Detection of Simultaneous Self-Administration of Testosterone and Epistosterone in Healthy Men

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Combined self-administration of testosterone (T) and epitestosterone (ET) by athletes counteracts the efficiency of the corresponding urinary glucuronides (G) ratio, (T/ET)G, as an indicator of T abuse. I therefore propose 5-androstene-3β,17α-diol (5A3β17α), the immediate metabolic precursor of ET, as a new reference compound for the expression of relative excretions of T and ET. Thus (T/5A3β17α)G and (ET/5A3β17α)G become potential criteria to indicate joint administration of T and ET, since their respective threshold values (2.5 and 1.5) are exceeded even when (T/ET)G remains below the critical value of 6.

Indexing Terms: steroid metabolism/glucuronide metabolites/5-androstene-3β,17α-diol/abused drugs/sports medicine

Detection of exogenous testosterone (T), administered orally or as injectable preparations, requires a quantitative analytical procedure such as gas chromatography–mass spectrometry (GC-MS) performed on the most specific urinary metabolite of T, the glucuronide (G). The critical point of analyzing unimpaired urine samples for doping control has imposed the use of a relative urinary excretion. The ratio (T/ET)G (ET, epitestosterone, 17α-hydroxy-4-androsten-3-one) was found to be adequate, and a cutoff point of 6 was adopted by the Medical Commission of the International Olympic Committee (Los Angeles, 1982), according to work by Donike et al. (1). Although the average (T/ET)G ratio found by several laboratories (1–4) was between 0.9 and 1.6 in a population of healthy men and male adolescents, in some rare cases healthy males may have (T/ET)G ratios exceeding the threshold value, without any supply of exogenous T (5–8). Therefore, some complementary criteria have been suggested, such as the urinary TG/luteinizing hormone (LH) ratio (2) and the serum T/17-hydroxyprogesterone ratio (2). We demonstrated recently (8) that it is advisable to take into account the two excretion forms of ET, the glucuronide (ETG) and the sulfate (ETS), and to use the ratio TG/(ETG+ETS). Indeed, a high (T/ET)G ratio may be due to decreased ETG output, which can be compensated for by increased ETS excretion, resulting in a normal TG/(ETG+ETS) ratio.

Because the joint misuse of T and ET may lead to false-negative test results, the guidelines of the international Olympic Committee for 1992 recommend that urinary ETG concentrations >150 µg/L be noted as abnormally high and therefore suspicious. Thus there is a need for an objective urinary test to detect surreptitious ET administration that is less dependent on urine flow than is the ETG concentration limit.

Information is scarce on the biochemical pathway(s) leading to production of ET in general, and particularly production in the testis. Until now, there is no evidence of conversion of androstenedione to ET in the testis because no testicular 17α-hydroxysteroid dehydrogenase activity has been detected. An interesting proposal was formulated by Weusten et al. (9) on the basis of in vitro experiments with human testicular microsomes. These authors suggested that biosynthesis of 5-androstene-3β,17α-diol (5A3β17α) occurs as a byproduct of 16α-3α-isomerase action, which converts pregnenolone into 5,16-androstadien-3β-ol by a single competitive mechanism. The identification of 5A3β17α in human testis tissue (10) and its gonadal secretion (11), as well as the significant correlation between ET and 5A3β17α concentrations in human spermatic vein plasma (12), are all pertinent arguments in favor of the suggestion that this androstenediol is a substrate for 3β-hydroxysteroid dehydrogenase/4β-isomerase and thus is a potential precursor of ET. The present study was undertaken to demonstrate that urinary excretions of ETG and TG relative to 5A3β17αG are potential indicators of simultaneous T and ET misuse in sport.

Materials and Methods

Subjects

Men and male adolescents (Tanner stages 3 to 5, assessed by conventional methods, n = 90), ages 15–30 years (mean 22.2, SD 5.2) with regular recreational sports activity and no access to anabolic steroids, volunteered for studies to establish reference values of urinary excretions of ETG, TG, 5A3β17αG, and their ratios (T/ET)G, (T/5A3β17αG), and (ET/5A3β17αG). Five sedentary men (ages 22, 35, 41, 49, and 55 years) volunteered for a pharmacokinetic/metabolic study of simultaneous oral absorption of T and ET preparations. All subjects were in general good health, were taking no medications, and had no signs of endocrine, hepatic, intestinal, or renal disorders. Informed consent was obtained, and the procedure followed was in accordance with the Helsinki declaration of 1975, as revised in 1983.
Drugs and Administration Protocol

Testosterone undecanoate (TU) was obtained as Pancell
tosterone from Organon, Saint-Denis, France. Epites
costerone undecanoate (ETU) was prepared by esterifi
cation of ET with undecanoyl chloride in dry toluene
containing a trace of pyridine; subsequent workup
yielded the pure oily compound, which was dissolved
in oleic acid (27 g/L). At 0800, just before breakfast, each
subject swallowed two capsules, one containing 40 mg of
TU (equivalent to 25.36 mg of T) and the other 1.57 mg
of ETU (equivalent to 1 mg of ET). Unesterified ET was
administered in 0.2 mL of an ethanolic solution (5 g/L)
deposited on a piece of sugar, which was dissolved and
then swallowed. There was a delay of at least 48 h
between two successive drug administrations, either
(TU + ET) or (TU + ETU).

Urinalysis

Timed or 24-h urine collections were made; samples
were collected at four intervals after the drug ingestion
at 0800 (time zero): 0–2 h, 2–4 h, 4–10 h, and 10–24 h.
Basal excretion rates were measured in urine voided 1 h
before dosing. Excretion volumes were measured and a
20-mL aliquot was frozen at −20°C until analysis. Cre
atinine was determined by fluorometry with radiative
energy attenuation of the creatinine–picrate complex
(REA/TDxFLx kit; Abbott Labs., North Chicago, IL).

Steroids

Unlabeled steroids and [3,4,13C]testosterone were
from, respectively, Steraloids (Wilton, NJ) and Euriso
top (Saint-Aubin, France). [1a,2a-2H]Epitestosterone
(ET-d2) was prepared as described previously (13);
[2,2,3,4,4,6,2H]5-androstone-3β,17α-diol (5A3β17α-d6) was
synthesized by alkaline deuterium exchange of ET
(13) and subsequent borodeuteride reduction of the cor
responding enol acetate, according to Beller and Gal
lagher (14). The crude compound was purified by chro
matography on a 30 x 1 cm Florisil column (Floridin,
Pittsburgh, PA) eluted with chloroform/ethanol (95/5, by
vol). The iso 
to 
pe composition was 61.5% d4, 30.2% d6, 8.1% d6, and <0.1% d0.

GC-MS with Stable Isotope Dilution

Instrumentation. Gas chromatography was performed
on a fused silica capillary column [30 m x 0.32 mm
(i.d.)] coated with RSL-300 stationary phase (35%
phenylmethyl silicone, film thickness 0.20 μm), in
stalled in the oven of a gas chromatograph equipped
with a glass solid injection system, and heated at an
isothermal temperature (between 220 and 240°C). The
column was directly coupled to the source (electron im
pact mode) of a quadrupole mass spectrometer (Model
1010 T; Nermag, Argenteuil, France) that was operated
under normal ionization and mass filter settings. Data
processing was performed with a PDP 11 computer (Di
gital Equipment Corp., Maynard, MA) and Sider soft
ware (Nermag).

Analytical method. Glucuronides were analyzed with
out preliminary separation of minor amounts of noncon
jugated androgens, according to the procedure described
previously (8). Briefly, 0.2 mL of urine (or less when the
excretion concentrations were high, after T and ET ad
ministration) was incubated overnight at 37°C with 10
U of β-glucuronidase from Escherichia coli in 0.5 mL of
0.2 mol/L phosphate buffer, pH 6.8. 13C-labeled T, ET-d2,
and 5A3β17α-d6 were added in known amounts (close to
those of the native androgens). Extraction and liquid
chromatography on Sephadex LH-20 (Pharmacia, Upp
sala, Sweden) swollen in a mixture of dichloromethane/
methanol (95/5, by vol) afforded two successive frac
tions, one containing T and ET and the next one
androstenediol. Quantification was performed by se
lected ion monitoring, in a first run at nominal masses
m/z 680 and 682 for the bisheptafluorobutrate of T
and ET and in a second run at m/z 461 and 467 for the
bis(tert-butyldimethylsilyl ether) of 5A3β17α. Quantita
tive results were calculated according to equations out
lined previously (15).

The accuracy of GC-MS determinations with stable
isotope dilution is principally based on permanent cor
rection of procedural losses, on the specificities attained
by the high resolution GC column and the selected ion
detection, and on the purity of the steroids used as
primary standards. Within- and between-assay CVs
were, respectively, 3–4% (n = 6) and 4–6% (n = 12).

Statistical Analysis

Results are expressed as geometric means, since the excretion
data in the reference population are positively
skewed and they have lognormal distributions. Means
of log-transformed data were compared by the t-test.

Results

Reference values of urinary excretions and excretion
ratios are given in Table 1. Because urine samples for
doping control under field conditions are always un
timed, a relative unit (micrograms of free steroid per
gram of creatinine) is used for the expression of andro
gen excretions to render excretion data less dependent
on urine flow. Tentative threshold values have been
established by adding a safety interval of 4.5 SD to the
mean ratios. The corresponding threshold values for
(T/ET)G, (T/5A3β17αG), and (ET/5A3β17αG) are then
6.0, 2.5, and 1.5. Normal ranges are all below these
proposed maximum allowable values.

Simultaneous oral administration of TU + ETU or
TU + ET affords (T/ET)G ratios that remain mostly
below the critical value of 6 (Fig. 1A). In those cases,
however, (T/5A3β17αG) ratios exceed the cutoff value
and demonstrate their usefulness for efficient detection
of T administration (Fig. 1B).

Finally, the proof of ET or ETU administration is
displayed by the (ET/5A3β17αG) ratio (Fig. 1C), which
exceeds the threshold value of 1.5 at most of the time
intervals during the 24-h time course after drug ad
ministration. In all cases, excretion concentrations of ETG
are higher after ET than after ETU administration.
The possibility of reversed peripheral or hepatic conversion of ET into 5Aβ17α may be excluded because none of the ET administrations produced any significant increase of urinary 5Aβ17α excretion. Indeed, this excretion had an average (±SE) concentration of 71.79 (±23.24) μg/24 h for the five volunteers on the day before ET administration. On the day of oral ET absorption, the corresponding average 5Aβ17αG excretion was 72.66 (±25.59) μg/24 h.

Discussion

On basis of current knowledge of androgen biosynthesis in eugonadal men, it is clear that when T and ET are administered jointly and proportionally to their respective physiological production rates (~25/1 for T/ET productions), the efficiency of the urinary (T/ET)G ratio as an indicator of T misuse may be severely obscured. This was first shown by Kicman et al. (2) after an intramuscular injection of T and ET enanthates into a single volunteer, and it is clearly demonstrated here for the combined oral administration of T and ET undecanoates to several subjects (Fig. 1A). In such cases the urinary TG/LH ratio, which is independent of ET administration, has been suggested as an indicator of exogenous T supply (2), but its general acceptance in doping analysis will necessitate adequate standardization of the urinary LH immunoassay. Moreover, the TG/LH ratio cannot be used in female athletes, who are allowed to suppress LH with contraceptives.

It seemed preferable to investigate the potential of the immediate metabolic precursor of ET as a new reference compound for the expression of relative TG excretions. Elsewhere, I showed that 5Aβ17α is secreted by the testis (12) and that its urinary excretion as the glucuronide is significantly depressed upon long-term T enanthate administration (8). Thus, as for ET, 5Aβ17α production is down-regulated through the negative feedback exerted by exogenous T on the pituitary LH production.

In a study on the effects of long-term and high-dose intramuscular T enanthate administration (8), Matsu-moto and I showed a significant decrease in (ET/5Aβ17α)G as a result of sharper decline of ETG excretion. Under those circumstances, one may anticipate that the (ET/5Aβ17α)G test of ET administration may be less sensitive. In this study, however, the short T peaks produced by a single oral dose of TU are not sufficient to suppress gonadotropins (16), and most probably the increased (T/5Aβ17α)G ratios of Fig. 1B are entirely due to increase in TG output, without decrease of 5Aβ17αG. The pattern of urinary TG concentrations (with a maximum reached between 2 and 6 h after ingestion of TU) closely resembles the pattern for serum T concentrations (17).
Esterification of ET to ETU does not lead to higher urinary excretions of ETG; on the contrary, (ET/5A3β17α)G values are always lower after ETU administration than after ET ingestion (Fig. 1C). This may be due to the action of intestinal 5a-reductase converting ETU into the 5α-reduced undecanoate, similar to what has been described for TU (18). This hypothesis is corroborated by the significantly higher excretions of 5α-androstane-3α,17α-diol glucuronide upon ETU dosing (results not shown). No significant difference, however, could be observed between ET and ETU administrations when analyzing plasma ET concentrations, which were in the 50 to 200 ng/L range, with a small peak value at 1 to 3 h after drug absorption. This is due to the very high metabolic clearance rate of ET (19). On the other hand, plasma ETG increased to higher concentrations after ET administration (1 to 10 μg/L range) than after ETU administration (1 to 3 μg/L range) in the 1- to 3-h interval after drug ingestion.

False-positive (T/ET)G test results, although seldom encountered (20), are a real problem for laboratories in charge of doping analysis. It seems to be related to an enzyme defect, favoring ETS rather than ETG excretion (8 and unpublished results). Whether this has any effect on (T/5A3β17α)G and (ET/5A3β17α)G is now under investigation in a population of subjects with physiologically high (T/ET)G ratios (4–12).

In conclusion, urinary excretions of TG and ETG relative to 5A3β17αG are potential indicators of T and ET administration. Whether critical values of (T/5A3β-17α)G and (ET/5A3β17α)G should be established with a safety interval different from the 4.5 SD chosen in this study will have to be decided when more pharmacokinetic data on T and ET administration become available. Unfortunately, none of the ratios [(T/ET)G, (T/5A3β17α)G, or (ET/5A3β17α)G] is efficient as a long-acting indicator of oral T and ET misuse.

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References