Human Nutritional Supplements in the Horse: Dehydroepiandrosterone versus Androstenedione: Comparative Effects on the Androgen Profile and Consequences for Doping Analysis

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Abstract

Dehydroepiandrosterone (DHEA) and androstenedione are weak androgens, which need conversion to more potent testosterone in order to enhance anabolic action. Consequences of oral dosing at 1 mg/kg on the urinary and plasma androgen profile of mare and gelding have been evaluated with an analytical method involving conjugate fractionation and selective hydrolysis, group separation, and quantitation by gas chromatography–mass spectrometry with selected ion monitoring of trimethylsilyl ethers. Peak levels of testosterone total conjugates in urine (range 300–6000 pg/L) were attained a few hours after dosing. Renal clearance was fast, so the testosterone detection period lasted only 20 to 33 h, the longest time being generated by androstenedione. The urinary testosterone/epitestosterone ratio for detection of exogenous testosterone in the mare was inoperative after DHEA administration because there was a concomitant increase of epitestosterone, which thereby acted as a masking agent.

Introduction

According to regulations under the Dietary Supplement Health and Education Act, dehydroepiandrosterone (DHEA, 3β-hydroxy-5-androsten-17-one) and androstenedione (4-androstene-3,17-dione) have been considered dietary or nutritional supplements since 1994 and are therefore available in the United States as over-the-counter drugs. Legal or illegal importation into the European Union is made possible by purchase orders transmitted via the Internet. It has thus become feasible to orally supplement human and equine individuals with variable and/or continuous doses in order to attain hypothetical enhancement of athletic performance that is claimed in misleading advertisements issued by the manufacturers.

DHEA and androstenedione have low affinity for the androgen receptor; they are therefore considered as weak androgens and potential prohormones for testosterone, which is the most potent endogenous anabolic steroid involved in muscle development (1). Besides being key intermediates in the biosynthesis of biologically potent androgen, DHEA and androstenedione are also along that route precursors for estrogen production (2,3). Interest in oral androstenedione administration to young men as a means of enhancing bioavailable testosterone is rather recent (4–7). The consensus emerging from these studies is that the bioconversion to testosterone is quantitatively of minor importance, with little or no influence on muscle strength.

The influence of oral DHEA loading on the urinary androgen excretion profile in the human species (8–10) and in the horse (11) has been addressed by several authors. The consequences of androstenedione ingestion have been mainly investigated in man by analysis of plasma androgens (4–7), and urinary determinations remain scarce (12). No work has yet been published on putative physiological effects of DHEA and androstenedione suplementations in the equine species.

A literature survey has thus indicated that the vast majority of physiological and analytical consequences of DHEA and androstenedione ingestions have been investigated in the human field; therefore, we performed the present study in order to demonstrate the comparative efficiency of DHEA and androstenedione for bioconversion to testosterone and ensuing urinary metabolites in the castrated male (gelding) and female (mare) horse.

Experimental

Administration protocol

Four thoroughbred horses were engaged in this excretion study which was approved by the local Ethical Committee for
Animal Studies. Two females (age 9 and 11 years, weight 575 and 475 kg) and two geldings (age 4 and 8 years, weight 485 and 465 kg) were selected. DHEA (administered to the oldest of each gender) and androstenedione (administered to the youngest of each gender) were pure reference compounds, obtained from Steraloids (Wilton, NH). The final dosage was 1 mg/kg body weight and was orally administered in two capsules, each containing half of the final dose. Basal androgen levels were assessed in urine and blood collections made at the time of drug administration and two days previously. Post-administration times of sampling were at 1, 2, 4, 7, 9, 12, 18, 24, 36, and 48 h. Twenty-milliliter volumes of blood were drawn in heparinized tubes and centrifuged, and the plasma was frozen immediately. Urine volumes, in the 100–250-mL range, were stored frozen until analysis.

Materials

Reference steroids, other than DHEA and androstenedione, were obtained from Steraloids (Wilton, NH) or Sigma (St. Louis, MO): androsterone (3α-hydroxy-5α-androstan-17-one) and its β3-epimer (epiandrosterone), etiocholanolone (3α-hydroxy-5β-androstan-17-one) and its β3-epimer (epiandrosterone), etiocholanolone, 5-androsten-3β,17α-diol and its 17β-epimer, 5α-androstane-3β,17α-diol and its 17β-epimer. Stable isotope-labeled analogues of testosterone, epitestosterone, and their corresponding sulfates, 4-androstene-3,17-dione, 5-androsten-3β,17α-diol, 5-androsten-3β,17β-diol and 5α-androstane-3β,17β-diol, were prepared according to procedures outlined previously (13,14). Deuterium-labeled DHEA sulfate was a gift from Professor Simon Gaskell (University of Manchester, U.K.). DEAE-Sephadex A25 (chloride) and Sephadex LH-20 were the liquid chromatography supports, produced by Pharmacia-Upjohn (Uppsala, Sweden). Solid-phase extraction cartridges loaded with 500 mg Bond-Elut C18-HF (octadecyl silica) were obtained from Varian Associates (Harbor City, CA). β-Glucuronidase (EC 3.2.1.31) from Escherichia coli (200 I.U./mL) was supplied by Boehringer Mannheim (Mannheim, Germany). Trimethylsilyldisiloxane (TMSI) and N-methyl-N-trimethylsilyltrimfluoracetonamide (MSTFA) came from Fluka (Buchs, Switzerland). Organic solvents and other usual chemicals were of analytical grade and obtained from common international suppliers.

GC–MS

GC–MS analyses were carried out using a Hewlett-Packard 6890 GC (HP Analytical Division, Waldbronn, Germany), equipped with a HP 7673 autosampler and coupled to an HP 5973 mass selective detector. Instrument control and data processing were performed with an HP Vectra XA Computer and ChemStation software. GC separation was achieved on an HP-5 Trace (5% phenylmethylpolysiloxane) fused-silica column (30 m × 0.25-mm i.d., 0.25-μm film thickness) operated with a helium inlet pressure of 108 kPa and temperature programming: 130°C for 0.5 min, ramped at 5°C/min to 260°C, ramped at 20°C/min to 310°C and held for 3 min (run time = 32 min). Injections of 2-μL samples were made at 275°C in the splitless mode (0.75 min) into a split-splitless injection port with an inner silanized glass liner containing silanized glass wool, and the transfer line was heated at 280°C. The ion source was operated in the electron impact mode with 70 eV electron energy, and the electron multiplier was set to 400 V above the automatic tuning voltage.

Analytical method

Internal standard addition. The concentrations of labeled internal standards in urine and plasma samples were in ranges similar to those of native compounds. An heterologous internal standard (6α-methyl-testosterone) was added at variable concentrations in urine samples (depending on the expected analyte concentration, with a range at 200–1000 μg/L) and at fixed concentration in plasma (2 μg/L).

Solid-phase extraction. Urine sample sizes were variable, depending on the expected concentration range: 5-mL volumes for baseline and late-time collections and 2-mL volumes for early- and middle-time collections. Plasma sample volume was 4 mL. Solid-phase extraction was performed with reversed-phase cartridges mounted on a Benchmate Workstation (Zymark, Hopkinton, MA). Conditioning, loading and elution were done as follows: 4 mL of methanol, 4 mL of water, sample aspiration, rinsing with 5 mL of water and elution with 6 mL of methanol. Eluates were evaporated at 60°C with a TurboVap VL Evaporator (Zymark).

Nonconjugate fraction in plasma. Solid-phase extracts were dissolved in 1 mL phosphate buffer (0.1M, pH 6.5) and extracted with 3 mL of a mixture of n-hexane/diethyl ether (1:1, v/v).

Conjugate fractionation by ion-exchange chromatography. Small disposable DEAE-Sephadex columns (20 × 5 mm) were packed in 80% aqueous methanol (methanol/water, 8:2). Glucuronides (together with minute amounts of nonconjugated androgens, in the case of urine) were eluted with 3 mL 0.03M LiCl in 80% aqueous methanol, sulfates were recovered with 2.5 mL 0.3M LiCl in 80% aqueous methanol. Eluates were evaporated at 60°C with TurboVap VL Evaporator.

Enzyme hydrolysis of glucuron conjugates. Extraction residues were dissolved in 1 mL phosphate buffer (0.1M, pH 6.5) and incubated with 12 I.U. of β-glucuronidase during 1 h at 55°C. The unconjugated and deconjugated steroids were extracted with 3 mL of a mixture of n-hexane/diethyl ether (1:1, v/v). The organic phase was dried with anhydrous sodium sulfate, and the solvent was evaporated under a nitrogen stream at 60°C.

Methanalysis of sulfoconjugates. Extraction residues were dissolved in 1 mL methanolic mixture (1M HCl and 1M LiCl in methanol) and heated for 1 h at 55°C. Neutralization was then performed with saturated aqueous NaHCO3 and methanol was evaporated in TurboVap VL Evaporator. Solvent extraction was similar to the glucuronide fraction.

Group fractionation by partition chromatography. Reusable columns (200 × 5 mm) were packed with Sephadex LH-20, swollen in a mixture of dichloromethane/methanol (95:5, v/v). After deposition on top of the column of the extraction residue, dissolved in 0.25 mL of dichloromethane/methanol mixture, the first 3 mL of eluent were discarded. Diketones and monohydroxy-ketones, with or without a double bound, were recovered in the next 2.5 mL. Diols were eluted in the next 4 mL.

Derivatization and selected ion monitoring. Trimethylsilyl-enol-trimethylsilyl ethers were made by dissolving final dry residues, which had been transferred into appropriate injection
vials, in 30 μL derivatization reagent (MSTFA containing 0.2% TMSI, v/v) and by subsequent heating at 70°C for 30 min. Molecular ions used for quantitation were m/z 430 (androsten-dione), m/z 432 (DHEA, testosterone, epitestosterone, androstenedione-d2), m/z 434 (androsterone, etiocholanolone, epiandrosterone, epietiocholanolone, DHEA-d2, [13C2] testosterone, epitestosterone-d2, 17α- and 17β-androstenediols), m/z 437 (17β-androstenediol-d3), m/z 440 (17α-androstenediol-d6), and m/z 446 (6α-methyl-testosterone). Fragment ions used for quantitation were m/z 241 (5α-androstan-3β,17β-diol and its 17β-epimer) and m/z 244 (17β-androstenedioli-d3).

Quantitation. Response factors of the mass selective detector for standard mixtures containing equal amounts of analyte and internal standard were measured. Corrections for isotope contributions were made when necessary. Ion abundance ratios of analyte versus internal standard, in the range 0.2-2.5, had a linear relationship with corresponding concentration ratios. Concentrations were calculated by linear extrapolation according to equations outlined previously (15). Intra- and interassay coefficients of variation for urine and plasma determinations were in the 4-9% range. Concentrations were expressed in micrograms of free steroid/L. Accuracy was evaluated by standard additions made to a urine pool containing 50 μg/L of all analytes and to a plasma pool spiked with 0.5 μg/L of testosterone and androstenedione. Equations relating added to found steroid had slopes not significantly different from 1. The limits of quantitative detection were in the range 0.2-1 μg/L for urinary analytes and 0.05 μg/L for plasma estimations of testosterone and androstenedione.

Results and Discussion

Urine
Sulfoconjugates constitute the largest proportion of urinary androgens in the equine species, with the position on the steroid nucleus and the stereochemistry of the hydroxyl-groups as determinant factors for conjugating enzyme affinity. The glucuronide fraction is, however, far from negligible and may be preponderant under specific circumstances, such as for the 17α-hydroxy-metabolites. Sulfates and glucuronides were analyzed separately because specific enzyme hydrolysis of glucuroconjugates and the selective isolation of the sulfate fraction with subsequent methanolysis afforded cleaner extracts which were much more appropriate for subsequent GC–MS quantitation. Analytical data will, however, be presented as total conjugates (i.e., glucuronides + sulfates + minute amounts of nonconjugated androgen) because these are preferentially taken into account in the rules established by horseracing authorities for doping control purposes.

The time course of urinary testosterone in the gelding is displayed in the upper panel of Figure 1. The areas under the curves demonstrate higher conversion to testosterone after androstenedione administration, which is probably due to preferential hepatic conversion of androstenedione into testosterone, whereas DHEA is for a large part converted by a similar enzyme and by 17α-hydroxysteroid dehydrogenase/17α-reductase into the corresponding 5-androstenediols. The official testosterone concentration threshold for geldings of 20 μg/L was exceeded during a 24- to 36-h delay.

A similar pattern was observed in the mare (middle panel of Figure 1), where the recently established official testosterone
concentration threshold for mares of 55 μg/L was exceeded during a somewhat shorter period (19–23 h). The older testosterone threshold involving the testosterone/epitestosterone ratio of corresponding concentrations (T/E = 12, lower panel of Figure 1) was exceeded during 28 h after androstenedione administration. However, the T/E ratio remained well below 12 after DHEA administration because the conversion rate of DHEA to epitestosterone was much higher than for androstenedione. Thus, DHEA administration could not be evidenced with the T/E = 12 criterion. This is one of the reasons why a concentration threshold for testosterone in the female horse has been recommended, the other reason being the difficulty to quantitate low epitestosterone excretion accurately and precisely, generated as a consequence of negative feedback on pituitary luteinizing hormone production by exogenous testosterone. The highest testosterone concentrations attained after these oral supplementations of DHEA and androstenedione were at least one order of magnitude higher than those observed after intramuscular administration of testosterone ester at similar doses to female horses (16), but the washout period was of course drastically reduced.

The profiles of other significant urinary androgens were rather similar in the two genders, with highest excretions at a few hours post-administration (Table I). Urinary DHEA sulfate attained high levels after DHEA administration, but urinary androstenedione remained low because no conjugation has yet been proven for this diketone. Among the other 17-ketosteroids, etiocholanolone and epiandrosterone were the most abundant. Androstenediols and androstanediols, which are final products in the metabolic pathways regulated by enzymatic conversions prior to excretion, were always at high concentrations, with preferential 17α-reduction. Return to baseline excretion levels was rapid and mostly in the 48–60 h range. In general, increments above basal excretions were found higher in the gelding, who has no gonadal production of androgen, in contrast to the mare where ovarian testosterone production has been proven (17).

Concerning the detection period of biologically active androgen (mainly testosterone) generated by these nutritional supplements, it should be kept in mind that they have been devised for continuous oral administration at supra-physiological doses (1–5 mg/kg range), and therefore the delay of detectability may be implemented above the data found after a single loading.

**Plasma**

Time courses of the principal circulating androgens after dosing with DHEA or androstenedione are displayed on Figures 2 and 3. They indicate that significant amounts of these two steroids escape first-pass hepatic inactivation and arrive in the bloodstream as bioavailable DHEA, androstenedione, and testosterone. This means that supplementation of the horse with DHEA or androstenedione has the potential of producing biologically active androgen, which becomes available for anabolic action and thereby for possible physical performance enhancement. Unconjugated testosterone increase was quantitatively rather modest in both genders, and this is probably related to high metabolic clearance rate with rapid conversion to testosterone sulfate, which is a consequence of the absence of sex hormone binding globulin in the equine species.

Conversely, unconjugated DHEA attained high levels which were superior to the corresponding conjugate concentrations. In the horse, baseline plasma concentrations of DHEA and its sulfate are approximately two orders of magnitude lower than in primates, including humans. Therefore, DHEA supplementation in the equine would be potentially more beneficial than in young men (18) and more similar to the effects evidenced in cases of DHEA deficiency due to high age-related decline in humans (19). Thus, DHEA, which has no direct anabolic action, becomes available for intracrine conversion to testosterone and estrogen, besides its proper actions as a multifunctional steroid (with minor effects on immune and central nervous system and on the...
Contradictory data have been reported on the androgenic-anabolic effects due to androstenedione ingestion by eugonadal young men (4–7), where the failure of androstenedione intake to raise circulating testosterone significantly has been ascribed to hepatic and peripheral aromatization in muscle and adipose tissue. Our results in the horse, however, demonstrate that the rise of circulating testosterone was counteracted by strong concomitant conversion to the sulfate ester, which was a proof of efficient enteral absorption of androstenedione. Estrone and 17α-estradiol, which are particularly abundant in the equine species, were not quantitated in this study because it was anticipated that supplementary estrogen, generated by oral DHEA or androstenedione administration, would be without significance at the anabolic level.

In conclusion, as long as nutritional supplements such as androstenedione, DHEA, and other non-controlled steroids will be marketed freely for human consumption, there will subsist a temptation to misuse these over-the-counter drugs for anabolic purposes in racehorses. This study has demonstrated the efficiency of gastrointestinal absorption, leading to considerable first-pass inactivation in the liver, but also to enhancement of circulating biologically active androgen. Detection of misuse, based on screening and quantitative confirmation analysis of urinary total testosterone conjugates, meets the present doping control requirements.

References


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