Reprint from

RECENT ADVANCES IN DOPING ANALYSIS (10)

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Sport und Buch Strauß, Köln, 2002

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In: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck (eds.) Recent advances in doping analysis (10). Sport und Buch Strauß, Köln, (2002) 51-60

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Disposition of Androstenedione and Testosterone Following Oral Administration of Androstenedione to Healthy Female Volunteers; Influence on the Urinary T/E Ratio

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Introduction

Androstenedione (4-androstene-3,17-dione) is not considered a controlled medicine in most countries and is readily available as a food supplement, marketed as a 'prohormone' that can increase blood testosterone concentrations for anabolic purposes. Androstenedione is classed as an anabolic agent as defined by the International Olympic Committee (IOC) Anti-Doping Code and as such is banned in most sports. Usually the substance is marketed as capsules purporting to contain 50, 100 or 300 mg of androstenedione with the manufacturers' recommendation being 100 to 300 mg per day for muscle growth and improvement in training.

Androstenedione is secreted endogenously by the adrenal cortex and the gonads, and a small amount is also produced by peripheral conversion. Androstenedione does not itself bind to the androgen receptor but it is extensively metabolised to testosterone. In women about 60% of the circulating testosterone is derived from the metabolism of androstenedione [1]. It follows that androstenedione supplements may be used by sportswomen and female bodybuilders in an attempt to raise their endogenous testosterone to supraphysiological levels for anabolic purposes. In healthy men oral administration of androstenedione has little influence on the plasma testosterone concentration [2, 3], this being moderated by the large amount of the hormone produced by the testes.

There is a paucity of data regarding the amount of testosterone produced from oral administration of androstenedione to women as most published studies on androstenedione administration are based on male volunteers[2, 3]. A limited study has been performed where

three plasma samples were collected from two women (unknown age and health) after oral administration of 100 mg of androstenedione [4].

The objectives of this pilot study were three-fold. Firstly the effect of a single oral dose of 100 mg androstenedione to women on plasma testosterone and androstenedione concentrations was investigated. Although this dose was insufficient to raise serum testosterone concentrations in men [2, 3] it was thought likely to raise circulating testosterone in women considerably. Secondly, the method of detection used in routine testing, namely the testosterone to epitestosterone (T/E) ratio, was evaluated to determine the efficacy of such tests. Thirdly, the use of isotope ratio mass spectrometry (GC-C-IRMS) as an alternative technique for detecting the administration of this hormone was investigated. A placebo controlled double-blind crossover administration study was designed to evaluate these objectives.

Experimental

Preparation of androstenedione for oral administration

Androstenedione (Sigma, Poole, UK) was found to contain approximately 1 % w/w testosterone by GC-MS (HP6890-5973 GC-MS, HP-1 column, cross linked polymethylsiloxane) (*m/z* 432). To counter the suggestion that this may exaggerate any increase in blood testosterone it was purified by flash chromatography over aluminium oxide with cyclohexane:ethyl acetate as the mobile phase (50:50 v/v) prior to use. The recovered product was recrystallized from hexane and ethyl acetate (9:1 v/v) and purity determined by GC-MS and UV spectrophotometry. The amount of testosterone was below the limit of detection after purification (equivalent to less than 0.1% w/w).

Hard gelatin capsules were filled accurately (± 5%) with either 400 mg of lactose (placebo) or 400 mg of a triturated mixture containing androstenedione (100 mg) and lactose (300 mg), before being coded and passed to the investigators.

Administration study

Three healthy female volunteers (20, 23 and 25 years, 60, 59 and 60 kg) were recruited; the age range 18-34 was chosen to encompass the age of the majority of competing athletes. The effect of androstenedione on a foetus is unknown; inclusion criteria were therefore that all volunteers were using the oral contraceptive and that urine samples taken before commencing were tested negative for hCG.

The King's College London Research Ethics Committee approved the project and volunteers gave their informed written consent.

Each volunteer received a capsule containing either placebo or androstenedione with the alternative formulation (androstenedione or placebo) being given at least two weeks later to ensure total wash-out of the drug. A cannula was inserted into the cubital vein and blood samples were taken immediately prior to, and then every 15 minutes after administration of the capsule with 13 samples being collected up to the 8 hour point and two samples taken by venepuncture at 24 h and 30 h post administration. Basal urine samples were also taken at 24 h and 10 min before administration. The volunteers consumed 100 mL of water per hour for the first 8 hours to ensure that a urine flow was maintained and to avoid dehydration. Post administration samples were taken every two hours as total collections (0-2 h, 2-4 h, 4-8 h, 8-10 h, 10-12 h) up to twelve hours, then as single collections at 24 and 30 h.

The blood samples were immediately spun at 1200 × g for 15 minutes before the plasma was removed and frozen. The pH and specific gravity of the urine samples were measured before being frozen. All samples were stored at –40 °C until analysis.

Sample Analysis

The plasma samples were analysed for androstenedione and total testosterone (free testosterone, and protein bound (SHBG and albumin) testosterone) using commercial radioimmunoassay kits (DPC Ltd, UK). The cross-reactions of androstenedione and testosterone-glucuronide with the testosterone assay were found to be negligible (testosterone-glucuronide = 0.01 %, androstenedione = 0.16 %).

Pharmacokinetic parameters were calculated from the data obtained. The initial half-life was calculated from the log plot of the first three or four points after the C_{max}, whilst the terminal half-life was determined using linear regression of the final four points of each volunteer (6, 8, 24 and 30 h) from a plot, using log (conc.) versus time. The slope calculated equals the k_{elim} and from this the $t_{1/2}$ could be calculated ($t_{1/2}=0.693/k_{elim}$). The area under the curve (AUC) between 0 and 24 h was calculated using the trapezoid approximation.

For the evaluation of the concentration of testosterone, epitestosterone and androstenedione in urine the method was essentially that as described previously [5] and used with the Donike [6, 7] method of analysis of the trimethylsilyl derivatives by selected ion monitoring gas-chromatography-mass spectrometry (*m/z* 432, 430). A stock solution of the

following was prepared in water with all components at a concentration of 1 mg/L: testosterone, epitestosterone and androstenedione. This stock solution was diluted to produce calibrants at: 0, 0.75, 1.5, 3, 6, 12.5, 25, 50, 100, 150 ng/mL of each analyte. An aliquot of 2 mL of each calibrant was extracted along with 2 mL of each sample. A weighted linear regression line was fitted to enable quantification. The T/E ratio was calculated from the concentrations. Following extraction and derivatisation using the method described the samples were injected onto the GC-MS (HP-1).

Previous work has suggested 6β-OH metabolites of androstenedione (6β-OH androsterone, 6β-OH epiandrosterone and 6β-OH etiocholanolone) as indicative of administration, following analysis of separate fractions of the urine (free, glucuronide and sulphate) [8]. Thus in addition to the above, the peak height of these metabolites (free plus aglycone steroids) were monitored and compared to that of the trideuterated testosterone internal standard.

Further to the analysis by conventional GC-MS methods it was decided to analyse the urine samples using GC-C-IRMS, initially by the method described by Aguilera [9] based on the analysis of androsterone and etiocholanolone. This method is not thought to be as useful for sports samples as the methods using an endogenous reference compound, as baseline samples are required for comparison. However this approach does have the advantage that small amounts of sample are required and it is relatively straightforward for preliminary observations (it is anticipated that further analyses be run using these samples in the future). To evaluate the isotope ratio difference between the placebo and androstenedione administrations, the mean control δ values of androsterone and etiocholanolone were subtracted from the corresponding treatment values.

Results and discussion

Following the administration of androstenedione, plasma concentrations of androstenedione and testosterone increased rapidly (Figures 1 and 2 respectively). Plasma androstenedione increased from a mean value of 5.6 nmol/L to a maximum of 181 nmol/L (range 150-222 nmol/L) within 75 minutes of administration. The plasma androstenedione concentrations had returned to the control levels by thirty hours in two volunteers, whilst in one volunteer by 24 h.

The plasma total testosterone concentrations increased from a mean endogenous concentration of 0.3 nmol/L to a mean maximum concentration of 29.6 nmol/L, again within 75 min. The plasma testosterone concentrations had returned to control values by 24 h post androstenedione administration.

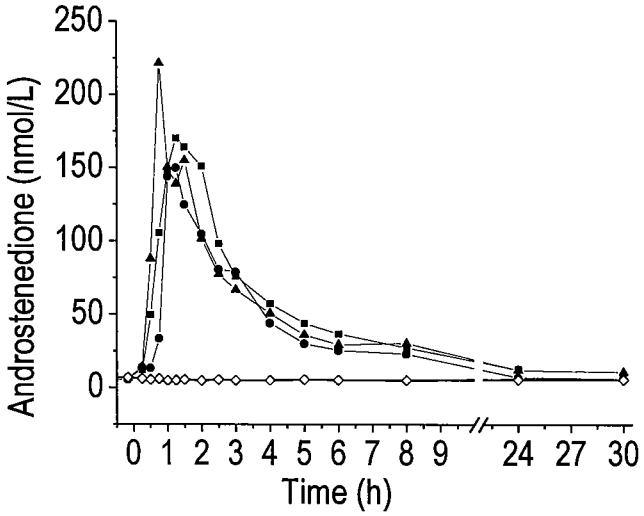

Figure 1. Plasma androstenedione concentrations for three volunteers post administration of 100 mg androstenedione with the mean control values (◊).

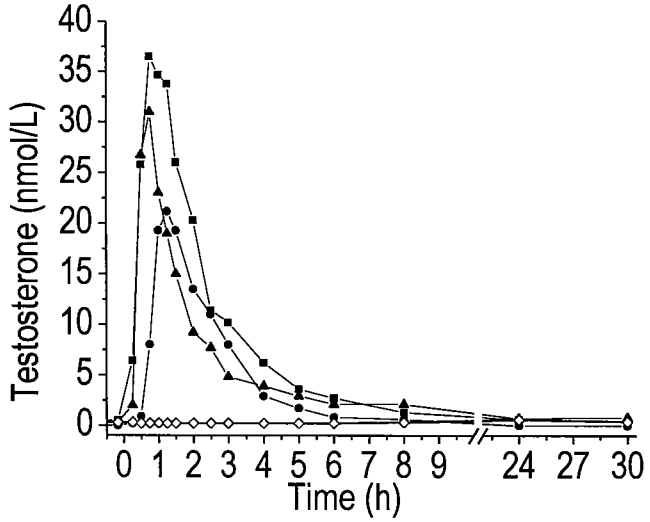

Figure 2. Plasma testosterone concentrations post administration of 100 mg androstenedione also showing mean control values (◊).

These data agree with that published by Mahesh and Greenblatt [4]. In that study a rapid increase in plasma testosterone concentration (between 4 and 7 times basal) was observed which was still augmented at 90 min, when the sampling stopped. Their results also showed an increase in androstenedione at 60 minutes and 90 min, with the baseline and 30 min samples being undetectable.

The pharmacokinetic parameters for both androstenedione and testosterone following administration of androstenedione are summarised in Table 1. The time curves approximate to biphasic elimination for both androstenedione and testosterone, giving two half lives, the initial and terminal. The comparison of the AUCs shows the relative amounts and exposure of each analyte present over the time course of the investigation.

Parameter	**Androstenedione**	**Testosterone**
C_{max} (nmol/L)	180 (150-220)	30 (21-36)
T_{max} (min)	45, 75, 75	45, 45, 75
Initial $t_{1/2}$ (h)	4.30 (4.1-4.5)	2.76 (2.4-2.9)
Terminal $t_{1/2}$ (h)	28 (25-33)	20 (11-31)
AUC(0-24 h)$_{drug}$ (nmol.h/L)	810 (680-880)	76 (50-98)
AUC Ratio (androstenedione/placebo)	6	12

Table 1. Pharmacokinetic parameters (mean and range).

The urinary T/E ratio time course profiles for the three volunteers are displayed in Figure 3, together with the IOC laboratory reporting threshold value of 6. The urinary T/E increased from a control mean of approximately 1 to a mean maximum of 15 at 6 h, returning to the basal value by 30 h. The T/E ratio remained above the reporting threshold of 6 only between 4 and 10 h in two volunteers but up to 24 h for the other subject.

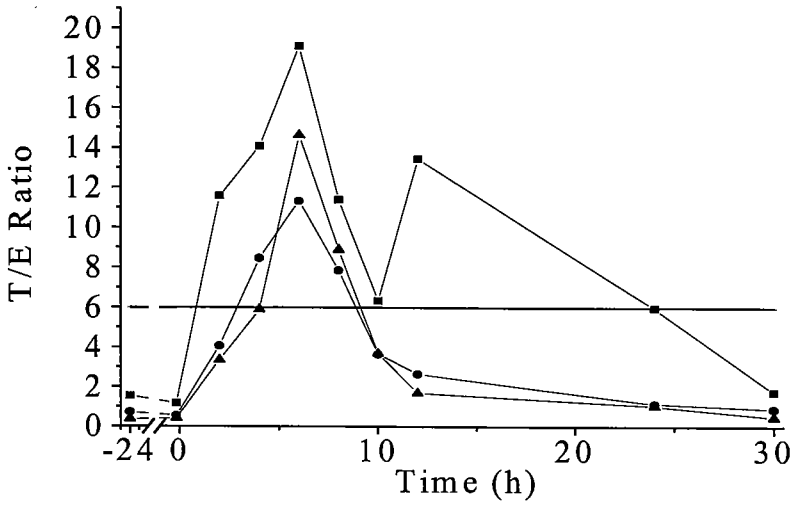

Figure 3. T/E ratio profiles for the three volunteers post androstenedione administration, together with the IOC laboratory reporting threshold value of 6, also showing –24 h samples.

The mean testosterone excretion rate for the first 8 h post androstenedione administration was greater than 50 μg/h compared to a mean value of 0.24 μg/h following placebo administration (Figure 4). An increase was also observed in urinary epitestosterone excretion following the administration of androstenedione, which was sufficient to attenuate the increase in the T/E ratio for the samples up to at least 6 h. The mean epitestosterone excretion rate for the 8 h following androstenedione administration increased from 0.26 μg/h to a mean of greater than 8 μg/h.

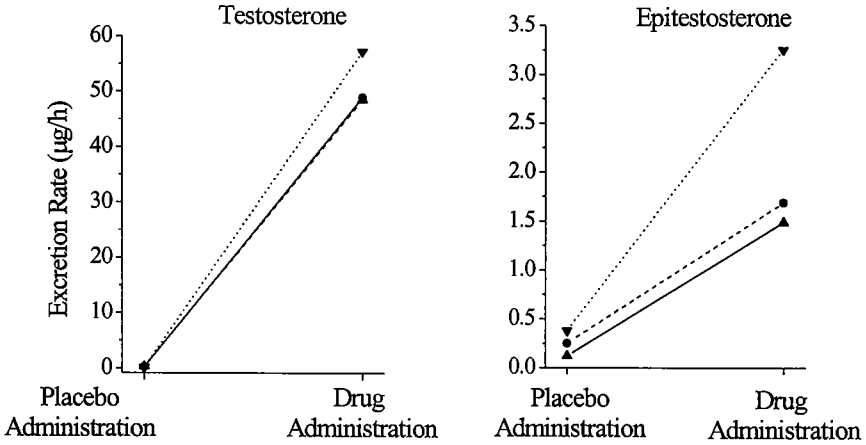

Figure 4. The mean 8 h excretion rates of testosterone and epitestosterone comparing androstenedione (100 mg) and placebo administration.

Upon analysis of 6β-OH androsterone, 6β-OH epiandrosterone and 6β-OH etiocholanolone a clear rise in the amount of all three of these metabolites was observed, lasting around 12 h. Further development work is required if these metabolites are to be of diagnostic value.

Following analysis of the samples using GC-C-IRMS the data was plotted as the individual profiles using the isotope ratio (δ) values of androsterone and etiocholanolone versus time (etiocholanolone traces shown in Figure 5) showing androstenedione and placebo administrations. These clearly show an immediate effect on the delta values, which remained below the values observed in the placebo phase for the duration of the study.

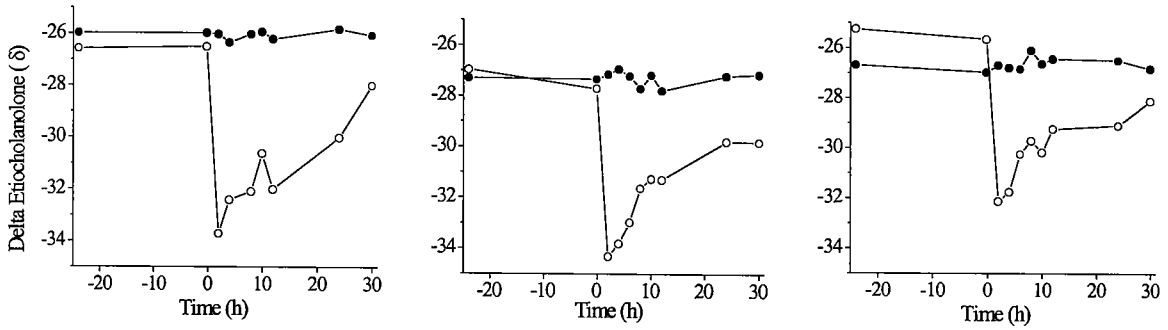

Figure 5. Individual plots of delta values (δ $^{13}C/^{12}C$ ‰) of etiocholanolone for drug (○) and placebo (●) administrations.

Once the mean of the control week values had been subtracted from the post androstenedione values a graph was plotted as shown in Figure 6. The dashed lines represent arbitrary decision thresholds, which have been set at a difference from the mean of 2.5 or 3. These values were chosen to be outside the normal range for individuals and instrumental variability. The mean δ (parts per mille) values following placebo administration were –26.02 ‰ (s=0.81) and–26.74 ‰ (s=0.55) for androsterone and etiocholanolone respectively. The 12 hour sample had differences from control value of greater than 3 ‰ from the mean placebo value in all subjects. If these were to be the criteria chosen then a detection window of greater than 12 h and up to 24 h would be obtained.

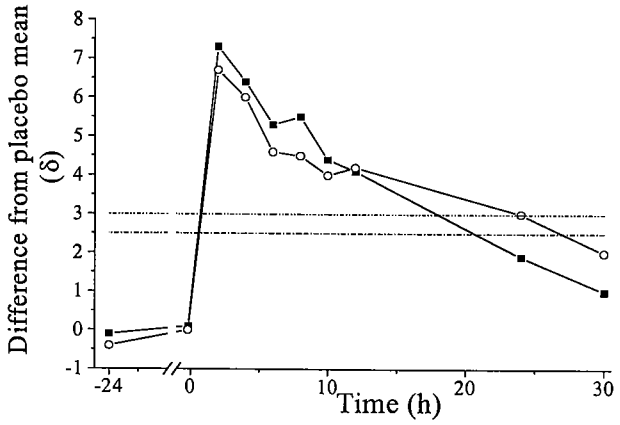

Figure 6. Mean delta values for androsterone (■) and etiocholanolone (○), after subtraction of corresponding placebo administration values.

Conclusion

Following administration of androstenedione, plasma testosterone concentrations of all three volunteers were elevated to a supra-physiological concentration for a female and the concentrations were in the upper end of the normal adult male physiological range (10-35 nmol/L) [10]. Although the rise in plasma testosterone lasted for approximately 8 h, if 100 mg was taken daily it would be sufficient to produce the effects that would aid female athletes. Chronic use of androstenedione could lead to the virilizing effects associated with androgens becoming apparent in females, including increased hirsutism, steroid acne and deepening of the voice [11], as well as other undesirable effects associated with supraphysiological concentrations of testosterone.

Despite the large increase in plasma testosterone and urinary testosterone excretion, the use of urinary T/E ratio as an indication of androstenedione abuse is limited as the window of detection is approximately 6 h. For greater retrospectivity the use of GC-C-IRMS would appear to be advantageous, but will ultimately depend on the decision criteria selected. If the absolute δ values of androsterone and etiocholanolone are to be used in a single untimed urine sample then it is important to agree what constitutes a positive sample, whether using an absolute cut-off value or other decision criteria. The androsterone and etiocholanolone method shown here indicates a window of detection of greater than 12 h, which is at least twice the length of detection window found using the T/E ratio. However, to improve doping control of androstenedione it would be necessary to increase unannounced out of competition testing, as

12-24 h is not a very large window of opportunity of detection. IRMS analysis of androstanediols using 5β-pregnanediol as an internal reference compound might further increase the detection time of doping with androstenedione.

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