved. Obviously these difficulties are the same as those encountered for endogenous anabolic steroids [7]. In that case, the steroid profile analysis, especially the gas chromatography–mass spectrometry (GC–MS) determination of the urine ratio of testosterone to epitestosterone (T/E) [7–11] has been extensively studied before being introduced into well-established screening procedures in doping analysis and a T/E ratio higher than four is currently used to identify suspicious samples [12]. However, there remains a need to perform isotope ratio mass spectrometry (IRMS) analysis as an extension of the GC–MS steroid profile to discriminate between biological and synthetic origins and unequivocally decide on the misuse of endogenous anabolic steroids [12–19]. In this respect, the IRMS method focuses on the metabolites of testosterone namely androsterone (A) and etiocholanolone as target compounds (TCs) and on the metabolites of hydrocortisone and cortisone, namely 11keto-etiocholanolone (11KE) and 11-hydroxyetiocholanolone (11OHE) [20], as endogenous reference compounds (ERCs) [12,21,22]. The results are reported as consistent with administration of testosterone or its

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precursor provided that the $\delta^{13}C$ (‰) value measured for the target metabolites differs by 3 delta units or more from that of the ERC [12]. Although the metabolism of cortisone and hydrocortisone is well documented, it has never been investigated as a screening marker of hydrocortisone and cortisone misuses. The objectives of this work were thus twofold. First, the effects of hydrocortisone and cortisone administration on the urine ratio of tetrahydrocortisol (THF) as the main metabolite of both HC and C [20] to tetrahydro-11-deoxycortisol (THS) as the metabolite of the exclusive precursor of endogenous glucocorticoids were investigated and compared to the physiological values of a control group. Focusing on the ratio of THF to THS was the result of a preliminary study where the advantage of monitoring the ratios of THF, cortisol, cortisone, 11OHE and 11KE to THS was examined. Second, the opportunity of using the measured THF to THS ratio to identify suspicious samples was compared to IRMS determination according to the method and the criterion officially recognised for ascertaining the misuse of testosterone and its precursors except that 11KE and 11OHE were used as target compounds and A as an ERC.

2. Materials and methods

2.1. Chemical and reagents

All solvents and reagents of analytical grade purity were purchased from Fluka (Buchs, Switzerland) and Carlo Erba (Milan, Italy). Bakerbond spe™ 200 and 500 mg octadecyl C_{18} disposable extraction columns were obtained from JT Baker (Phillipsburg, NJ, USA). β-Glucuronidase from *Escherichia coli* in a 50% glycerol solution (pH 6.5, 140 U/ml at 37 °C) was supplied by Roche Diagnostics GmbH (BoehringerManheim, Germany). N-methyl-N-trimethyl-silyltrifluoroacetamide (MSTFA) was obtained from Machery-Nagel (Duren, Germany), ammonium iodide (NH_4I) was from Merck (Darmstadt, Germany), and dithioerythritol (DTE) from Sigma (St. Louis, MO, USA). Steroid standards of 17a-methyltestosterone (17-MeT used as internal standard, ISTD) and all the steroids considered in the present study were obtained from Sigma, St. Louis, MO, USA. BondElut® SPE cartridges were obtained from Varian, Harbor City, CA, USA. The water used was obtained using a Milli-Q® water purification system purchased from Millipore (Bedford, MA, USA). Acetic anhydride was purchased from Aldrich Chemical Company Inc. (Milwaukee, WI, USA), with a $\delta^{13}C$ isotopic value of −48.5‰. The mixture of four alkanes, C_{10} (*n*-decane), C_{11} (*n*-undecane), C_{12} (*n*-dodecane) and C_9COOCH_3 (*n*-methyl-decanoate) was supplied by Chiron AS (Trondheim, Norway).

2.2. Subjects and collection of urines samples

The administration trials were approved by the local ethical committee and informed consent to participate in them was obtained from all subjects. The control group used for establishing the cut-off value of the urinary THF/THS ratio consisted of two hundred Caucasian athlete volunteers (100 males and 100 females). A single spot urine sample was collected from each between 8 a.m. and 8 p.m. Samples were collected three times (morning, noon, and evening) within the day before the initial administration and at post-treatment times close to each over an appropriate period for recovery.

Eight healthy male volunteers received a single oral dose of 10 mg HC (hydrocortisone Roussel) and a single intramuscular injection of 100 mg HC (hydrocortisone hemisuccinate Roussel), with 1 week between each treatment. The post-treatment collection times were approximately 2, 4, 6, 9, 12 and 24 h after oral and intramuscular administrations.

Additionally, one healthy female volunteer received orally, in the morning, on two different occasions, 1 week apart, a single dose of C (cortisone acetate Roussel, 40 mg) and subsequently a single dose of HC (hydrocortisone Roussel, 40 mg). The post-treatment collection times were approximately 2 h (C4), 3 h (C5), 5 h (C6), 6 h (C7), 9 h (C8), 11 h (C9), 12 h (C10), 17 h (C11), 22 h (C12) and 34 h (C13) after C treatment, and 1 h (HC4), 2 h (HC5), 3 h (HC6), 4 h (HC7), 5 h (HC8), 6 h (HC9), 9 h (HC10), 10 h (HC11), 11 h (HC12), 14 h (HC13), 19 h (HC14), 22 h (HC15), 26 h (HC16), 28 h (HC17), and 30 h (HC18) after HC treatment.

All these samples were used for the GC–MS analysis but only samples coming from the woman were additionally analysed for GC–C–IRMS analysis.

2.3. Sample preparation for SIM GC–MS analysis and instrumentation parameters

Preparation of urine samples and GC–MS analysis of urinary glucuronide steroids were performed similarly to a previously reported method [23]. Briefly, it consists of addition of 17 methyl testosterone as an internal standard, to aliquots of 2 mL of urine, hydrolysis with β-glucuronidase from *E. coli*, TBME extraction on a solid phase extraction SPE–C_{18} column, formation of trimethylsilyl derivatives and GC–MS analysis in the single ion monitoring mode. Acquisition was carried out in selected ion monitoring (SIM) of the following fragment ions *m/z* 301 (ISTD); *m/z* 636 (THF); *m/z* 548 (THS). The urine THF/THS ratio was calculated from the peak areas of the detected signals obtained after calibration of the GC–MS instrument with a reference mixture of steroids as it is commonly performed in sports drug testing.

2.4. Isolation of steroids from urine for GC–C–IRMS analysis

Urine (10 mL) samples were adjusted to pH 7.0 (with K_2CO_3 and CH_3COOH) and treated with 50 μL of β-glucuronidase for 1 h at 55 °C. After incubation, the mixture was centrifuged in glass tubes at 2500 rpm for 5 min, and the deconjugated steroids were extracted with methanol using a C18 column (200 mg). The eluate was evaporated until dry under a nitrogen stream for 30 min at 55 °C. Acetylation of the extract was carried out in 50 μL of pyridine and 50 μL of acetic anhydride at 60 °C for 1 h incubation. The reaction medium was evaporated to dryness and subsequently the residue was redissolved in 3 mL of acetonitrile:H_2O (50:50, v/v). The solution was applied to a C_{18} column (500 mg). Fraction F1 containing the corticosteroids (11KE and 11OHE) was obtained by elution with 12 mL of acetonitrile:H_2O (50:50, v/v). Finally fraction F2 containing the ERC (A) was obtained by elution with 12 mL of acetonitrile:H_2O (75:25, v/v). The derivatized steroids of fractions F1 and F2 were spiked with 10 and 80 μL of an internal standard (IS, 5α-androstan-3β-ol acetate, 0.20 mg/mL in methanol), respectively. Then, both fractions F1 and F2 were evaporated to dryness and finally dissolved in 50 μL of hexane. After adjusting the hexane volume or IS concentration in each sample, this solution was analysed by GC–MS and then by GC–C–IRMS.

2.5. GC–MS analysis of derivatized GC–C–IRMS extracts

GC–MS-EI analyses of the acetylated derivatives from the extracted urines were performed on a Hewlett-Packard 6890 chromatograph coupled with a HP 5973 quadrupole mass selective detector (MSD). GC separation was achieved on a DB-17 MS column (30 m × 0.25 mm i.d., 0.25 μm film thickness) from J&W Scientific (Folsom, CA, USA) operated with a helium inlet pressure of 125 kPa. The oven temperature was increased from 70 °C (1 min) to 270 °C (12 min) at 30 °C/min, then to 300 °C (3 min) at 10 °C/min. Injections of 1 μL samples from each fraction were made at 280 °C in the splitless mode using an HP 7673 auto-sampler. The analyses were performed in the mass range 50–450 at ionization energy of 70 eV.

2.6. GC–C–IRMS analysis

The $\delta^{13}C$ of A, 11KE, 11OHE and IS were determined in each of the urine samples coming from the female volunteer. These measurements were obtained using an IsoPrime isotope ratio mass spectrometer via a GC–C Combustion interface (GV, Manchester, UK) coupled to an Agilent 6890 gas chromatograph. Chromatographic separations were achieved on a DB-17 MS fused silica capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) from J&W Scientific. The injector temperature was set to 280 °C. The combustion oven temperature was set to 850 °C and the oxidative catalyst consisted of copper oxide pellets in quartz tubing. Reference carbon dioxide gas pulses (20 s duration) were introduced at six different times during the course of the chromatographic separation.

The volume of injection was 1–2 μL, depending on the concentrations of the compounds of interest. The extracts were injected once in splitless mode. For the calibration of the reference gas with the alkane mixture of known $\delta^{13}C$-values, the oven temperature was increased from 80 °C (1 min) to 270 °C (7.0 min) at 15 °C/min. The GC conditions for the analysis of the acetylated derivatives (fraction F1 and F2) are as follows: the oven temperature was increased from 70 °C (1 min) to 271 °C at 30 °C/min, then to 281 °C at 0.6 °C/min (3 min) and finally to 300 °C at 5 °C/min, and maintained at the final temperature for 5 min.

The results were expressed as $\delta^{13}C$ (‰) depletion values that were calculated from $\delta^{13}C$ (‰) – values for acetylation [24].

2.7. Statistical analysis

XLSTAT 2006 (Addinsfoft™) was used to compute descriptive statistics for the purpose of selecting suitable discrimination limits for the THF/THS ratio. To normalize the data, the variable was log transformed and the Shapiro–Wilk test ($\alpha < 0.05$) was used to evaluate the fit of the transformed data to a normal distribution. Log-transformed THF to THS ratios after drug administration were compared to their baseline values by ANOVA for significant differences. Statistical significance in each experiment was determined using a one-way analysis of variance (ANOVA), a value of $P < 0.05$ was considered significant.

3. Results

As shown in Fig. 1a and b, there was a logarithmic normal frequency distribution of the THF to THS ratio values for men and women in the control group. A significant sex-related difference in