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POSSIBLE INDICES FOR THE DETECTION OF THE ADMINISTRATION OF DIHYDROTESTOSTERONE TO ATHLETES

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Summary—Dihydrotestosterone (DHT) can be used by an athlete as an anabolic steroid to evade the current International Olympic Committee approved drug tests. To investigate the possibility of a method for its detection, the heptanoate ester of DHT was administered to two male subjects (150 mg i.m.). Urine samples, collected before and after the injection, were subjected to enzymatic hydrolysis and the excretion rates of DHT, 5α-androstane-3α,17β-diol (3α-diol) and testosterone (T) were determined by radioimmunoassay. Relative changes in the excretion of DHT, 3α-diol, 5α-androstane-3β,17β-diol (3β-diol), 5β-androstane-3α,17β-diol (5β-diol), T and epitestosterone (17α-hydroxyandrost-4-en-3-one; Epi-T) were determined by gas chromatography–mass spectrometry (GC–MS). Following administration of DHT, the urinary excretion rates of DHT, 3α-diol and 3β-diol increased when compared to those of T, Epi-T, 5β-diol and luteinizing hormone (LH). Concentrations of DHT in the plasma increased whereas those of T, LH and follicle stimulating hormone decreased. The changes following such modest doses of DHT suggest that these ratios of urinary hormones may be used for the detection of doping with DHT.

INTRODUCTION

At present, only an untimed urine sample is available from an athlete to determine whether doping with prohibited substances has occurred. All that is required for detecting the use of synthetic anabolic steroids is the unequivocal identification of the parent compound or its diagnostic metabolite in the urine. In the case of natural hormones, where they or their metabolites are normally present in urine, detection depends upon the effect of administration upon the system controlling their endogenous secretion and the consequent alterations in the pattern of steroid excretion. For example, the administration of supraphysiological doses of testosterone (T) to men results in the inhibition of luteinizing hormone (LH) release and, as a consequence, inhibition of the secretion of T and other testicular steroids. The resulting decrease in the urinary excretion rates of LH and epitestosterone (17α-hydroxyandrost-4-en-3-one; Epi-T), together with the increase in that of T (from the injection), is reflected by elevated ratios of T/Epi-T and T/LH in urine [1]. A T/Epi-T ratio greater than six is not allowed under the regulations of the International Olympic Committee (IOC) [2] and may result in disqualification of the athlete and, possibly, disciplinary action. In the U.K. the ratio of T/LH is measured in addition to that of T/Epi-T.

Administration of dihydrotestosterone (DHT), an active metabolite of T, also inhibits LH secretion [3], but, because of the reduced excretion of T as well as of LH and Epi-T, it is unlikely to alter the above ratios significantly. For this reason alone, DHT would seem a likely candidate for use by athletes. Furthermore, DHT may be regarded as more potent than T since it is known to bind more strongly to the androgen receptor than does T [4]. Under normal circumstances the concentration of circulating T in men is as much as ten times that of DHT, so that in skeletal muscle T is the predominant ligand bound to the androgen receptor. In contrast to muscle, sexual tissue contains much greater 5α-reductase activity which converts T to DHT. In these tissues it is DHT that

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is bound by the receptor thus amplifying the effect of circulating T. It may be argued, therefore, that high plasma concentrations of DHT (e.g. following DHT administration) would have a greater effect in muscle compared to sexual tissue thereby giving DHT a greater myotrophic/androgenic ratio than T.

In the human, reduction of the A ring of testosterone results in either an α or β orientation of the added hydrogen at C5. This is essentially irreversible, so interconversion between 5α and 5β isomers does not occur. Consequently, DHT can give rise only to 5α products, while T can yield both 5α and 5β compounds. It follows that administration of DHT should give rise to 5α-metabolites while suppressing the endogenous production of those 5β-metabolites which originate from T or other testicular precursors (Fig. 1).

To examine this premise, the excretion rates of DHT, androstane-$3\alpha,17\beta$-diol (3α-diol), T and LH were determined by high pressure liquid chromatography (HPLC) and/or radioimmunoassay (RIA) following the administration of DHT as its heptanoate ester, to two normal subjects. To expand on the promising results obtained, the ratios of these and other related steroids [i.e. 5α-androstane-$3\beta,17\beta$-diol (3β-diol), 5β-androstane-$3\alpha,17\beta$-diol (5β-diol), T and Epi-T] were also measured by gas chromatography–mass spectrometry (GC–MS).

EXPERIMENTAL

Materials

Steroid standards were obtained from Sigma Chemical Co. (Poole, Dorset). β-Glucuronidase (Helix pomatia, code 22867), used for hydrolysis of urine samples prior to GC–MS analysis, was purchased from Serva Feinbiochemica GmBH and Co. (Heidelberg, Fed. Rep. Germany). β-Glucuronidase from Patella vulgata (used prior to HPLC–RIA analysis) was prepared by the method of Brooks [5]. Both β-glucuronidase preparations contained some sulphatase activity.

DHT-17-heptanoate was prepared by the catalytic condensation of equimolar amounts of DHT and heptanoic anhydride [6]. The crude product was purified [7] by countercurrent extraction and column chromatography. Follow-

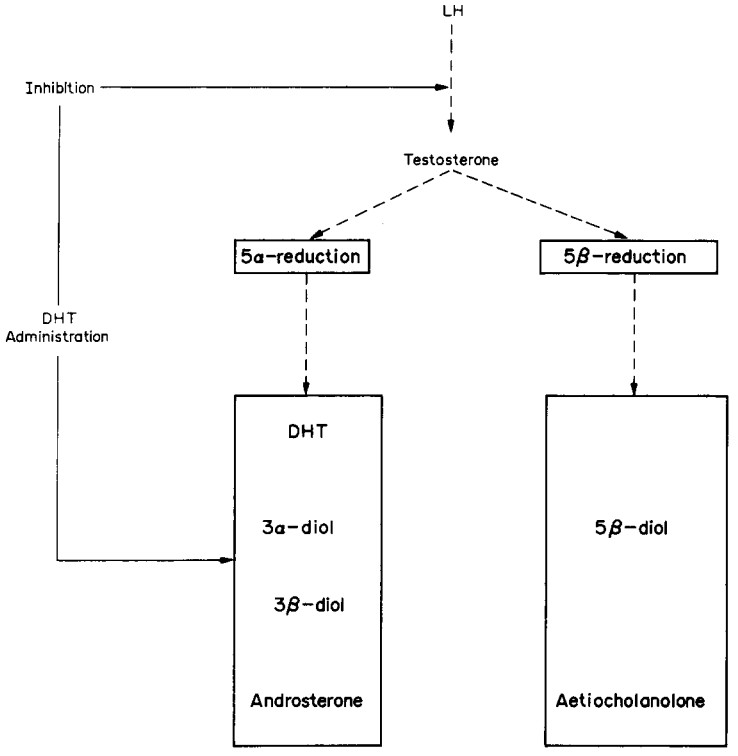

Fig. 1. The 5α-reduced and 5β-reduced metabolites of T. DHT administration would be expected to give rise to DHT, 3α-diol, 3β-diol and androsterone in urine while suppressing the production of 5β-diol and aetiocholanolone.

ing recrystallization from aqueous methanol (twice) and aqueous acetone (twice), the ester was dissolved in sesame oil and passed through a sterile 0.22 μm filter into 1 ml ampoules to give a final concentration of DHT heptanoate of 75 mg/ml (as estimated by GC).

Rabbit antiserum, raised against 5α-androstane-3α,17β-diol-7-*O*-carboxymethyloxime-bovine serum albumin, was used for the RIA of T, DHT and 3α-diol. The antiserum was obtained from Dr D. Bulbrook, Department of Chemistry and Pathology, Hope Hospital, Salford, England. [1,2-^{3}H]5α-androstane-3α,17β-diol (59 Ci/mmol, Amersham Int., Bucks., England) was used as tracer. Cross-reactions at 50% displacement of the total tracer bound were: 3α-diol 100%, DHT 87% and T 43%. Scintillation counting was performed on a 1211 minibeta counter using a scintillant "Optiphase", both obtained from Pharmacia Wallac U.K. Ltd, (Milton Keynes, England). The HPLC system used consisted of a Rheodyne 7125 sample injector, PU 4010 isocratic pumps and a PU 4020 u.v. detector purchased from Phillips (Cambridge, England). A Spherisorb C18 (5 μm) pre-column (4.6 mm i.d. $\times$ 30 mm), also supplied by Phillips, was used in series with a Nova Pak C18 (4 μm) radial compression cartridge (8 $\times$ 100 mm) housed in a RCM100 module, supplied by Waters Associates (Harrow, Middlesex, England). HPLC grade solvents were obtained from BDH (Poole, Dorset, England).

The gas chromatograph–mass spectrometer (HP-5890A/5970B) with autosampler (HP-7673A) and HP-1 methylsilicone column (25 m, 0.2 mm i.d., film thickness 0.1 μm) was supplied by Hewlett-Packard Ltd (Wokingham, Berks., England).

Drug administration

Two male volunteers (subject G, aged 28 years and B, 65 years), having given informed consent, each received an injection of DHT heptanoate (150 mg in 2 ml sesame oil). 24 h urine collections were made prior to the injections (administered at time 0) and continued for 4 weeks afterwards. Additionally, each void of the bladder during a basal 24 h period was collected from both subjects. 24 h urine collections were also taken from 13 normal men (aged 20–50 years). An aqueous solution of merthiolate (10% w/v) was added to all collections (0.5 ml per 24 h collection), which were then stored at 4°C. Blood samples were taken immediately before the injections (time "0", basal) and at the same time (10:00 h) on previous (basal) and subsequent days. Blood was collected in tubes containing heparin, mixed gently and centrifuged. The plasma was stored at -70°C until analysed.

Hormone measurements

Measurements of LH, FSH and oestradiol in plasma were performed by the Supra-regional Assay Service at St Thomas' Hospital, London using their own laboratory produced RIA methods.

HPLC–RIA of DHT, T and 3α-diol (described in detail elsewhere [7]) was as follows: a tenth of each 24 h urine collection was diluted to 200 ml, a portion of which was applied to a primed C18 Bond Elute cartridge (100 mg, Jones Chromatography, Hengoed, Wales). The cartridge was washed with water (2 $\times$ 0.5 ml) and dichloromethane (0.5 ml) before steroid conjugates were eluted with methanol (2 $\times$ 0.5 ml). The methanol was evaporated and the extracts dissolved in acetate buffer (0.75 ml, 0.3 M, pH 4.5) containing β-glucuronidase enzyme (Patella vulgata, 3000 Fishman Units). The reconstituted extracts were incubated overnight at 37°C and then extracted with dichloromethane (4 ml). The organic phase was washed with sodium hydroxide solution (0.5 ml, 0.1 M) and water (2 $\times$ 0.5 ml) before being evaporated to dryness. The extracts were dissolved in HPLC mobile phase and injected onto the HPLC column. A mobile phase of aqueous acetonitrile (50% v/v) was used at a flow rate of 1 ml/min. Fractions of eluant corresponding to T, 3α-diol and DHT were collected blind by reference to the internal standard (11β-hydroxyandrostenedione) which was monitored at 240 nm. Each fraction was evaporated to dryness (in a vacuum desiccator, overnight at 37°C) and reconstituted in phosphate buffered saline (PBS, 2.5 ml, pH 7.4) containing bovine serum albumin (0.1% w/v). Duplicate portions (100 μl) of each fraction were assayed against appropriate standard curves by incubating with 3α-diol antiserum (diluted 1:20,000 in PBS, 200 μl) and tracer in PBS (10 nCi, 100 μl) for 1 h at room temperature followed by 1 h at 4°C. Free steroids were then removed with charcoal and antibody-bound label estimated by scintillation counting. Calculated excretion rates were corrected for procedural losses, derived from the mean analytical recoveries of tritiated analogues of DHT, T and 3α-diol (Amersham Inter-

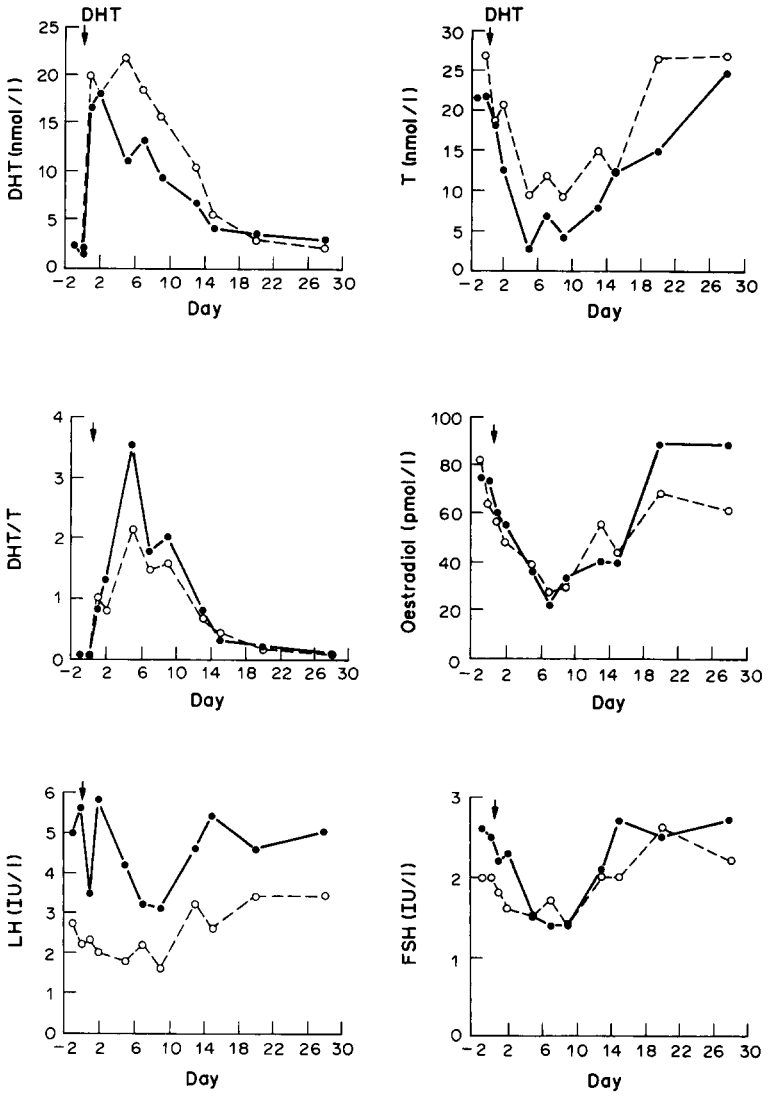

Fig. 2. Plasma concentrations of DHT, T, oestradiol, LH and FSH, and the ratio of plasma concentrations of DHT to T, before (day −1 and 0) and after the administration of DHT heptanoate (150 mg, i.m. on day 0), in two normal adult male subjects, B (—●—) and G (– –○– –).

national, Bucks, England) from hydrolyzed urine.

Plasma samples (1 ml) were extracted with ether (10 ml) by vortex mixing for 10 min. The ether was then evaporated and the extract dissolved in HPLC mobile phase. A portion was injected onto the HPLC. HPLC conditions were as above, but only the T and DHT fractions were collected and assayed. Sample extracts from each subject were measured on the same RIA for which the intra-assay precision (C.V.) was 10% for T, 12% for 3α-diol and 14% for DHT.

GC–MS analysis of hydrolyzed urine was performed using the method of Cowan *et al.* [8]. The steroids were converted to their bis-trimethylsilylether (bis-TMS) derivatives by the addition of a 50 μl mixture of *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide, iodotrimethylsilane and dithioerythritol (1000:2:2, by vol) under nitrogen and heating at 60°C for 15 min. [9]. To the cooled derivatives, dry dodecane (50 μl) was added and an aliquot (1 μl) was analysed by selective ion monitoring GC–MS. The flow rate of the helium carrier gas was 0.7 ml/min. The temperatures of the injection port and transfer line were 250 and 275°C, respectively. The oven temperature was programmed at 180°C for 1 min, followed by a 2°C/min rise to 250°C and then by a 5°C/min rise to 280°C. Selected ions were monitored using a 50 ms dwell time. The abundance of the

Table 1. Normal urinary excretion rates (nmol/24 h) of T, DHT and 3α-diol in 13 normal men

	T	DHT	3α-Diol
Mean	241	109	459
SD	159	68	250
Range	80–609	28–243	83–933

ions at a mass to charge ratio (m/z) of 272 for the mono-trimethylsilyl ether derivatives of androsterone/aetiocholanolone were monitored and were required to be $<1\%$ of that of the abundance of ions at m/z 434 for the androsterone/aetiocholanolone bis-TMS derivatives to confirm satisfactory derivatization. The abundance of selected ions for the peaks of the bis-TMS derivatives of T and Epi-T (m/z 432); DHT (m/z 434); and 3α-diol, 5β-diol and 3β-diol (m/z 436) were measured and, for steroids of interest, the abundance ratios of these ions were calculated. Normal ratios were established using untimed urine samples taken from 36 athletes competing in sports with a low incidence of steroid abuse. GC–MS analysis of these samples showed no indication of the presence of drugs banned by the IOC.

RESULTS

The injection of DHT heptanoate resulted in a rapid increase in plasma DHT, a concomitant fall in plasma T and, as a consequence, a large (30-fold) increase in the plasma ratio of DHT/T in both subjects (Fig. 2). Concentrations of LH, FSH and oestradiol in plasma also declined, although the proportional decreases in the gonadotrophins (≈50%) were less than those for T (≈80%). T, LH and FSH all tended towards normal pre-injection values after a period of about two weeks, as circulating DHT returned towards normal.

Table 2. Variation of urinary ratios of 3α-diol/T and DHT/T during a 24 h period in 2 normal men

Collection period	3α-diol / T	10 × DHT / T
Subject G		
17:00–21:00	1.0	2.3
21:00–24:00	1.0	2.3
24:00–08:30	1.2	3.9
08:30–12:15	1.0	3.7
12:15–17:00	1.0	3.1
Subject B		
17:00–22:10	0.9	3.3
22:10–06:00	0.7	2.6
06:00–08:15	1.1	4.3
08:15–11:40	1.5	7.0
11:40–17:00	1.2	4.5

Urinary steroids determined using HPLC–RIA.

The substantial suppression of the urinary excretion rates of T (Fig. 3), 5β-diol and Epi-T meant that the proportional increases in the ratios shown in Fig. 4 were greater than those observed in the excretion rates/24 h of 3α-diol and DHT (as measured by RIA). These ratios remained above the normal ranges determined from 36 athletes for up to 2 weeks after the administration of DHT. The excretion rates of 3α-diol and DHT were greater than their basal values for a similar period (Fig. 3), but barely exceeded the normal ranges established (Table 1). Evidence of small circadian variations

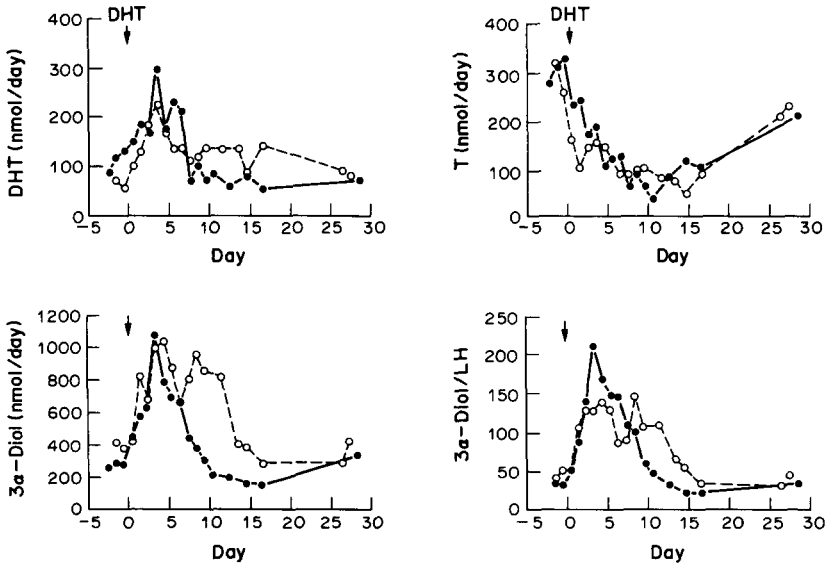

Fig. 3. Effect of DHT heptanoate administration (150 mg, i.m. on day 0) on the daily excretion rates of DHT, T and 3α-diol, and the ratio of the rates of excretion of 3α-diol to LH, in two normal male subjects, B(—●—) and G (--○--).

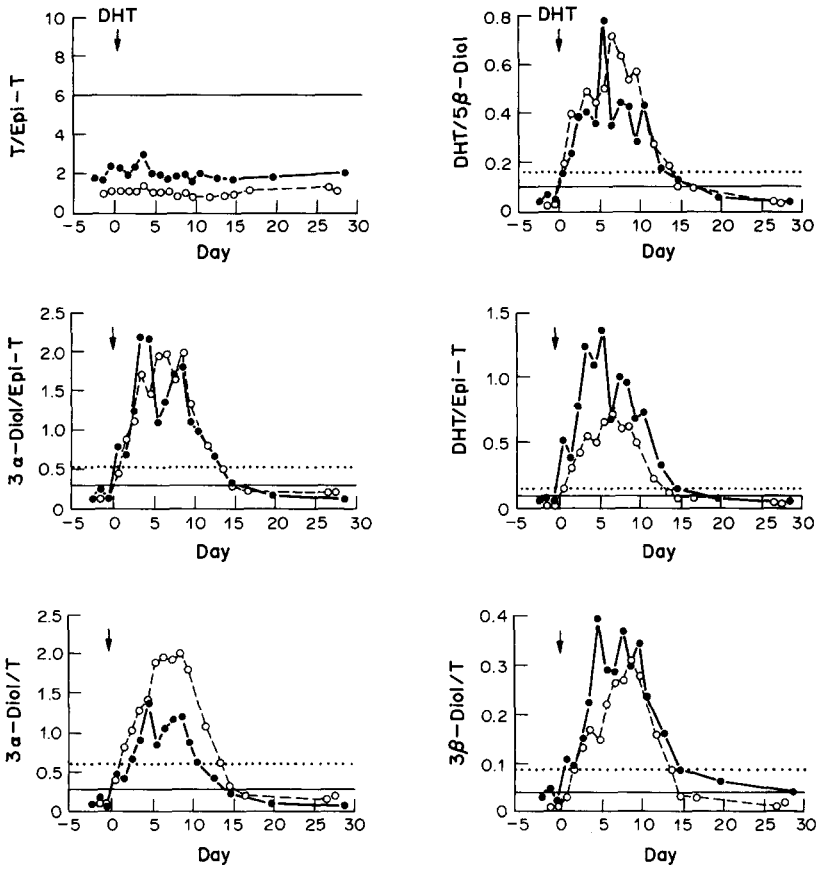

Fig. 4. Effect of DHT heptanoate administration (150 mg, i.m. on day 0) on the abundance ratios of the molecular ions for the peaks of the bis-trimethylsilyl ether derivatives of T and E (m/z 432); DHT (m/z 434); and 3α-diol, 5β-diol and 3β-diol (m/z 436), as determined by selective ion monitoring GC–MS. Mean (—) and mean + SD (· · ·) of normal males ($n = 36$) are shown as horizontal lines, except for the ratio T/Epi-T where the IOC cut-off limit of 6 is shown.

in the ratios of 3α-diol/T and DHT/T was found in both subjects (Table 2).

Following the injection of DHT to subject B, the excretion rate of LH declined steadily, reaching a minimum of 50% of basal on day 9. Although this fall was not as apparent in subject G, the ratios of DHT/LH and 3α-diol/LH increased in both subjects. The ratio of 3α-diol/3β-diol showed little change in either subject, whereas the ratio of 3α-diol/DHT showed a small initial decrease but had returned to normal by day 5.

DISCUSSION

Increased plasma concentrations of DHT following the injection of DHT heptanoate were accompanied by decreases in the concentrations of LH and T in plasma (Fig. 2). This is consistent with the action of supraphysiological doses of DHT in suppressing the release of LH and the consequent decline of testicular steroidogenesis [3, 10]. These changes were in accord with increases in the urinary excretion rates of DHT and its metabolites 3α-diol and 3β-diol and reductions in those of LH and T (and by inference those of Epi-T and 5β-diol). This is to be expected if DHT is metabolized to only 5α-metabolites and suppresses the hypothalamic–pituitary–gonadal axis (Fig. 1). Accordingly, the urinary ratios of the above 5α-metabolites to any of T, Epi-T, 5β-diol or LH rose substantially, following the administration of DHT (Fig. 4).

Although the excretion rates of Epi-T, 3β-diol and 5β-diol were not measured directly, approximations can be extrapolated from their ratios (as measured by GC–MS) to those steroids for which the excretion rates were measured (3α-diol, T, DHT). However, a different preparation of β-glucuronidase was used for sample analysis by GC–MS compared to that for HPLC–RIA. Nevertheless, even with use of the same β-glucuronidase preparation, the

efficiencies of hydrolysis of different steroid glucuronides are not the same [11]. It follows that while consistency of hydrolysis conditions is required for precise comparison of samples, this does not guarantee accuracy in the estimated ratio.

By day 11, plasma concentrations of DHT had declined sufficiently to allow plasma LH and T to increase. Consequently, the urinary ratios of 5α-metabolites to 5β-metabolites or T decreased (Fig. 4) as the excretion rates of the DHT metabolites declined toward pre-injection values and those of T (and Epi-T and 5β-diol) rose. The concurrent increases of plasma concentrations of gonadotrophins and T from maximal suppression by DHT, while the concentrations of DHT were still 2–4 times basal, implies that circulating DHT probably plays only a small part in the regulation of LH and FSH in normal men. Nevertheless, as these concentrations of DHT (5–8 nmol/l) were only a third of the basal concentrations of T measured in the two subjects, the potency of DHT *per se* in suppression of LH and FSH may be greater than that of T.

Although the falls in plasma T correlated well with the decreases in the urinary excretion rates of T, the relative changes in the ratio of DHT/T in hydrolyzed urine were not as great as those in plasma (Figs 2 and 4). This was because the relative increase in the excretion rate of DHT did not match that of plasma DHT. This, in turn, may be due either to the suppression of an endogenous source of urinary DHT which is distinct from circulating DHT, or the further metabolism of DHT in urogenital tissue as has been suggested for both DHT and 3α-diol [12].

If it is assumed that the injection of DHT heptanoate was the sole source of 3α-diol and DHT excreted during the subsequent 2 weeks, then the proportions of DHT recovered from hydrolyzed urine as DHT and 3α-diol were 1.7 to 3.2% and 0.4 to 0.8%, respectively. Much of the rest of DHT is probably metabolized to androsterone (3α-hydroxy-5α-androstan-17-one). Even so, the ratio of androsterone: aetiocholanolone (3α-hydroxy-5β-androstan-17-one) in hydrolyzed urine as measured by GC–MS rose from 0.5 to only 1.0 (subject B) and 0.7 to 1.1 (subject G), presumably because of the continued contribution to both metabolites from the adrenal androgens dehydroepiandrosterone and androstenedione. These adrenal steroids may also contribute to urinary 3α-diol excretion; stimulation of the production of adrenal steroids by the administration of adrenocorticotrophic hormone can cause an increase in urinary excretion of 3α-diol without affecting plasma concentrations of 3α-diol [13]. Such an adrenal source of urinary 3α-diol or DHT may contribute to the variation of ratios (estimated by HPLC–RIA) over a 24 h period (Table 2).

As judged by the lack of significant change in the ratio of T/Epi-T following administration of DHT (Fig. 4), the profound fall in the urinary excretion rate of T was matched by that of Epi-T. This confirms earlier observations [1] that the excretion rate of Epi-T is decreased when the hypothalamic–pituitary–gonadal axis is suppressed. Thus, it is apparent that administration of DHT bypasses the IOC regulations concerning the ratio of T/Epi-T. Similarly, the ratios of T/LH were not grossly altered. In contrast the ratios of DHT/LH and 3α-diol/LH were increased (Fig. 3).

DHT is available as proprietary preparations which are to be administered sublingually or sublabially. It has been claimed in the 'Underground Steroid Handbook II' that one such preparation, Pesomax, was a "popular DHT derivative used at the Seoul Olympic Games" that "didn't get tested positive" [14]. It follows that rapid introduction of tests for DHT abuse would be desirable. Results presented here suggest that distortion of normal steroid excretion patterns may form the basis of such a test. However, the large alteration of urinary parameters demonstrated in individuals receiving DHT must be viewed against the range of values in a normal population. Although the maximum excretion rates of DHT and 3α-diol observed after injection of DHT heptanoate to both subjects barely exceeded the range of normal values observed (Table 1), they were well above the mean values for over a week. On the other hand, the excretion rates of T fell to below the normal range and it was this contribution to the ratios of 3α-diol/T and DHT/T that was largely responsible for their abnormality following the administration of DHT.

The ratios shown in Fig. 4 for both subjects following the administration of DHT, remained above the appropriate normal ranges for similar periods of around 10 days. It is pertinent that Epi-T may be a better denominator for urinary ratios than T, because Epi-T is not available as a pharmaceutical preparation. Nevertheless, non-proprietary preparations of aqueous suspensions of Epi-T for direct introduction into

the systemic circulation and esters of Epi-T for i.m. injection [1] should not be overlooked.

The dose of DHT contained in the injection given was low (equivalent to 104 mg DHT), but even after 2 weeks plasma DHT was still elevated. Reduced excretion of T contributed considerably to maintaining urinary DHT/T and 3α-diol/T ratios even when the excretion rates of DHT and 3α-diol were declining. Serial injections at weekly intervals, even at such low doses, would have a cumulative effect in causing more complete suppression of LH secretion and hence of testicular steroidogenesis, resulting in considerable augmentation of these ratios. In view of the small proportion of DHT excreted as urinary DHT and 3α-diol, monitoring changes in more than one parameter seems advantageous. Indeed, reliance on just one ratio has been shown to be limiting in other instances [1]. GC–MS allows such steroid profiling and the steroids discussed here could be measured with only slight modification to existing IOC methods.

The results reported here are sufficiently encouraging to warrant further study of these ratios as a basis for defining DHT abuse. At the very least, the characteristic GC profile would provide a screening technique to identify samples worthy of further investigation. The best method to confirm DHT administration is as yet unclear, but significant changes in the ratio of $C^{13}:C^{12}$ of excreted T has been shown to occur following injection of pharmaceutical T [15] and may be relevant to other steroids.

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