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Effects of Oral Administration of Androstenedione on Plasma Androgens in Young Women Using Hormonal Contraception

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Androstenedione as a dietary supplement has been targeted at the sporting community, but there are limited data regarding its effects on plasma androgens in young women. A double-blind, cross-over study was undertaken involving 10 women (20–32 yr) using hormonal contraception. Because contamination of supplements has been reported, an in-house oral formulation was prepared containing purified androstenedione, the control being lactose only. After oral administration of a single dose of androstenedione (100 mg), blood was collected frequently up to 8 h and at 24 h. Maximum plasma androgen concentrations observed between volunteers were well above the upper limit of reference ranges for women, being 121–346 nmol/liter for androstenedione, 14–54 nmol/liter for testosterone (T), 11–32 nmol/liter for 5α-dihydrotestosterone, and 23–90 nmol/liter for 3α-androstanediol glucuronide. The free androgen index and T concentration changed in a similar manner. The mean change in area under the plasma concentration-time curve (0–24 h), compared with control data were: androstenedione approximately 7-fold, T approximately 16-fold, 5α-dihydrotestosterone approximately 9-fold, and 3α-androstanediol glucuronide approximately 5-fold; the mean conversion ratio of androstenedione to T was 12.5% (range 7.8–21.6%). Increases in T area under the plasma concentration-time curve were correlated with SHBG concentration ($r = 0.80$; $P = 0.005$). Formulation characteristics and SHBG levels appear to be important factors when considering plasma androgen increases after acute androstenedione administration. (*J Clin Endocrinol Metab* 89: 6030–6038, 2004)

ANDROSTENEDIONE IS WIDELY marketed as a dietary supplement or prohormone that increases the concentration of circulating testosterone (T), and as a consequence there is the suggestion that it results in increased lean body mass, strength, sporting performance, energy, and libido. Sales advertising appears to be mainly targeted at gym users, particularly body builders, with directions that desired effects can be gained by taking 100–300 mg androstenedione daily. It appears to be at least as widely used as legally controlled anabolic steroids (1–3), *i.e.* 3% of gym users in one region of the United States have admitted to using androstenedione (4). Androstenedione is exempt from control as a medicine or food additive because of the U.S. Dietary Supplement Health and Education Act of 1994. However, in March 2004 the Food and Drug Administration, launched a White Paper on the Health Effects of Androstenedione, together with accompanying documentation (Ref. 5; also see http://www.fda/gov/oc/whitepapers/andro.html). The FDA considers that these products may increase the risk of serious health problems because they are converted in the body to T, which is an androgenic and anabolic steroid. Furthermore, they consider that the requirements under the Dietary Supplement Health and Education Act to support that such dietary supplements are "not to be deemed adulterated" have not been met. As part of its public health mission, the FDA is committed to removing unsafe products, and therefore it has recently sent warning letters to 23 companies asking them to cease distributing androstenedione as dietary supplements.

The majority of published scientific work on the effects of androstenedione administration focus on men (6–13), in whom the steroid (50–300 mg daily) has modest or no effect in raising circulating T, and with these dose regimens, androstenedione supplementation appears to be ineffective in increasing lean mass and strength as shown by metaanalysis (14). In contrast, there have been few reports on the effects of androstenedione after administration to women. The conversion ratio for blood-borne androstenedione to T approximates 14% in both men and women, but the endogenous amount converted accounts for about half the circulating T in the female (15, 16). Hence, in women, a 100-mg dose has the potential to increase the relatively low concentrations of plasma or serum T, as shown in a limited study (two subjects) in the 1960s (17) and more recently in an investigation on postmenopausal women (18). In the FDA White Paper (5), under the section, Effects in women: predominant elevations in androgens, the results of two androstenedione acute administration studies are summarized: the study by Leder *et al.* (18) and our preliminary report describing the effects of

Abbreviations: AI, Androgen index; AUC, area under curve; C_{max}, maximum observed steroid plasma concentration; CV, coefficient of variation; DHT, dihydrotestosterone; D_3T, trideuterated (16,16,17α-^{2}H) T; GC-MS, gas chromatography-mass spectrometry; HRT, hormone replacement therapy; QC, quality control; T, testosterone; T-Gluc, testosterone glucuronide; t_{max}, maximum time.

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administration to young women (19). The latter age group is more likely to use androstenedione in an attempt to improve sporting performance or for body sculpture, and there are few publications regarding effects of administration on plasma androgens (19, 20).

In contrast to the study by Brown *et al.* (20), an inclusion criterion for our study was that volunteers were using oral contraception because in countries in which androstenedione supplements are most likely to be available, the use of oral contraceptives is prevalent. In addition, we wished to minimize the possibility of androstenedione being administered to pregnant women and potential androgenization of the fetus.

Subsequent to our preliminary report (19), we have now extended our investigation, providing further insights into the handling of oral androstenedione in the female, and here we report additional clinically relevant plasma androgen measurements and pharmacokinetic data, together with an assessment of T conjugate concentrations by mass spectrometry. Furthermore, we have characterized the particle size of the purified formulation we prepared by employing scanning electron microscopy. Previous studies investigating the effects of androstenedione administration on men and women have either not reported SHBG levels or have not highlighted this feature, which we believe is an important factor to consider when assessing the degree of androgen exposure, especially because the use of estrogen-containing oral contraceptives would be expected to raise SHBG levels (21, 22). We therefore examined whether the increase in plasma T area under curve (AUC) after androstenedione administration to the female volunteers was related to their SHBG concentration.

After androstenedione administration, plasma concentrations of androstenedione, total T (nonconjugated T, both free and bound to plasma proteins), 5α-dihydrotestosterone (DHT), and 3α-androstanediol glucuronide were compared with that after placebo administration. T and SHBG values were used to calculate the free androgen index (AI) (23). Because only a small amount of T is thought to enter the general circulation after oral administration of androstenedione (15), differential extraction of plasma total T from its glucuronide and sulfate conjugates was also performed for comparative purposes, at a limited number of time points due to the complexity of the analytical procedure.

Materials and Methods

Purification

The androstenedione (Sigma-Aldrich Chemical Co., Poole, UK) was found to contain approximately 0.9% (wt/wt) T by analysis of *bis*-trimethylsilyl ether derivatives (24, 25), using a method essentially as previously described employing gas chromatography-mass spectrometry (GC-MS) operating in selected-ion monitoring mode (ions *m/z* 430 and 432) (26). The T impurity was removed, with the intention of achieving a level of contamination of less than 0.1%, as frequently required for related substances in pharmaceuticals. For purification the purchased material was subjected to flash chromatography (27) using aluminum oxide [Brockman grade I, 0.05–0.15 mm (pH 7.0)] as the stationary phase and cyclohexane to ethyl acetate (1:1 vol/vol) as the mobile phase. Fractions of the eluent were collected, with a small portion of each being spotted onto thin-layer chromatography plates (Polygram ALOX N/UV_{254}; 0.2 mm thickness; Sigma-Aldrich Chemical Co.), together with standards of T and androstenedione. The plates were developed using the same mobile phase and the spots visualized by examination under UV light (254 nm). Eluent fractions found to contain only androstenedione were pooled, the solvent removed by evaporation, and the resulting solid recrystallized from a mixture of hexane/ethyl acetate (9:1 vol/vol). After purification of androstenedione, no peak corresponding to T was observed in the ion chromatogram (see *Results*).

Formulation

To aid dispersion and hence subsequent dissolution of the steroid, androstenedione was ground and triturated with lactose (androstenedione/lactose, 1:3 wt/wt) using a pestle and mortar. A weighed portion of the resulting powder was analyzed by UV spectrophotometry (240 nm) to ensure the mixture was homogenous. In addition, scanning electron microscopy was employed to establish the possible extent of reduction in particle size, in comparison with the starting mix. Capsules were then prepared containing either 400 mg of the triturated mixture, corresponding to a dose of 100 mg androstenedione, or 400 mg lactose (placebo); these were then coded and placed in sealed containers before being passed to the study investigators to maintain the integrity of the double-blind trial.

Study volunteers

Our local research ethics committee for human studies granted approval for the investigation, and the volunteers recruited (n = 10) gave their written informed consent. Criteria for volunteer participation included being in good general health, aged between 20 and 32 yr, and currently using oral contraceptives (see Table 1 for subject details). Exclusion criteria were a positive test for urinary human chorionic gonadotropin at 24 h before each administration or notification recommending exclusion by a participant's physician. None of the volunteers was taking any other medication during the study or had taken any medication that may have compromised the study in the preceding month.

TABLE 1. Volunteer anthropometrical data and oral contraceptive therapy details

Volunteer	Age (yr)	Height (m)	Mass (kg)	BMI (kg/m^2)	Contraceptive		
					Progestogen	Estrogen	UK trade name
1	20	1.72	60.3	20.4	Norethisterone acetate (1 mg)	Ethinylestradiol (20 μg)	Loestrin 20
2	20	1.67	63.5	22.8	Cyproterone acetate (2 mg)[a]	Ethinylestradiol (35 μg)	Dianette
3	25	1.77	65.0	20.8	Levonorgestrel (150 μg)	Ethinylestradiol (30 μg)	Microgynon 30
4	23	1.58	59.0	23.6	Desogestrel (150 μg)	Ethinylestradiol (30 μg)	Marvelon
5	24	1.72	78.0	26.4	Levonorgestrel (150 μg)	Ethinylestradiol (30 μg)	Ovranette
6	27	1.52	56.6	24.5	Levonorgestrel (150 μg)	Ethinylestradiol (30 μg)	Microgynon 30
7	22	1.66	59.4	21.6	Desogestrel (150 μg)	Ethinylestradiol (30 μg)	Mercilon
8	32	1.50	50.0	22.2	Levonorgestrel (150 μg)	Ethinylestradiol (30 μg)	Ovranette
9	21	1.78	69.9	22.1	Norethisterone[b]	Ethinylestradiol (35 μg)	Trinovum
10	24	1.60	57.2	22.3	Norethisterone acetate (1 mg)	Ethinylestradiol (20 μg)	Loestrin 20

[a] Dianette contains the antiandrogen cyproterone acetate (2 mg), which also has progestational activity.
[b] Trinovum is a triphasic contraceptive containing ethinylestradiol (35 μg) in all tablets, and varied quantities of norethisterone: 500 μg, 7 d; 750 μg, 7 d, and 1 mg, 7 d.

Study protocol

The investigation undertaken was a double-blind, cross-over study, with a minimum washout period of 2 wk between the administrations (19). Each of the 10 volunteers received a single unmarked capsule containing either the androstenedione (100 mg) formulation or the placebo at 1000 h. Blood (10 ml) was collected into glass tubes containing heparin via a cannula inserted into a forearm vein immediately before administration (0 h) and then at 15-min intervals up to 1.5 h followed by 2, 2.5, 3, 4, 5, 6,and 8 h post administration with a final sample taken by venipuncture at 24 h. After centrifugation, plasma was removed and frozen in 0.5 ml aliquots at −40 C until required for analysis.

Analysis of plasma

Immunoassay kits were used according to manufacturers' procedures to measure total T (Coat-A-Count, DPC, UK), androstenedione (Coat-A-Count, DPC), 3α-androstanediol glucuronide (Active, Diagnostic Systems Laboratories Inc., Webster, TX), 5α-dihydrotestosterone (Active, Diagnostic Systems Laboratories), and SHBG (DPC Immulite). The literature provided with the commercial kits included reference intervals; for androstenedione 1.4–9.4 nmol/liter in women aged 18 to 51 yr; for T, nondetectable to 1.0 nmol/liter for women taking oral contraceptives and for 3α-androstanediol glucuronide 1.0–11.5 nmol/liter for premenopausal women. Although no reference interval was provided for DHT in the kit insert, it has been reported to be between 0.17 and 1 nmol/liter (28). Validation data for each of the immunoassays were quoted in the accompanying information sheets, including specificity data for various steroids. Cross-reaction for the major androgen metabolite androsterone was negligible in the androstenedione and T assays (0.017 and 0.004%, respectively) and nondetectable as the glucuronide conjugate in the DHT and androstanediol assays. The T assay is not affected by variations in SHBG concentration. The cross-reaction of T in the androstenedione assay was stated to be 1.49%. We assessed the cross-reactivity of T glucuronide (T-Gluc), DHT, and androstenedione in the T assay and also performed some other additional validation studies (see *Results*). Some androstenedione and DHT samples were above their respective highest calibration points, and therefore the linearity (dilution recovery) of the assays were assessed (see *Results*).

The free AI was calculated by dividing the total T concentration by that of SHBG and multiplying the quotient by 1000 for each sample (23).

Differential extraction of T conjugates using solid-phase extraction cartridges (C_{18}) with appropriate elution solvents (methanol to water compositions), followed by deconjugation using β-glucuronidase [*Escherichia coli*; 5000 Fishman U/ml in 0.1 M phosphate buffer (pH 6.8)] and solvolysis, was employed (29) on selected plasma samples (75 min and 5 h post administration) to separate total T, T-Gluc, and sulfate ($T\text{-}SO_4^-$) before quantification by GC-MS analysis. An internal standard mixture containing trideuterated (16,16,17α-^{2}H) T (D_3T) and its conjugates, D_3T-Gluc and $D_3T\text{-}SO_4^-$ (all at ~10 nmol/liter) was added to each sample before analysis. Calibrants were prepared in water for T (concentrations: 3.5, 17.4, 34.7, 69.4, 174, 347 nmol/liter), $T\text{-}SO_4^-$ (concentrations: 2.7, 13.6, 27.2, 54.4, 136, 272 nmol/liter), and T-Gluc (concentrations: 2.1, 10.7, 21.4, 42.9, 107, 214 nmol/liter). After extraction each fraction was dried over desiccant (P_2O_5 and KOH) under vacuum for at least 30 min before analysis as the steroid *bis*-trimethylsilyl ether derivatives (24). After derivatization, 20 μl dodecane were added (containing 5α-cholestane at 25 mg/liter as an external standard to monitor injection reproducibility), and 1 μl of the solution was analyzed by GC-MS (HP 6890–5973), incorporating a methylsilicone column (HP-1). The temperature program used has been previously described (30), with selective ion monitoring of the individual analytes (T, *m/z* 432, 417; D_3T, *m/z* 435, 420; 5α-cholestane, *m/z* 372). Analyte concentrations were interpolated from calibration curves (weighting $1/x^2$), constructed from the ratio of the peak height of the derivatives of T to D_3T *vs.* concentration.

Data analyses

Repeated-measures ANOVA was employed to analyze the difference between treatment and control values at each time point for all the analytes using SPSS software (SPSS Inc. Chicago, IL). Only where a statistically significant difference was found ($P \leq 0.05$) was the Student's *t* test then applied for paired samples to locate the differences at specific time points.

The maximum observed steroid plasma concentrations (C_{max}) and the time to attain them (t_{max}) were obtained by inspection of the individual plasma concentration *vs.* time curves. The $AUC_{0-8\ h}$ and $AUC_{0-24\ h}$ were calculated for each steroid and volunteer by the linear trapezoid approximation.

Results

Androstenedione purification and formulation

Analysis of the purified androstenedione showed no detectable T (limit of detection < 0.1%, wt/wt), unlike the original material (Fig. 1). No chromatographic peak greater than 0.1% of the androstenedione signal was observed. Furthermore, full-scan MS data showed only characteristic ions for the *bis*-trimethylsilyl derivative of androstenedione (*m/z* 430, 415, 234). Quantitation by UV spectrophotometry of the formulated androstenedione was consistent with the target amount of 95–105 mg androstenedione per capsule. Analysis of the androstenedione and lactose mixture by scanning electron microscopy showed a considerable reduction in particle size after grinding and trituration (Fig. 2).

Assay validation data

Immunoassays. The cross-reactivity of the T assay was determined at 50% displacement of the activity measured in the zero calibrant (50% B/B_o) and found to be 2.8% for DHT but negligible for T-Gluc (0.01%). Cross-reactivity with androstenedione was 0.16%, and this was not sufficient to interfere significantly with the T analyses after androstenedione administration. Between-assay coefficients of variation (CVs) (n = 10) were 9.5, 8.5, and 7.3% at plasma T concentrations of 5.4, 12.6, and 35.4 nmol/liter, respectively. Because the

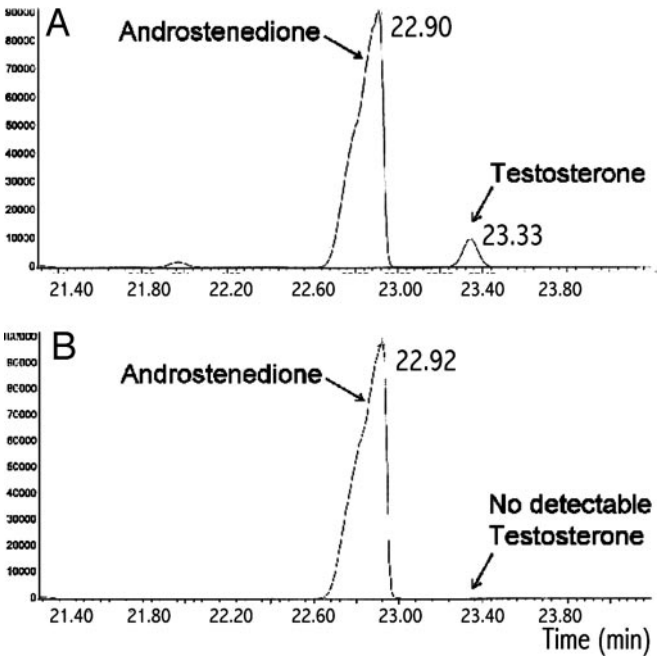

FIG. 1. Ion chromatograms of the molecular ion (m/z 432) of the *bis*-TMS derivatives of T and the isotope of androstenedione before (A) and after (B) purification of androstenedione. A peak was not observed at time 23.33 min in chromatogram B. Note: The peak corresponding to the isotope of androstenedione is front-tailed as a result of the vast excess of androstenedione injected in order to analyze for impurities.

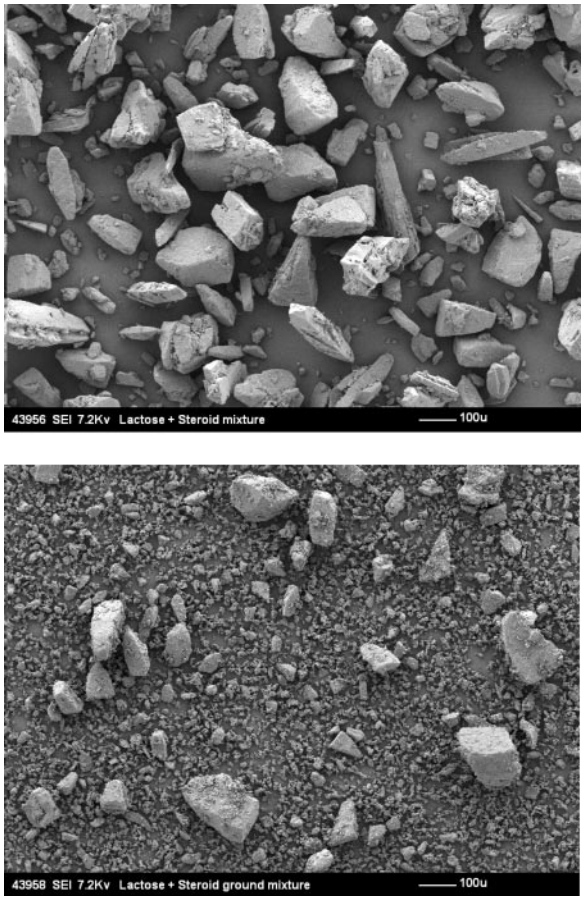

FIG. 2. Scanning electron microscope images of androstenedione and lactose mixtures both before (*top*) and after (*lower*) grinding and trituration. In the *upper scan*, the needle-like crystals are the steroid and the amorphous particles are the diluent (lactose). The 100-μm scale indicates the considerable decrease in particle size.

measured T in the samples was considerably lower in concentration than androstenedione, any increase in androstenedione values due to cross-reactivity (of 1.49%) with T would be marginal, and thus no correction was applied. The between-assay CVs (n = 12) were 9.6 and 10.1% at plasma concentrations of 4.6 and 12.6 nmol/liter, respectively. The accuracy of dilution for samples above the calibration range was examined by addition of 258 nmol/liter of androstenedione to control samples, yielding a recovery of 111% after a 10-fold dilution.

The DHT between-assay CV (n = 7) was 13.5 and 17.5% for plasma concentrations of 0.39 nmol/liter and 1.6 nmol/liter, respectively. Four- and 8-fold dilution of a sample to which DHT had been added (14 nmol/liter) gave a dilution recovery of 109 and 112%, respectively. For androstanediol glucuronide, the between-assay CV (n = 10) was 3.7 and 2.6% for plasma concentrations of 5.1 nmol/liter and 19.8 nmol/liter, respectively.

Within-assay imprecision for SHBG measurement was 2.4, 6.1, and 4.1% at 9.9, 33, and 64 nmol/liter, respectively. At similar concentrations, the between-assay imprecision was 6.4, 8.8, and 7.9%.

Measurement of T conjugates. For the analysis of T conjugates, two different quality control (QC) samples, A and B (pooled plasma from women to which T, T-Gluc, and T-SO_4^- had been added) were analyzed with each batch of plasma samples. The results were as follows (n = 7): QC A; T mean 6.8 nmol/liter, CV 8.3%; T-Gluc mean 12.1 nmol/liter, CV 17.5%; T-SO_4^- mean 8.2 nmol/liter, CV 10.3%; QC B: T mean 20.5 nmol/liter, CV 8.6%; T-Gluc mean 48.8 nmol/liter, CV 8.5%; T-SO_4^- mean 14.3 nmol/liter, CV 12.3%), all calibration lines had $r > 0.99$.

Plasma steroid profiles

The mean plasma concentration *vs.* time profiles of T, androstenedione, DHT, and androstanediol glucuronide are shown in Fig. 3, and the pharmacokinetic parameters derived from an examination of the individual volunteer profiles are presented in Table 2. The differences between treatment and control plasma concentration values were highly significant for the above hormones ($P \leq 0.0005$) by repeated-measures ANOVA. After treatment, the increase in plasma concentrations of these steroids was rapid and remained augmented, all showing similar time-course profiles, over approximately the first 8 h. Subsidiary tests showed that plasma androstenedione concentrations were significantly different from those of control values at 15 ($P \leq 0.05$), 30, and 45 min ($P \leq 0.01$) and 1–24 h inclusive ($P \leq 0.001$); for plasma T concentrations at 30 min ($P = 0.014$) and between 45 min and 8 h inclusive ($P \leq 0.002$); for DHT from 15 min to 8 h inclusive ($P \leq 0.01$); for 3α-androstanediol glucuronide at 75 min ($P \leq 0.05$) and between 1 and 24 h inclusive ($P \leq 0.01$).

There was no significant difference ($P = 0.5$) between SHBG measurements for the treatment and control period (Fig. 4). The profile of the free AI was similar to that observed for total T during the treatment period (Fig. 3). The differences between treatment and control values were highly significant ($P \leq 0.0005$), the subsidiary test locating differences between 30 min and 8 h after androstenedione administration (all differences with $P \leq 0.005$).

Dispositional data for each steroid, derived from the examination of the individual plasma concentration *vs.* time curves (AUCs) are presented in Table 2. A typical logarithmic-transformed concentration plot is shown in Fig. 5, illustrating the biphasic nature of the profile for both androstenedione and T after treatment and the parallel decline in concentration of the two steroids. Plasma concentrations of androgens were still significantly raised at 8 h after androstenedione administration, and therefore AUC values over the sampling period of the study ($AUC_{0-24\ h}$) were calculated (Table 2), together with $AUC_{0-8\ h}$ to allow particular comparison with data from a study by Leder *et al.* (18). Comparison of the AUCs between the treatment and control study periods was used to provide an index of exposure after androstenedione administration. The ratio of the $AUC_{0-24\ h}$ (values in *parentheses* are the $AUC_{0-8\ h}$ ratios) of the treatment to control period for each individual showed a mean increase for androstenedione of 7.3 (14.8)-fold; T of 15.7 (44.7)-fold; DHT of 9.4 (27.3)-fold; and 3α-androstanediol glucuronide of 4.7 (7.3)-fold. The ratios of the T $AUC_{0-24\ h}$ to androstenedione $AUC_{0-24\ h}$ for each volunteer (corrected for the corresponding areas for the placebo values), expressed as a percentage, yielded a mean value of 12.5% (range 7.8–21.6%). A correlation (r = 0.80; $P = 0.005$) was observed between the treatment $AUC_{0-24\ h}$ for T, corrected by subtraction of control

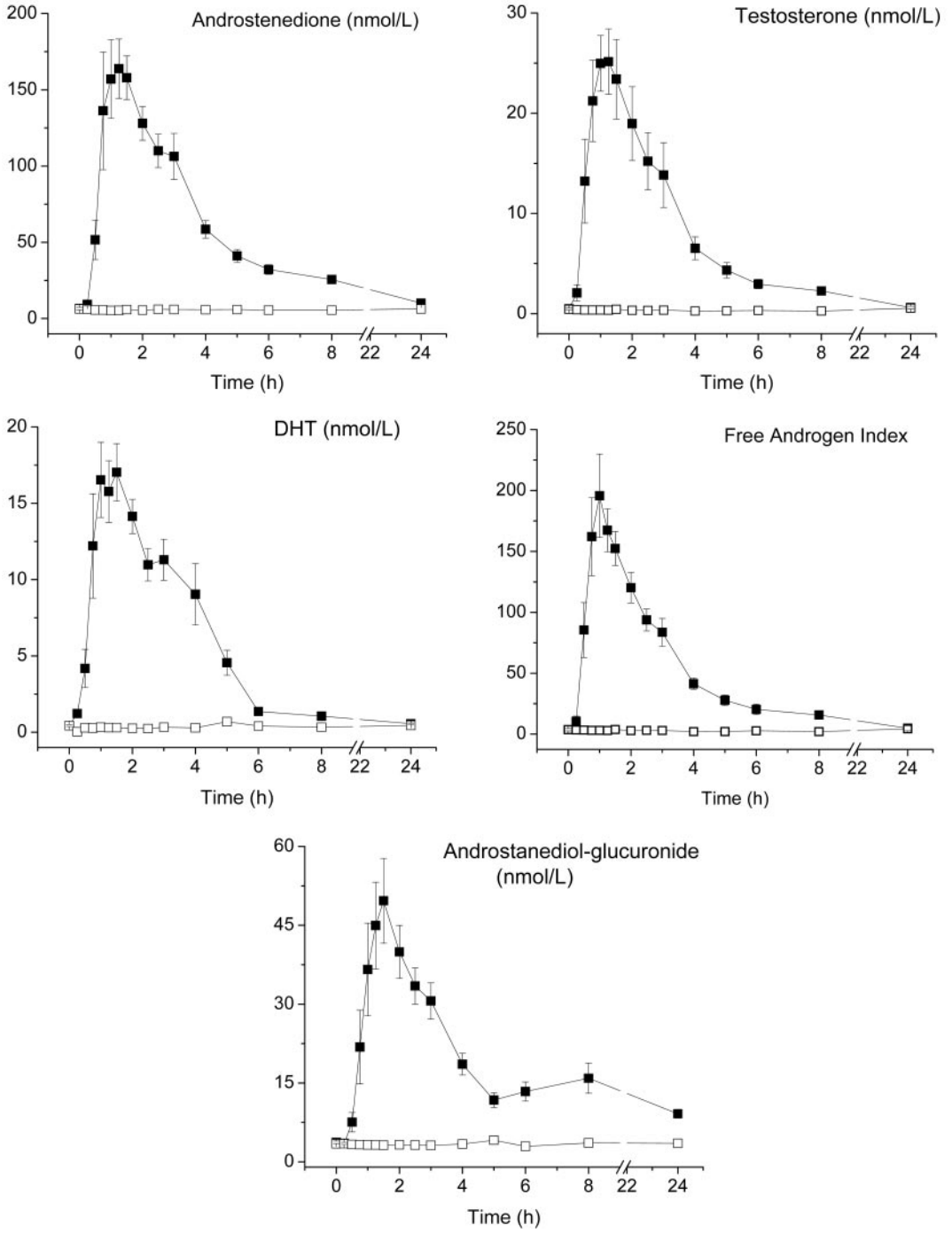

FIG. 3. Plasma steroid hormone concentrations and derived free AI (mean ± SEM) after oral administration of androstenedione (■) or placebo (□) in 10 healthy young women. Note: SEM values were too small to be displayed for the placebo intervention. The plasma concentration-time profiles of androstenedione and T and derived data are reproduced from a Technical Brief published in Clinical Chemistry (reproduced from Ref. 19). Permission granted by the American Association for Clinical Chemistry.

values, and SHBG concentration (Fig. 6). As expected, there was no significant correlation between SHBG and androstenedione ($r = 0.32$; $P = 0.36$).

T conjugates

The respective mean (SEM) control values of T concentrations after differential isolation of the conjugates at 75 min and 5 h were: T 1 (0) and 1.4 (0.3) nmol/liter; T-Gluc 6.4 (5.4) and 3.2 (1.5) nmol/liter; and T-SO_4^- 4.2 (1.2) and 4.7 (2.4) nmol/liter. After treatment the values at 75 min and 5 h, respectively had increased to: T 21.2 (3.7) and 3.0 (0.8) nmol/liter; T-Gluc 56.1 (11.6) and 5.7 (1.9) nmol/liter; and T-SO_4^- 18.8 (4.0) and 8.8 (2.7) nmol/liter (Fig. 7). Compared with control data, T was found to be significantly elevated at both

TABLE 2. Disposition of plasma androgens after oral administration of androstenedione (100 mg) to healthy female volunteers (n = 10)

Parameter	Analyte			
	Androstenedione	T	DHT	3α-Androstanediol glucuronide
C_{max} (nmol/liter)				
Mean (SD)	204 (76)	30.5 (12.4)	21.1 (6.8)	52.2 (25.6)
Range	121–346	14–54	11–32	23–90
t_{max} (h)				
Mode	0.75	0.75	1.0	1.5
Range	0.75–3	0.5–3	0.75–4	1–3
$AUC_{0-8\ h}$ (nmol·h/liter)				
Treatment mean (SD)	562 (150)	76 (36)	54.3 (15.3)	172 (52)
Range	346–676	37–142	34.4–90.0	87–228
Control mean (SD)	46 (19)	2.6 (2.4)	2.3 (1.0)	27 (9.4)
Range	28–81	1.1–8.2	1.0–4.5	16–44
$AUC_{0-8\ h}$ ratio (treatment/control)				
Mean (SD)	14.8 (7.8)	44.7 (29.2)	27.3 (10.7)	7.3 (3.4)
Range	4.6–27	7.1–94.6	14.6–49.9	2.5–12.2
$AUC_{0-24\ h}$ (nmol·h/liter)				
Treatment mean (SD)	847 (195)	98 (46)	70 (2)	358 (105)
Range	500–1200	49–195	47–118	203–551
Control mean (SD)	140 (64)	9.3 (7.7)	9 (4)	84 (27)
Range	82–277	3.4–22.7	4.2–17.8	46–131
$AUC_{0-24\ h}$ ratio (treatment/control)				
Mean (SD)	7.3 (3.6)	15.7 (8.9)	9.4 (4.9)	4.7 (2.0)
Range	2.4–12.5	3.3–27.3	3.6–21.4	1.8–8.1

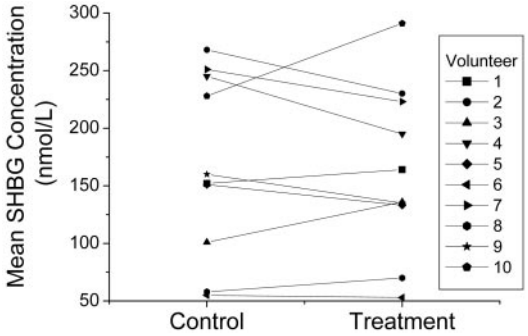

FIG. 4. Mean SHBG concentrations on the control and treatment days.

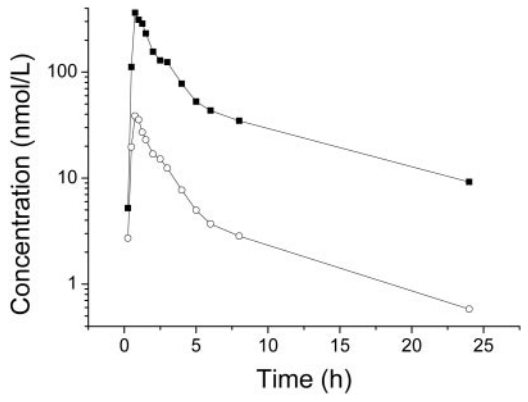

FIG. 5. Logarithmic-transformed plasma concentration *vs*. time plot for androstenedione (■) and T (○) from a typical volunteer after treatment, showing the biphasic nature of elimination and the essentially parallel decline of the two hormones.

75 min ($P < 0.001$) and 5 h ($P \leq 0.05$), T-Gluc at 75 min ($P < 0.01$) and 5 h ($P < 0.05$) and T sulfate at 75 min ($P < 0.01$) and 5 h ($P < 0.05$).

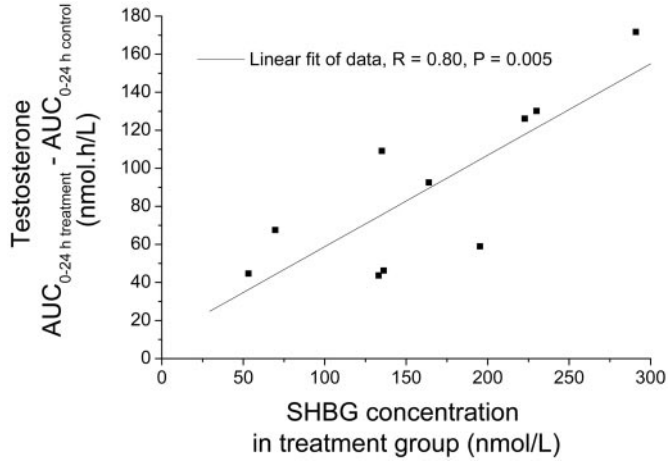

FIG. 6. Correlation between mean treatment SHBG concentration (nmol/liter) and increase in plasma testosterone exposure ($AUC_{0-24\ h}$ treatment value minus the $AUC_{0-24\ h}$ control).

Discussion

Steroid preparations marketed as nutritional supplements have been found to contain undisclosed steroids. In the case of androstenedione, supplements have been found to be contaminated with other androgens, such as 19-norandrostenedione or T or, alternatively, contain no androstenedione at all (18, 31). A large number of products are available, and, because no regulatory standard exists, these have the potential to result in highly variable steroid plasma profiles. In addition, we are not aware of any published data concerning the significance of particle size and formulation on the absorption of androstenedione. In an attempt to avoid the above problems, we decided to prepare and characterize a formulation. This approach also had the additional advantage that a placebo formulation could not be distinguished visibly from the drug, thus preserving the double-blind

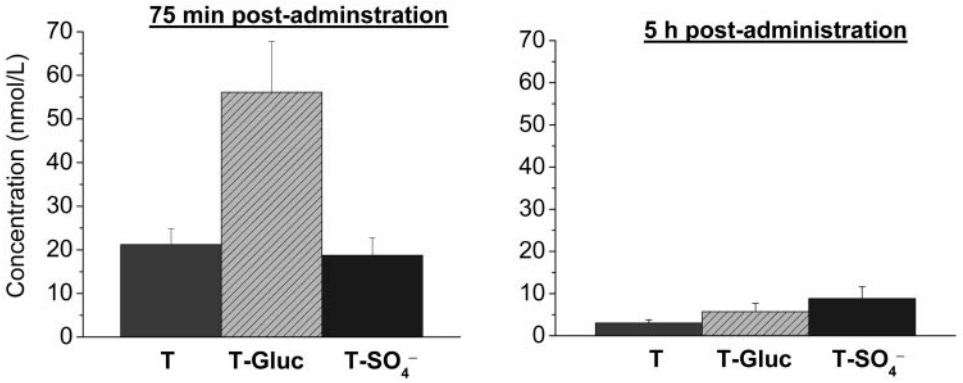

FIG. 7. T, T-Gluc, and T sulfate (T-SO_4^-) concentrations as determined by GC-MS at 75 min and 5 h post androstenedione administration, all of which were significantly greater than the corresponding control values (see text).

study design. However, the purchased androstenedione was found to contain a small amount of T (0.9%, wt/wt), and, to eradicate the possibility that this might have caused any increase in exposure to either the steroid or its metabolites, the androstenedione was purified, the T content being reduced to less than 0.1% (wt/wt) (Fig. 1). The purified androstenedione was reduced in particle size when ground and triturated with lactose, as confirmed by examination of the resultant powder by scanning electron microscopy (Fig. 2). Androgens are hydrophobic, and mixing with lactose reduces aggregation of the steroid both before administration and in the gut. Thus, the formulation of the steroid with lactose would be expected to aid dispersion and subsequent dissolution of the microparticulate drug.

After treatment, the mean increase in exposure (as measured by increases in $AUC_{0-24\ h}$ to androstenedione was approximately 7-fold, and that of T was greater than an order of magnitude, compared with the control period; the mean plasma T increased to supraphysiological concentrations (much greater than the reference range upper limit of 3.5 nmol/liter in women) (28). Plasma concentrations of the androgens measured were still noticeably greater than control values at 8 h (and small but statistically significant differences were still observed at 24 h for plasma androstenedione and 3α-androstanediol glucuronide).

The large increases in exposure to androstenedione and T in the present study contrast somewhat with those reported by Leder *et al.* (18), in which postmenopausal women received 100 mg oral androstenedione. In the latter investigation, the sampling period was for 12 h, but the mean serum androstenedione concentration was comparable with baseline values at 10 h and the T concentrations were no longer statistically different from baseline; the mean percentage increases in AUC for androstenedione and T, compared with the control group, were approximately 250 and 460%, respectively. By contrast, in the present study, the ratio of $AUC_{0-8\ h}$, expressed as a percentage, was considerably larger, at 1480 and 4470% for androstenedione and T, respectively, in comparison with control values (the corresponding 24-h values being 730 and 1580%; Table 2). Although Leder *et al.* used a separate control group for their study, in contrast to our cross-over, placebo-controlled study design, it is unlikely that this would account for the large difference in the AUCs between the investigations. Moreover, Brown *et al.* (20) administered placebo, 100 and 300 mg of androstenedione to young women not using oral contraceptives, the results indicating some similarity to those found by Leder *et al.* (18). However, it is difficult to comment further, given that Brown *et al.* curtailed their study after 4 h, when serum androstenedione and T concentrations appear to be approaching maximum observed steroid plasma concentrations and also that the AUC values that they quote with placebo administration do not appear to agree with their presented figures. As an adjunct, concerns have been raised over the accuracy of immunoassays for T measurement (32, 33), based on studies by Wang *et al.* (34) and Taieb *et al.* (35), but the Coat-A-Count nonautomated RIA (DPC) has been found to show the best agreement with values determined by liquid chromatography tandem mass spectrometry (34), although this immunoassay overestimates (>20%) serum T in the female reference range (<3.5 nmol/liter). Of note, we used the Coat-A-Count RIA (DPC) for T as did Leder *et al.* (18) and Brown *et al.* (20), thus eliminating bias of measurement when comparing values between studies. We consider that the overestimation of low levels of T by immunoassay is very small, compared with the large differences between the control and high treatment values observed in our study.

Assuming there is no large difference in steroid metabolic pathways between pre- and postmenopausal women, then the difference in androstenedione AUCs found between studies may be rationalized by the differences in androstenedione products (note: for T the concentrations of SHBG also need to be considered; see later text); in the study by Leder *et al.* (18), androstenedione obtained in bulk powder form was placed directly into capsules whereas in our study it was first ground and triturated with lactose. This explanation is further supported by the fact that the plasma androstenedione exposure in the current study was also larger than that observed in some studies in which the equivalent dose of the steroid (not ground and triturated), rather than our formulated product, has been administered to healthy men (6, 7). As an independent check of the veracity of the increase in plasma androstenedione, we compared the GC-MS responses (in selected-ion monitoring mode) to androstenedione in samples at 75 min (modal t_{max}) and found that the mean quotient in response between treatment and control samples (~34-fold increase) was comparable with that observed by immunoassay (Fig. 3). Further studies would indicate the significance of variation in androstenedione particle size on androgen plasma concentrations.

Additionally, we observed very large increases in concentrations of DHT (an androgen not measured in other androstenedione administration studies), a metabolite that binds with greater affinity to the androgen receptor than T (36, 37). The large and contemporaneous increase in androstanediol glucuronide can be considered to arise as a result of metabolism of DHT to another potent androgen receptor ligand, 3α-androstanediol, albeit it also results from metabolism of the glucuronide conjugate of DHT itself (Ref. 38 and references therein). The increase in the free AI reflected that observed for total T concentration because there was no change in SHBG concentrations within subjects over the study period, the free AI being regarded as a surrogate for free T measurement. Plasma estrogens were not measured because all the volunteers were using oral contraceptives containing ethinylestradiol, a cross-reacting steroid known to elevate estradiol results in immunoassays to a considerable extent.

Oral contraceptive therapy containing ethinylestradiol raises plasma concentrations of SHBG (22). Because hepatic clearance of T is inhibited when it is bound to this β-globulin, the increased exposure to plasma total T after acute administration of androstenedione was expected to be related to the SHBG concentration. Indeed, this supposition is supported by the strong positive correlation between increased T exposure (as measured by difference in $AUC_{0-24\ h}$, Fig. 6) and the concentration of SHBG. Whereas direct comparison of exposure to plasma androstenedione can be made among various studies (androstenedione binds with low affinity to SHBG, and hence its clearance rate is unlikely to be affected by variation in SHBG concentration), when comparing total T exposure, it is also important to allow for large differences in SHBG. Leder *et al.* (18) assessed the acute hormonal effects of androstenedione on postmenopausal women to assess its potential for hormone replacement therapy (HRT), but SHBG values were not reported. Postmenopausal women, who have abstained from HRT for several months before administration of androstenedione, are likely to have considerably lower SHBG levels, compared with premenopausal women using combined oral contraceptives containing ethinylestradiol (21, 22). The SHBG concentrations observed in the present cohort, and consequent additional T binding capacity, is anticipated to contribute noticeably to the increase in exposure to the total plasma T observed in the present study, compared with that of Leder *et al.* (18), androstenedione formulation variations between the two studies notwithstanding. Despite not standardizing the volunteers on the same oral contraceptive, which we believe would have been an unreasonable request, the $AUC_{0-24\ h}$ of total T between individuals with treatment gave a relative SD of 47%, a variation considerably smaller than might be expected if the type of mixed contraceptive used was an important factor. It is noteworthy that, in keeping with the law of mass action, there is no contradiction between the increase in the concentration of SHBG-bound T and a concordant increase in free T concentration, as might be reasonably surmised by the increased AI.

The mean ratio of the $AUC_{0-24\ h}$ of plasma T to that of androstenedione was calculated to be 12.5%, a value within the conversion ratio of 12–14% for blood-borne androstenedione to T, the conversion ratio being defined as the concentration ratio of T/androstenedione resulting from iv infusion of labeled androstenedione (15, 16). Nonetheless, caution must be exercised in comparison of these results, given the differences in route of administration, dose, and methodology employed. With regard to the proportion of phase II metabolites of T formed, it is important to consider first-pass metabolism. Horton and Tait (15) reported that after oral administration of ^{14}C-labeled androstenedione (total mass infused into the stomach over a 2-h period was 0.03 μg; mass calculated from the infusion dose of 4 μCi with a specific activity of 137 μCi/μg), the steroid enters the liver in which a large proportion is metabolized to T and subsequently T conjugates, with only a small amount of unconjugated T entering the general circulation. With such efficient hepatic conversion of androstenedione to T conjugates, it might be expected that after a 100-mg oral dose of androstenedione, the plasma concentration of T-Gluc and/or sulfate would greatly exceed that of unconjugated T. To examine this hypothesis, we determined the concentrations of the glucuronide and sulfate conjugates of T at 75 min and 5 h after treatment, 75 min being approximately the t_{max} and 5 h being well into the elimination phase, for unconjugated T. However, plasma concentrations of T conjugates did not greatly surpass those of unconjugated T in these samples, T-Gluc being 2- to 3-fold greater. The significance of this data is difficult to discern, taking into account the parallel pathways of glucuronidation and sulfation, but one or more of the metabolic pathways may be saturated.

Chronic administration of large doses of androstenedione to women is expected to result in sustained supraphysiological concentrations of T and DHT. As a result, the plasma concentrations of SHBG would be expected to decrease considerably, thus increasing the concentration of free T and thereby enhancing androgenic effects. Androstenedione administration also results in increased estrogen concentrations (18), but any agonist effect on SHBG synthesis is likely to be largely outweighed by the antagonistic effect of raised T concentration.

There is some interest in the potential value of oral androstenedione as a medicine, compared with traditional HRT in postmenopausal women (18). Long-term studies are required to elucidate whether the oral steroid is of therapeutic benefit, with judicious consideration as to the preparation of the formulation and the dose regimen required, thereby controlling the amount of T formed for androgen supplementation. The degree of exposure to T, and also DHT and androstanediol, in women after oral androstenedione will also depend on the subject's SHBG level. Because androstenedione has been categorized as a dietary supplement, some may construe that it is safe to use. This notion may explain, at least partially, why some nutritional magazines recommend administration of about 1000 mg prohormones per day and make no distinction between the size of dose for men and women (Geyer, H., personal communication). Even allowing for possible differences between androstenedione preparations, women chronically administering large doses of androstenedione, without medical supervision, is a cause for concern due to increased androgen exposure, although it would be unethical to prove that adverse effects, such as virilization, can result because of androstenedione administration. Given the good reasons for the restricted sale of T, the

findings from this investigation support similar restrictions on the sale of androstenedione.

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