On the origin of physiologically high ratios of urinary testosterone to epitestosterone: consequences for reliable detection of testosterone administration by male athletes

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Abstract

Testosterone administration to male athletes can be safely detected in the vast majority of cases by the urinary excretion ratio of testosterone to epitestosterone glucuronides (TG/EG), which may not exceed 6. Some rare cases of physiologically high TG/EG ratios (between 6 and 12) are encountered; these may be attributed to a dysregulation of the testicular secretions of epitestosterone which is decreased, and of epitestosterone sulphate (ES) which is normal or increased. Impaired hydrolysis of circulating epitestosterone sulphate by deficiency of a specific sulphatase acting on 17α-sulphates must also be considered as a possible reason for the decreased availability of epitestosterone for hepatic glucuronidation. Urinary excretions of conjugates and metabolites of testosterone and epitestosterone (expressed in nmol/mmol creatinine) have been determined by gas chromatography-mass spectrometry associated with stable isotope dilution, in a reference population of 90 healthy male subjects and in 12 subjects with chronic TG/EG>4. Urinary excretion ratios such as TG/(EG+ES), EG/ES and TG/5-androstene-3β,17α-diol glucuronide are shown to be efficient criteria which allow discrimination between physiologically high and pharmacologically high TG/EG ratios. A simple oral loading test with deuterium-labelled epitestosterone demonstrates the difference between hepatic and total epitestosterone metabolism clearly, particularly in subjects with physiologically high TG/EG in comparison with subjects with normal TG/EG.

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Introduction

Detection of xenobiotic androgen administration to athletes by gas chromatography-mass spectrometry (GCMS), performed on characteristic urinary metabolites, has gained considerable efficacy during the past decade. A trend might therefore exist towards the preferential abuse of endogenously produced testosterone, which is much more difficult to prove without contest. Because of the wide inter-individual variability of testosterone production and testosterone glucuronide excretion, the notion of setting a concentration cut-off was found to be unrealistic. A more reasonable approach is the use of relative urinary excretions which are independent of urine flow.

Introduced by Donike *et al.* (1983), the ratio of glucuronides of testosterone to epitestosterone (17α-hydroxy-4-androsten-3-one) has been accepted by the International Olympic Committee and most international sport authorities as the sole proof of testosterone administration when the critical value of 6 is exceeded. Although the average ratio of testosterone to epitestosterone glucuronides (TG/EG) found by several laboratories was between 0·9 and 1·6 in a population of healthy men and male adolescents (Donike *et al.* 1983, Dehennin & Scholler 1990, Kicman *et al.* 1990, Carlström *et al.* 1992), it has been reported that, in some rare cases, normal male individuals may have TG/EG ratios exceeding the threshold value without any supply of exogenous testosterone (Namba *et al.* 1989, Oftebro 1992, Raynaud *et al.* 1992, Dehennin & Matsumoto 1993). Some complementary criteria have therefore been suggested, such as the urinary TG/luteinizing hormone (LH) ratio (Kicman *et al.* 1990) and the serum testosterone/17-hydroxyprogesterone ratio (Carlström *et al.* 1992). The incidence of TG/EG>6 was evaluated to be less than 0·8% by Catlin & Hatton (1991). We confirmed this figure in a population of 144 healthy male subjects, where five in 1000 had TG/EG>6 and seven in 10 000 had TG/EG>9 (author's unpublished results).

Reports concerned with doping analysis often use testosterone/epitestosterone and testosterone/LH ratios, where testosterone and epitestosterone include glucuro- and non-conjugated androgen. Throughout this text, and to avoid confusion with sulphoconjugate excretion, TG/LH and TG/EG will be used, where G includes glucuro- and non-conjugated androgen.

In a recent work on the effects of long-term administration of testosterone enanthate on the urinary profile of

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androgen metabolites, we have briefly mentioned the importance of epitestosterone sulphate (ES) excretion and the potential use of the TG/(EG+ES) ratio for a better discrimination between physiologically high and pharmacologically high TG/EG ratios (Dehennin & Matsumoto 1993). We now wish to present further evidence hereof and also some more extensive results which indicate that subjects, with TG/EG>4 and free of any anabolic steroid supply, are characterized by normal TG/(EG+ES) ratios in conjunction with abnormally low EG/ES ratios.

Materials and Methods

Subjects and administration protocol of deuterated epitestosterone

Men and male adolescents (Tanner stages 3 to 5, assessed by conventional methods, $n=90$), aged 15–30 years (mean 22·2, S.D. 5·5) with regular recreational sports activity and free of any anabolic steroid supply, volunteered for the establishment of reference values of urinary excretions. All subjects were in good general health, were taking no medications and had no signs of endocrine, hepatic, intestinal or renal disorders.

Twelve sporting male subjects (Tanner stages 3 to 5), aged 14–26 years (mean 20·7, S.D. 1·2) and having TG/EG>4 without any anabolic steroid administration, were selected in the past 3 years. They trained regularly, without exhaustion on the day of urine collection. Three of these subjects had been monitored for TG/EG>6 ratios twice a year for 3 years and they gave informed written consent for a loading test with 1 mg deuterium-labelled epitestosterone administered orally at 0800 h just before breakfast. Procedures followed were in accordance with the Helsinki Declaration of 1975, as revised in 1983.

Urinalysis

Urine collections were either timed, or untimed as practiced under doping control conditions, and therefore excretion levels are expressed relative to creatinine concentration. When deuterated epitestosterone was administered, 24-h collections were fractionated at 4 time-intervals following the drug absorption at 0800 h (time zero): 0–2 h, 2–4 h, 4–10 h and 10–24 h. Excretion volumes were measured and an aliquot of 20 ml was frozen at −20 °C until analysis. Creatinine was determined by fluorometry with radiative energy attenuation of the creatinine–picrate complex (REA/TDxFLx kit; Abbott Laboratories, North Chicago, IL, USA).

Steroids

Unlabelled steroids and [3,4-^{13}C]testosterone ([^{13}C]testosterone) were of commercial origin, respectively from Steraloids (Wilton, NJ, USA) and Euriso-Top (Saint-Aubin, France). 17α-Hydroxy-4-oestren-3-one (hydroxyoestrenone) was a gift from Organon (Oss, The Netherlands). [1α,2α-^{2}H]Epitestosterone ([^{2}H]epitestosterone), [2,2,3,4,4,6-^{2}H]5-androstene-3β,17α-diol ([^{2}H]5-A-3β17α), [16,16,17-^{2}H]5α-androstane-3α,17β-diol ([^{2}H]5α-A-3α17β) and its similarly deuterated 5β-analogue ([^{2}H]5β-A-3α17β) were prepared as described previously (Dehennin *et al.* 1980).

[3,4-^{13}C]Testosterone 17-sulphate ([^{13}C]testosterone sulphate), [1α,2α-^{2}H]epitestosterone 17-sulphate ([^{2}H]epitestosterone sulphate) and 17α-hydroxy-4-oestren-3-one 17-sulphate (hydroxyoestrenone sulphate) were synthesized by esterification of the parent compounds (0·1 mmol) with chlorosulphonic acid in pyridine–dimethylformamide. Steroids in the neutralized and diluted reaction mixture were adsorbed on an Amberlite XAD-2 column (200 × 5 mm; Serva, Heidelberg, Germany). After washing with 10 ml water, a 4 ml fraction of methanolic eluate was submitted to anion exchange chromatography on a small DEAE-cellulose column (40 × 5 mm; Serva) packed in methanol. Sulphates were selectively retained, and after washing the column with 4 ml methanol, they were eluted as ammonia salts with 2 ml 2 mol ammonia/l in methanol.

Gas chromatography-mass spectrometry

Instrumentation Gas chromatography was performed on a fused silica capillary column (30 m × 0·32 mm) coated with RSL-300 (Bio-Rad/RSL, Eke, Belgium) stationary phase (35% phenylmethyl silicone, film thickness 0·20 μm), installed in the oven of a gas chromatograph, equipped with a glass solid injection system and heated at an isothermal temperature (between 200 and 240 °C). The column was directly coupled to the source (electron impact mode) of a quadrupole mass spectrometer (Model 1010 T; Nermag, Argenteuil, France) which was operated under normal ionization and mass filter settings. Data processing was performed with a PDP 11 computer (Digital Equipment, Maynard, MA, USA) and Sidar software (Nermag).

Analytical procedure

Glucuronide fraction In this fraction glucuronides were estimated without preliminary separation of non-conjugated androgens. The latter represent less than 3% of the corresponding glucuronides and remain within the experimental error range of the quantitative glucuronide estimations by selected ion monitoring (SIM) (Dehennin & Matsumoto 1993). Excretion of steroid conjugates is expressed in nmol/mmol creatinine.

Testosterone and epitestosterone Enzyme hydrolysis was performed with 10 IU (or 2000 Fishman units) of β-glucuronidase from *Escherichia coli* (Diagnostics Pasteur,

Marnes-la-Coquette, France) in 0·2 ml urine (or less for concentrations higher than 10 ng/ml) diluted with 0·5 ml phosphate buffer (0·2 mol/l, pH 6·8), and incubated for at least 4 h at 37 °C or at room temperature overnight. Known amounts of [^{2}H]epitestosterone and [^{13}C]testosterone, close to the endogenous amounts present in the sample, were then added. A mixture of n-hexane and diethyl ether (4:1, v/v) was used for extraction and the residue was purified by liquid chromatography on Sephadex LH-20 columns (200 × 5 mm; Pharmacia, Uppsala, Sweden), packed in the eluent which was a mixture of dichloromethane and methanol (95:5, v/v); the first 4 ml were discarded and the next 1·5 ml contained testosterone and epitestosterone. After derivatization to the bis(heptafluorobutyrates), the residue was taken up in 30 µl iso-octane and 3 or 6 µl were deposited on the solid injection needle. SIM was performed at nominal masses 680 and 682, and quantitative results were calculated according to equations outlined previously (Reiffsteck *et al.* 1982).

Androstanediols and androstenediol These glucuronides, present in 0·2 ml urine, were enzymatically hydrolysed as described for testosterone, but with a longer incubation time (24 h at 37 °C), and the following isotopic internal standards were added: [^{2}H]5α-A-3α17β, [^{2}H]5β-A-3α17β and [^{2}H]5-A-3β17α. Extraction was performed with a mixture of n-hexane and diethyl ether (3:2, v/v) and the residue was purified with the same chromatographic system as the one used for testosterone; the first 5·5 ml eluent were discarded and (androstanediols+androstenediol) were eluted thereafter with 2·5 ml. 5α-Androstane-3α,17α-diol, 5β-androstane-3α,17α-diol, 5α-A-3α17β, 5β-A-3α17β and 5-A-3β17α were converted to the bis(t-butyldimethylsilyl ether) and quantification was done by SIM at nominal masses 463 and 466 for androstanediols, 461 and 467 for androstenediol. All these androstanediol isomers had baseline separations and they did not interfere with any of the corresponding 3β-hydroxyepimers, which were not analysed here.

Sulphate fraction In this fraction, sulphates of testosterone and epitestosterone were analysed with preliminary separation of the corresponding glucuro- and non-conjugated compounds. Steroid sulphate concentrations are expressed in nmol/mmol creatinine. To 1 ml urine were added 20 ng [^{2}H]epitestosterone sulphate and 10 ng [^{13}C]testosterone sulphate. After addition of 3 ml methanol, precipitates were separated by centrifugation and superants were then fractionated on small columns (20 × 5 mm) of DEAE–Sephadex (chloride form) packed in methanol. Glucuro- and non-conjugated steroids were washed from the column with 4 ml methanol, and sulphates were eluted with 0·3 mol lithium chloride/l in methanol; 0·5 ml was first discarded and the next 1·5 ml contained sulphates. Solvolysis was carried out by dissolving the dry residue in 0·5 ml hydrochloric acid (1 mol/l) in methanol and heating at 60 °C for 4 h. Neutralization with 0·25 ml of a saturated aqueous sodium bicarbonate solution, evaporation to dryness, dissolution in 0·5 ml water and extraction with a mixture of n-hexane and diethyl ether (4:1, v/v) gave upon evaporation a dry residue which was taken up in 0·2 ml chromatography eluent and purified by Sephadex LH-20 chromatography as described above. Quantification was performed by SIM of the bis(heptafluorobutyrate) derivatives at nominal masses 680 and 682. There was exchange of the 2α-deuterium for protium in [^{2}H]epitestosterone sulphate under solvolysis conditions, but when corrections were made by solvolysing standard mixtures under the same conditions, recovery of added epitestosterone sulphate was quantitative.

Determination of glucuro- and sulphoconjugates of deuterium-labelled epitestosterone The deuterated urinary glucuro- and sulphoconjugates excreted after [^{2}H]epitestosterone dosing were determined as described for the non-labelled analogues, except that homologue internal standards were used, respectively hydroxyoestrenone and hydroxyoestrenone sulphate. Sample sizes were reduced when excretion rates were high. Corrections for isotope exchange in [^{2}H]epitestosterone sulphate during solvolysis were made. No significant *in vivo* isotope exchange occurred since variations of the ratio [$^2H_0$]/[$^2H_2$] of the corresponding isotopic species measured in the excreted [^{2}H]epitestosterone glucuronide remained within the experimental error range of the same ratio measured in [^{2}H]epitestosterone before oral administration. The excretion rates of deuterium-labelled epitestosterone conjugates are expressed in nmol/h.

Accuracy and precision The accuracy of GCMS determinations with stable isotope dilution is principally based on permanent correction of procedural losses, on the specificities attained by the high resolution GC column and selected ion detection, and on the purity of the steroids used as primary standards. For glucuronide determinations, standard additions of testosterone glucuronide were made and compared with those of equivalent amounts of non-conjugated testosterone; no significant differences were observed, thus indicating completeness of the enzyme hydrolysis step. As expected, precision was better in those cases where the corresponding isotopically labelled analogues were available. The coefficient of variation (C.V.) for interassay replicates (n=10) was between 3 and 4%. In cases where a homologue internal standard had to be used, variability increased significantly (C.V. was between 6 and 9%).

TABLE 1. Urinary excretions of steroid conjugates (nmol/mmol creatinine) and LH (IU/mmol creatinine) in a reference population (n=90) and in subjects (n=12) with chronic TG/EG>4

	EG	TG	ES	TS	5-A-3β17αG	5α-A-3α17αG	5β-A-3α17αG	5α-A-3α17βG	5β-A-3α17βG	LH
Reference population										
Mean	9·77	10·93	5·49	2·46	21·40	12·98	1·79	15·67	60·01	1·20
S.D.	7·60	6·99	2·82	1·98	12·90	11·57	1·60	11·60	40·01	1·01
Range	1·36–51·18	0·71–37·76	1·48–14·46	0·55–11·97	5·77–60·88	2·59–56·14	0·23–12·23	2·25–99·12	6·79–195·8	0·19–5·07
Chronic TG/EG>4 group										
Mean	2·44*	13·88	6·08	5·09*	13·32†	7·24*	0·90*	12·09	51·81	0·79
S.D.	0·84	4·63	2·64	3·18	4·74	5·06	0·80	3·41	23·87	0·63
Range	1·26–4·22	5·66–22·86	2·35–11·02	1·67–11·44	7·87–19·75	1·17–13·97	0·15–3·76	6·89–16·92	23·30–100·3	0·19–2·02

*P<0·001, †P<0·05 compared with reference values (t-test).
Abbreviations: E, epitestosterone; T, testosterone; G, glucuronide; S, sulphate; 5-A-3β17αG, 5-androstene-3β,17α-diol glucuronide; 5α-A-3α17αG, 5α-androstane-3α,17α-diol glucuronide; 5β-A-3α17αG, 5β-androstane-3α,17α-diol glucuronide; 5α-A-3α17βG, 5-α-androstane-3α,17β-diol glucuronide; 5β-A-3α17βG, 5β-androstane-3α,17β-diol glucuronide. Creatinine excretion (mmol/l) in the reference population was 11·76 ± 5·48 (mean ± S.D.) and in the subjects with chronic TG/EG>4, 12·36 ± 5·75.

TABLE 2. Urinary excretion ratios (without units, except TG/LH which is expressed in nmol/IU as by Kicman *et al.* 1990) in a reference population (n=90) and in subjects (n=12) with chronic TG/EG>4

	TG/EG	TG/(EG+ES)	TG/LH	TS/ES	EG/ES	TG/TS	EG/ 5-A-3β17αG	TG/5-A-3β17αG	5α-A-3α17αG/ 5β-A-3α17αG	5α-A-3α17βG/ 5β-A-3α17βG
Reference population										
Mean	1·42	0·82	13·24	0·49	1·99	5·68	0·50	0·63	9·46	0·33
S.D.	0·96	0·48	10·46	0·33	1·34	4·42	0·25	0·46	5·64	0·18
Range	0·08–4·34	0·06–1·99	0·74–58·10	0·13–1·87	0·29–6·36	0·36–34·55	0·10–1·19	0·06–2·43	0·82–27·4	0·09–0·96
Threshold value[a]	6·0	3·0	60	2·0			1·5	2·5		
Chronic TG/EG>4 group										
Mean	6·06*	1·67*	30·92*	0·83*	0·46*	3·53	0·19*	1·07*	11·96	0·27
S.D.	2·56	0·28	17·56	0·28	0·21	2·01	0·07	0·28	6·37	0·10
Range	4·01–12·59	1·20–2·19	6·61–55·20	0·23–1·28	0·18–0·78	1·53–8·38	0·11–0·28	0·60–1·60	2·53–24·83	0·11–0·48

*P<0·001 compared with reference values (t-test).
Abbreviations: T, testosterone; E, epitestosterone; G, glucuronide; S, sulphate; 5-A-3β17α, 5-androstene-3β,17α-diol; 5α-A-3α17αG/5β-A-3α17αG, ratio of glucuronides of 5α-androstane-3α,17α-diol to 5β-androstane-3α,17α-diol; 5α-A-3α17βG/5β-A-3α17βG, ratio of glucuronides of 5α-androstane-3α,17β-diol to 5β-androstane-3α,17β-diol.
[a]Threshold value = mean + 4·5 S.D.

Radioimmunoassay

Urinary LH was extracted by acetone precipitation and measured by radioimmunoassay using the 2nd International Reference Preparation of human menopausal gonadotrophin as reference standard (Kulin & Santner 1977). Storage time of urine samples at −20 °C before LH analyses did not exceed 1 month.

Statistical analysis

When appropriate, data were log transformed to achieve normal distribution and then compared by *t*-test and analysis of variance with the *F* test. Correlations were established with Spearman's rank correlation coefficient at the $P<0{\cdot}05$ significance level. Results are means ± S.D. or means ± S.E.M. when stated in figure legends.

Results

An overview of the principal urinary androgen metabolite levels is given in Table 1. Intercomparison of the reference population and the subjects with chronic TG/EG>4 indicates, in the latter group, a decreased output of all 17α-hydroxyandrogen glucuronides together with an increased excretion of sulphoconjugates and similar LH concentrations.

Characteristic ratios of urinary excretions are listed in Table 2. Some significant differences could be noticed between the two subject groups for most of the ratios. In the group with chronic TG/EG>4, threshold values, however, were only exceeded for TG/EG, while other important ratios such as TG/(EG+ES) and TG/5-A-3β17αG remained within normal ranges. Nevertheless all ratios with testosterone as numerator were higher in the TG/EG>4 group, thus confirming sustained testosterone production. The 5α/5β ratios of androstanediol isomers were not significantly different between the subject groups. Significant positive correlations existed for testosterone and epitestosterone between their respective conjugates. Data analysis also showed that TG/EG was negatively correlated with EG/ES in both subject groups ($r=-0{\cdot}33$, $P<0{\cdot}001$ in the reference population and $r=-0{\cdot}84$, $P<0{\cdot}001$ for the other subjects).

The EG/ES ratio was found to be of particular diagnostic importance for subjects with chronic TG/EG>4, where the average value was four times less than in the reference group and this denotes preferential sulphoconjugate excretion (EG/ES<1). This is also supported by the results of the loading test with deuterated epitestosterone which was devised as an indicator of hepatic epitestosterone metabolism. The average urinary excretions of [^{2}H]epitestosterone glucuronide attained a maximum of 30% of the orally administered dose, whatever the subject group (Fig. 1). The urinary excretion of [^{2}H]epitestosterone sulphate on the contrary was significantly higher in the TG/EG>4 group than in the normal group, but remained well below the level of the corresponding glucuronide excretion. During the loading test, simultaneous determinations of endogenous epitestosterone conjugates were made (Fig. 2). These indicate stable excretion rates in the

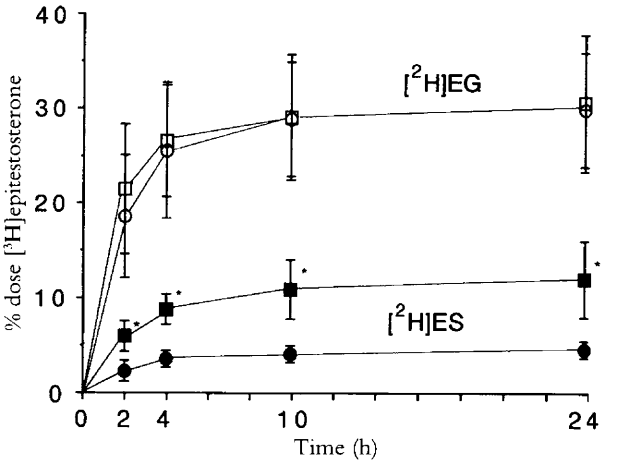

FIGURE 1. Time-course of deuterated epitestosterone ([^{2}H]epitestosterone) excretion (mean ± S.E.M.), as glucuronide ([^{2}H]EG) and as sulphate ([^{2}H]ES), after oral administration of 1 mg [^{2}H]epitestosterone at time zero (0800 h), in three subjects with normal ratios of testosterone to epitestosterone glucuronides (TG/EG; circles), and in three subjects with chronic TG/EG>4 (squares). *$P<0{\cdot}05$ compared with subjects with normal TG/EG (*t*-test).

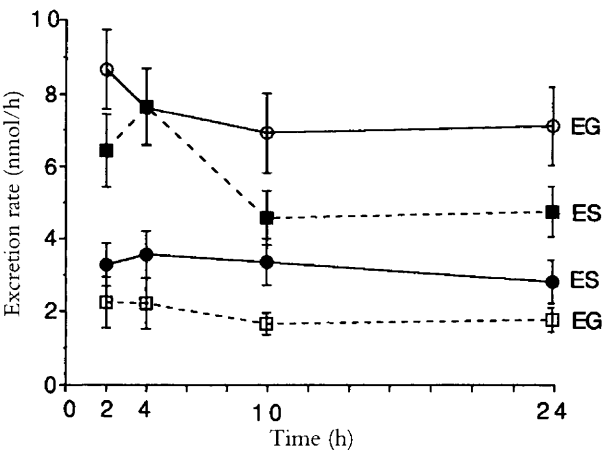

FIGURE 2. Excretion rates of endogenous epitestosterone as glucuronide (EG) and as sulphate (ES) measured during the deuterated epitestosterone loading test (drug administration at time zero) in the same subjects and at the same time-intervals as in Fig. 1. Values are means (± S.E.M.) in three subjects with normal ratios of testosterone to epitestosterone glucuronides (TG/EG; circles), and in three subjects with chronic TG/EG>4 (squares).

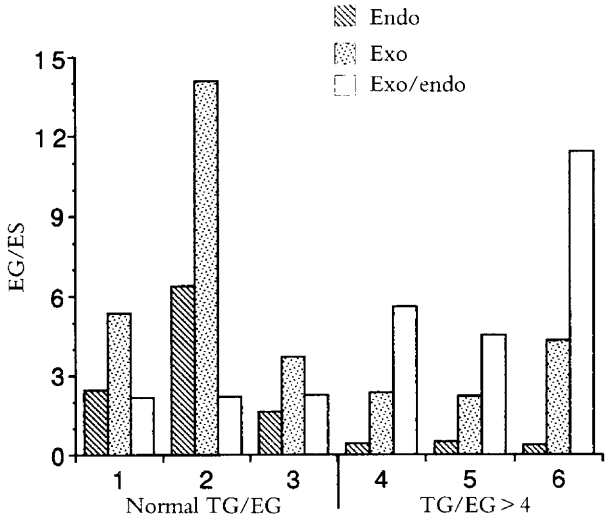

FIGURE 3. Excretion ratios of glucuro- to sulphoconjugate of epitestosterone (EG/ES) determined in 24-h urine samples from three subjects (1–3) with normal ratios of testosterone to epitestosterone glucuronide (TG/EG), and three subjects (4–6) with chronic TG/EG>4. Endo=EG/ES ratio for epitestosterone of endogenous origin. Exo=EG/ES ratio for epitestosterone of exogenous origin (oral administration of 1 mg deuterated epitestosterone).

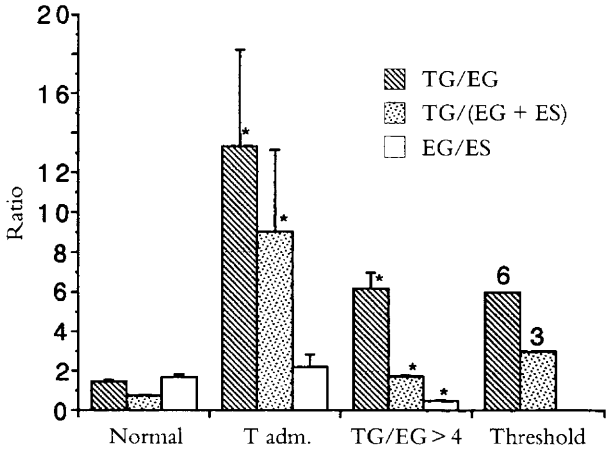

FIGURE 4. Means (± S.E.M.) of three characteristic excretion ratios found in the reference population with normal ratios of testosterone to epitestosterone glucuronide, (TG/EG; normal), in subjects (n=9) treated with 100 mg testosterone enanthate/week for 6 months (Dehennin & Matsumoto 1993) (T adm.) and in subjects with chronic TG/EG>4. TG/(EG+ES)=ratio of testosterone glucuronide to epitestosterone (glucuronide+sulphate), EG/ES=ratio of glucuro- to sulphoconjugate of epitestosterone. *P<0·001 compared with normal subjects (t-test). Threshold values are those mentioned in Table 2.

normal subjects, but a decline of these rates started in the afternoon in subjects with TG/EG>4, probably as a consequence of training in the morning. Differences between hepatic metabolism of exogenous epitestosterone (Fig. 1) and total metabolism of endogenous epitestosterone (Fig. 2) are demonstrated in Fig. 3. This figure displays glucuroconjugation as the preferential form of epitestosterone conjugation (EG/ES>1), with one important exception: conversion of endogenously produced epitestosterone to its sulphate was preponderant in subjects with chronic TG/EG>4 and caused an inversion of EG/ES to values below 1. The ratios of (EG/ES)exogenous to (EG/ES) endogenous (conjugate ratios of exogenous and endogenous epitestosterone) remained remarkably constant (=2) in subjects with normal TG/EG, but increased drastically (=5 to 11) in cases of chronic TG/EG>4.

Finally, Fig. 4 illustrates the diagnostic value of both the TG/(EG+ES) and the EG/ES ratios for a better discrimination between high TG/EG values, those consecutive to testosterone administration with TG/(EG+ES) above the cut-off value of 3, and those due to a physiological deficiency of epitestosterone glucuronide excretion with EG/ES<1 and TG/(EG+ES)<3.

Discussion

As urine samples for doping control under field conditions are always untimed, and also in order to render excretion data less dependent on urine flow, relative units (nmol/mmol creatinine for steroid conjugates and IU/mmol creatinine for LH) have been used.

Results regarding epitestosterone metabolism must be discussed with caution, keeping in mind that only secretions of epitestosterone and its sulphate by the human testis have been proven up to now. Data on adrenal secretion of sulpho- and non-conjugated epitestosterone are still awaited, since it was demonstrated in the early 1960s that adrenocorticotrophin stimulates urinary excretion of epitestosterone glucuronide (Tamm *et al.* 1966, Wilson & Lipsett 1966). Nevertheless, the most important finding of this study is the association of low EG/ES ratios (<1) with physiologically high TG/EG. Pharmacologically high TG/EG due to testosterone administration has no significant effect on EG/ES because the urinary excretions of both epitestosterone conjugates are equally reduced by testosterone-induced LH suppression (Dehennin & Matsumoto 1993). Primarily, as testicular secretion rates of epitestosterone and its sulphate are very similar in normal men (Dehennin 1993), a normal urinary EG/ES ratio is expected to be close to 1, or preferably somewhat higher since the metabolic clearance rate of sulphates is low. It is noteworthy that physiologically high TG/EG is never associated with EG/ES>1, while subjects with normal TG/EG and EG/ES<1 may be encountered, although seldom.

Physiologically high TG/EG is thus a consequence of sustained testosterone secretion coupled with impaired

epitestosterone secretion, the sulphate being maintained at a normal (or slight supranormal) secretory level. Subjects with TG/EG>4 may have a dysregulation between testicular secretion rates of epitestosterone and its sulphate, probably due to stimulated sulphotransferase activity in the testes. A similar trend can be observed for TG/TS, although this is much less pronounced because testicular secretion rates of testosterone and its sulphate are highly different (Dehennin 1993). The low epitestosterone glucuronide excretions in subjects with TG/EG>4 are by no means related to stimulation of epitestosterone metabolism by 5α- or/and 5β-reduction and 3α-reduction, since the glucuronide excretions of 5α-androstane-3α,17α-diol and its 5β-isomer are similarly reduced.

Deficiency of a specific sulphatase acting on circulating epitestosterone sulphate of glandular origin cannot be excluded as a possible reason for the decreased availability of epitestosterone for hepatic glucuronidation. Thus the plasma ratio of epitestosterone to epitestosterone sulphate may also be regulated by peripheral hydrolysis of the sulphate, similarly to that which has been observed for the plasma ratio of dehydroepiandrosterone to its sulphate (Zumoff & Bradlow 1980).

As suggested recently (Dehennin & Matsumoto 1993) on the basis of a study of excretion of epitestosterone conjugates in a smaller number of normal subjects, in subjects after long-term testosterone enanthate administration and in a single case of physiologically high TG/EG, the ratio TG/(EG+ES), which integrates both epitestosterone conjugates, is shown here to be appropriate data complementary to TG/EG measurements. All our subjects with physiologically high TG/EG values (between 4 and 12) had TG/(EG+ES) ratios below the threshold value of 3.

The excretion of testosterone glucuronide relative to 5-androstene-3β,17α-diol glucuronide is another candidate ratio for the detection of testosterone administration. All our subjects with chronic TG/EG>4 had TG/5-A-3β17αG values which remained below the cut-off point of 2·5. Another useful application of the glucuronide excretion of 5-androstene-3β,17α-diol (the immediate precursor of epitestosterone in the testicular biosynthetic scheme proposed by Weusten *et al.* 1989) is found in the ratio EG/5-A-3β17αG, which allows the detection of epitestosterone administration (Dehennin 1994). As these EG/5-A-3β17αG ratios decrease rather similarly in subjects with either physiologically high or pharmacologically high TG/EG, they do not have a diagnostic value for the differentiation of the latter two types of TG/EG.

Concerning TG/LH and TS/ES, these ratios are increased in subjects with chronic TG/EG>4, without attaining the respective threshold values. They are therefore helpful parameters in the establishment of a detailed urinary profile of androgen metabolites.

A simple oral loading test with 1 mg deuterated epitestosterone, a dose corresponding roughly to four times the daily production in man, indicates a 30% conversion to urinary epitestosterone glucuronide. This confirms the data of Wilson & Lipsett (1966) obtained upon intravenous injection of tritiated epitestosterone and proves that there is little or no metabolism of epitestosterone outside the liver. The main objective of this test was to demonstrate more explicitly the differences in epitestosterone metabolism between normal subjects and those with chronic TG/EG>4. First, there was no difference in the excretion rate of [^{2}H]epitestosterone glucuronide between normal subjects and those with physiologically high TG/EG (Fig. 1). This supports the hypothesis that the decreased excretion of epitestosterone glucuronide in subjects with chronic TG/EG>4, mentioned in Table 1, is due to impaired glandular epitestosterone secretion and/or to deficiency of sulphatase action on circulating epitestosterone sulphate. Then there are the excretion rates of [^{2}H]epitestosterone sulphate in subjects with physiologically high TG/EG which were higher than in normal subjects, but they remained much lower than the corresponding excretion rates of [^{2}H]epitestosterone glucuronide (exogenous EG/ES>1). This is in contrast to that which was observed for conjugates of endogenous epitestosterone (endogenous EG/ES<1) and therefore leads to (EG/ES)exogenous/(EG/ES)endogenous ratios significantly higher than the normal ratio. This is another characteristic of subjects with physiogically high TG/EG.

In conclusion, when urinary TG/EG ratios in the 6 to 12 range are found for the first time in subjects without any previous indication of normal ratios, then it is recommended that some complementary criteria such as the excretion of epitestosterone sulphate and 5-androstene-3β,17α-diol glucuronide should be taken into account. These excretions are of particular interest because they allow a better discrimination between physiologically high and pharmacologically high TG/EG ratios with the aid of ratios such as TG/(EG+ES), EG/ES and TG/5-A-3β17αG.

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