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Presence of endogenous prednisolone in human urine ☆

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ABSTRACT

The possibility of an endogenous presence of the glucocorticoid prednisolone has already been demonstrated in bovine and horse urine, with the aim of clarifying its origin in this matrix, which is used by official agencies for the control of illicit treatments. From this point of view, the endogenous nature of prednisolone could be a major topic in doping control of both amateur and professional human athletes. A study was therefore made on 34 human volunteers (13 males and 21 females; aged 22–62) to detect the presence of prednisolone in their urine by HPLC–MS^3. One of the volunteers underwent vernal allergy treatment with betamethasone for two subsequent years. An investigation was carried out with the aim of verifying if the suppression, and the circadian rhythm, of cortisol urinary levels could also apply to prednisolone. The results of the study show that prednisolone was present in the urine of all 34 volunteers, with a concentration very close to 100-times lower that of cortisol, with no dependence on gender. The same ratio (1/100) was observed in the prednisolone and cortisol levels detected during the 24 h together with the suppression of prednisolone by betamethasone treatment.

These data demonstrate the endogenous nature of low concentrations of prednisolone in human urine, and motivate further studies about the biosynthetic pathways of this corticosteroid and its relationship with stress in humans, as already described in cows.

1. Introduction

Prednisolone (11β,17α,21-trihydroxy-pregna-1,4-diene-3,20-dione) is a glucocorticosteroid with a similar structure to cortisol (11β,17α,21-trihydroxy-pregn-4-ene-3,20-dione), differing from this hormone by only one double bond at the C1–C2 position on ring A (Fig. 1). Prednisolone was first synthesised in 1955, through microbiological oxidation of cortisol [1]. Together with its prodrug prednisone, prednisolone is used both in human and veterinary medicine because of its anti-inflammatory and immunosuppressive activities [2,3]. The illicit use of prednisolone by athletes is monitored by the World Anti-Doping Agency (WADA), using in-competition controls for all corticosteroids administered by systemic (e.g. oral), non-systemic and non-topical (e.g. intra-articular, inhalation) routes [4] are performed. The Fédération Equestre Internationale (FEI™) considers prednisolone as a "controlled medication substance" [5], i.e. a substance that must not be "present in the horse's body during an event" [6]. In the field of animal husbandry, prednisolone use is permitted in the European Union, with the restriction to meet the maximum residue limits (MRLs) in edible tissues [7]; its presence in the urine of food-producing animals is not, however, provided.

Some studies show that a problem in finding of prednisolone in human, equine and bovine urine exists: this corticosteroid could in fact be the product of a microbiological dehydrogenation of cortisol after the collection of urine samples [8–11], and endogenous metabolic pathways could also be involved in bovine [12] and equine [13] animals. The analogies between the anabolic steroid boldenone (17β-hydroxy-1,4-androstadiene-3-one) and the glucocorticosteroid prednisolone have to be underlined: both steroids have a C1–C2 double bond; cortisol is released by the adrenal gland in response to stimulation by adrenocorticotropic hormone (ACTH); also androstenedione (4-androsten-3,17-dione; AED), the direct precursor of testosterone (17β-hydroxy-4-androsten-3-one) can be released by adrenals in response to the same stimulation [14]; and AED differs from the precursor of boldenone, androstadienedione (1,4-androstadiene-3,17-dione; ADD), in the same way that cortisol differs from prednisolone, i.e. the dehydrogenation of the carbon atoms in position 1 and 2. In a study carried out using GC/C/IRMS (gas chromatography/combustion/isotope ratio mass spectrometry), Piper et al. [15] showed that the $^{13}C/^{12}C$ isotope ratios of boldenone and its main metabolite 5β-androst-1-en-17β-ol-3-one were inconsistent with an exogenous origin in 11 out of 23 athlete urine samples

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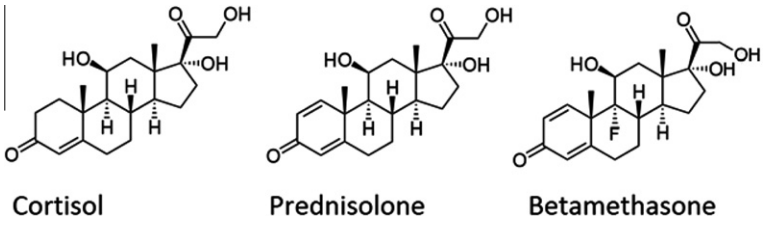

Fig. 1. Chemical structures of cortisol, prednisolone and betamethasone.

found positive in a 4-year period. As two of the 11 samples were collected from the same athlete within a one-month interval, the authors strongly affirmed the endogenous production of boldenone, likely in the gut, working as an "endocrine active side organ".

In the present work, it was hypothesised that prednisolone synthesis could be endogenous, similar to boldenone synthesis. The presence of the glucocorticosteroid in urine was therefore investigated in 34 healthy volunteers of both sexes in order to understand if the frequency of prednisolone detection and the correlation with cortisol urinary levels confirmed this supposition. The prednisolone urinary levels of a male volunteer, orally treated to prevent pollen allergy with betamethasone (9α-fluoro-11β,17α,21-trihydroxy-16β-methylpregna-1,4-diene-3,20-dione) on medical prescription, were also investigated, in two different years, before, during and after the treatment.

2. Experimental

2.1. Reagents and chemicals

Cortisol, prednisolone, betamethasone and methylprednisolone (as internal standard) were from Sigma–Aldrich (St. Louis, MO, USA); *tert*-butyl methyl ether was from Panreac (Barcelona, Spain); acetonitrile, methanol and formic acid were from J.T. Baker (Deventer, Holland). All solvents used were of HPLC grade. Water was freshly prepared with a Milli-Q Advantage A10 Ultrapure Water Purification System (Merck-Millipore, Darmstadt, Germany). Standard stock solutions (1 mg/mL) were prepared by dissolving the dry powder of each analyte in methanol; solutions were stored at −20 °C. Working solutions were prepared daily by diluting the stock solutions with methanol.

Bentelan® (effervescent tablets 0.5 or 1 mg–betamethasone 21-disodium phosphate 0.6578 or 1.316 mg equal to betamethasone 0.5 or 1 mg, respectively) was from Defiante Farmacêutica, S.A., Funchal, Portugal.

2.2. Study population

In total, 34 healthy individuals (33 did not use glucocorticoids, one was studied for two subsequent years both before, during and after a treatment with a glucocorticoid) participated in this study. The urine samples from the volunteers (13 males aged 30–61; 21 females aged 22–62) were analysed. All individuals provided one urine sample, except for two male and three female individuals who gave two samples, collected within a six-month interval. One individual, a 41-year old male, gave urine samples nine times in a 24-h period before, and once a day (at 10.00 A.M.) during an oral treatment with betamethasone (a medical prescription to prevent pollen allergy) and 12 and 15 h after the last intake of the corticosteroid; in a second trial in the subsequent year, urine was collected from the same subject 1 h before the first administration, at the moment (11:00 P.M.) and 7 h (6:00 A.M.) after each oral administration of betamethasone and over 3.5 days after the last administration at different intervals by urinary urges. The dosages were: one 0.5-mg tablet orally administered per day for 11 days at 10.00 A.M. (first trial) or one 1-mg tablet orally administered per day for 7 days at 11.00 P.M. (second trial).

All samples were analysed for the presence of prednisolone and cortisol; in the second trial concerning the betamethasone treatment, this corticosteroid was considered for analysis, too. All participants gave written informed consent.

2.3. Sample collection

All samples were collected in sterile urine containers. The ones from non-treated volunteers were immediately extracted and analysed, while the ones from the betamethasone-treated individual were placed in a freezer at −18 °C, transferred to the laboratory, thawed and processed, avoiding any further storage.

2.4. Sample preparation

Urine samples (5 mL) were spiked with the internal standard methylprednisolone to give a final concentration of 10 ng/mL. *Tert*-butyl methyl ether (5 mL) was added. After shaking in a vertical rotary shaker for 30 min, the sample was centrifuged at 3000*g* for 30 min. The upper organic layer was collected with a Pasteur pipette, transferred to a 10-mL glass tube and dried under vacuum in a centrifugal evaporator at a temperature of 45 °C. The residue was dissolved in 100 μL of the mobile phase (30% acetonitrile, 70% formic acid in a 0.1% aqueous solution) and transferred to vials for HPLC–MS^3 full-scan analysis. The injection volume was 5 μL.

2.5. Sample analysis

The HPLC–MS^3 analysis (and the problems involved) was described in detail in a previous work [13]. Briefly, an LTQ linear ion trap mass spectrometer equipped with an ESI source (Thermo Fisher Scientific, San José, CA, USA), connected to a Surveyor Autosampler and an MS Pump (Thermo Fisher Scientific, San José, CA, USA), was used. The chromatographic separation was performed at ambient temperature, in the isocratic condition, on a reversed-phase Sunfire® column (150 × 2.1 mm, 3.5 μm; Waters, Milford, MA, USA) equipped with a Sunfire C_{18} Guard Column® (2.1 × 10 mm i.d., 3.5 μm). The mobile phase consisted of a mixture of 70% water with 0.1% formic acid and 30% acetonitrile at a flow rate of 0.3 mL/min. Methylprednisolone (10 ng/mL) was used as the internal standard. The linear ion trap mass spectrometer was operated in negative electrospray ionisation mode (ESI-) under the following conditions: sheath and auxiliary gas (nitrogen) flow rates of 40 and 20 arbitrary units, respectively; spray voltage of 4 kV; ion transfer capillary temperature of 275 °C; capillary voltage of −6 V; tube lens offset of −50 V. Helium was used for collision-induced dissociation.

Prednisolone showed, in full scan MS, the very abundant and stable formate adduct 405 ($[M + HCOO]^-$). Consequently, this ion was used as precursor ion for the MS^2 analysis. The most abundant ion detected after the collision had m/z = 329, which was then used as a precursor for MS^3 fragmentation. The analysis was performed in full-scan mode (Table 1). The ESI–MS parameters had been