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Intramuscular administration of 5α–Dihydrotestosterone Heptanoate : Changes in the Urinary Hormone Profile

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Abstract

A recommended confirmatory procedure for detecting 5α–dihydrotestosterone (DHT) doping in male athletes proposes the use of the urinary concentration ratio of DHT to epitestosterone (EpiT) as the primary marker, and those of 5α–androstane-3α,17β-diol (5α–Adiol) to EpiT, luteinizing hormone (LH) and 5β–androstane-3α,17β-diol (5β–Adiol) as secondary markers. Here we investigate the effects on these markers of intramuscular (i.m.) administration of DHT heptanoate (250 mg) to 6 healthy men. Within 24 h of administration all four markers greatly exceeded our chosen discrimination limits, remaining above these limits for 10 to 14 days. All ratios returned to basal values by day 28. In contrast to percutaneous administration, 5β–Adiol excretion decreased, probably as a consequence of greater suppression of testicular steroidogenesis. The results were largely in agreement with those obtained after percutaneous administration, though augmented probably by the larger dose and the different route of delivery.

Introduction

In the context of doping with anabolic steroids in sport, recent attention has been focused on dihydrotestosterone (DHT), the active 5α–reduced metabolite of testosterone (T). A proposed confirmatory procedure (1) for detecting 5α–DHT administration in male athletes recommends the urinary concentration ratio of DHT/epitestosterone (EpiT) to be the primary marker of administration; 5α–androstane-3α,17β-diol (5α–Adiol: the main metabolite of DHT)/EpiT, 5α–Adiol/luteinising hormone (LH) and 5α–Adiol/5β–androstane-3α,17β-diol (5β–Adiol: a metabolite of T) were chosen as secondary markers. Discrimination limits of 2, 11.6, 112.4 and 4.3 respectively were determined; values exceeding these limits being indicative of an offence having taken place (1).

Having studied the effects of percutaneous DHT administration on these markers (1), we wanted to investigate the application of the test to the detection of DHT doping using other routes of administration. Some work has already been done to investigate the effects of oral (2, 3) and sublingual (4) administration on the urinary hormone profile. Intramuscular administration (i.m.) of esterified compounds prolongs the activity of steroids and investigations into the possible clinical use of DHT heptanoate (5) in replacement therapy found i.m. injection to give a prompt and sustained rise in DHT. Such a future clinical use would increase the risk of underground availability of licensed DHT compounds. Even without such a supply, esters of DHT would be relatively simple to synthesise by the underground chemist and could easily be formulated for i.m. delivery.

Our primary objective in this study was to quantify by gas chromatography-mass spectrometry (GC-MS) the changes in urinary steroid concentrations following i.m. administration of DHT heptanoate and thus determine hormone concentration ratios. We formulated a dose of 250 mg as this is equivalent in mass to licensed formulations of T heptanoate, e.g. Primoteston®, and administered the DHT ester to 10 healthy men. Heptanoyl chloride was used to synthesise the

heptanoate ester and a trideuterated analogue of 5α–DHT (D_3-DHT), synthesised by hydrogenation of D_3-T, was added to our internal standard mixture.

[A more detailed description of this work has been submitted for publication to Clinical Chemistry.]

Materials and Methods

Materials

Materials were obtained as described previously (1). D_3-EpiT was supplied by Ultrafine® chemicals, Manchester, UK.

Synthesis of DHT Heptanoate

DHT (25 g) was dissolved in dichloromethane (250 mL). Heptanoyl chloride (16 mL) was added, together with 4-(N,N-dimethylamino)-pyridine (3.75 g) as a catalyst. The flask was stoppered with an anhydrous calcium chloride tube, the entire apparatus protected from the light and the reaction mixture stirred magnetically at regular intervals. After one week the reaction mixture was washed with sodium hydroxide (2 x 75 mL, 1M), hydrochloric acid (2 x 75 mL, 1M) and then deionised water until an aqueous layer of neutral pH was obtained. Evaporation of the organic layer resulted in a yellow, waxy solid which was further dried by placing in a desiccator for several days. The product was recrystallised using acetone/water and characterised using electron impact full scan mass spectrometry (MS) with an ion-trap detector (ITD 800; Finnigan MAT, Herts, UK) coupled to a gas chromatograph (model 5890A: Hewlett-Packard) fitted with an HP-1 methyl silicone column. Purity of the compound was also assessed by nuclear (^1H) magnetic resonance spectroscopy (AMX 400 NMR Spectrometer). The DHT heptanoate was prepared for injection (250 mg; arachis oil and benzoyl alcohol;1 mL) at St Thomas' Hospital, London.

Synthesis of [16,16,17-$^2H_3$]-5α–DHT

[16,16,17-$^2H_3$]-T (20 mg) was dissolved in tetrahydrofuran (10 mL). A catalyst (palladium on activated charcoal; 20 mg) was added and the mixture hydrogenated for 2 hours whilst stirring. The reaction products, the 5α– and 5β– isomers of [16,16,17-$^2H_3$]-DHT were separated by means of their differing solubilities in acetonitrile/H_2O. The chemical and isotopic purity of this [16,16,17-$^2H_3$]-5α–DHT was assessed using full scan MS. The retention time/methylene units of the bis-TMS derivatives of D_3-5α– and D_3-5β–DHT (m/z 437) were 22.2 min/2621 MU and 17.0 min/2456 MU respectively.

Administration and Sample Collection

5α–DHT Heptanoate (250 mg i.m.) was administered to 6 male volunteers, aged between 23 and 28. 24 hour pooled urine samples were collected on days -2 to day 5, and on days 7, 10, 14, 21 and 28, the exception being the day of administration when the collections were divided into two 12 hour periods. Total volumes were recorded and the samples divided into appropriate aliquots. Urine samples for steroid analysis were stored at -20 °C and for LH analysis samples were frozen rapidly in liquid nitrogen and then stored at -70 °C.

Steroid Extraction

Steroid concentrations were determined according to the method described by Kicman *et al* (1): extraction using Isolute C_8 cartridges; hydrolysis by β-glucuronidase; extraction into diethyl ether; and conversion to trimethylsilyl ether (TMS) derivatives for selected ion monitoring (SIM) GC-MS. Quantification of steroids, also described previously, was achieved by ratioing the abundance of each steroid to that of its trideuterated analogue and referencing these peak height ratios to calibration curves constructed from aqueous standards run simultaneously with samples. For the steroids androsterone (A) and etiocholanolone (E), peak height ratios alone were determined and on urine samples that had been diluted 1:10 (v/v) with water prior to extraction. Calibrants and quality controls were the same as before; the trideuterated internal standard mixture consisted of

D_3-T, D_3-EpiT, D_3-DHT and D_3-5α–Adiol in amounts that, when added as 50 μL aliquots, gave final concentrations of 50, 50, 50 and 100 μg/L respectively.

LH Measurement

Urinary LH concentration was determined using the Serono® immunoenzymetric assay by direct measurement and following ultrafiltration according to the method described previously (1 and references therein).

Results

Synthesis of DHT heptanoate resulted in 91.6 % recovery (31.8 g) of crude product. Recrystallisation in acetone/water gave a white product that was considered chemically pure after subsequent analysis by both full scan MS and NMR.

Hydrogenation of D_3-T gave a 50.4 % yield (10.1 mg) of the combined 5α– and 5β– isomers of D_3-DHT: separation of the two isomers produced 4.19 mg of D_3-5α–DHT.

Validation of the steroid assay has been described elsewhere (1). The four quality controls analysed in each run (n=6 runs) showed a similar between-assay imprecision and gave urinary concentrations of steroid analytes within 2 SD of the mean values reported previously.

The hormone concentration ratios DHT/EpiT, 5α–Adiol/EpiT, 5α–Adiol/LH and 5α–Adiol/5β–Adiol shown previously to be suitable markers of percutaneous administration were similarly assessed in this study. All four ratios were found to increase rapidly, means for the group exceeding the respective discrimination limits of 2, 11.6, 112.4 and 4.3 within the first 24 h after injection, and remaining elevated above these limits until between days 10 or 14 (Fig. 1). All ratios had returned to basal by day 28.

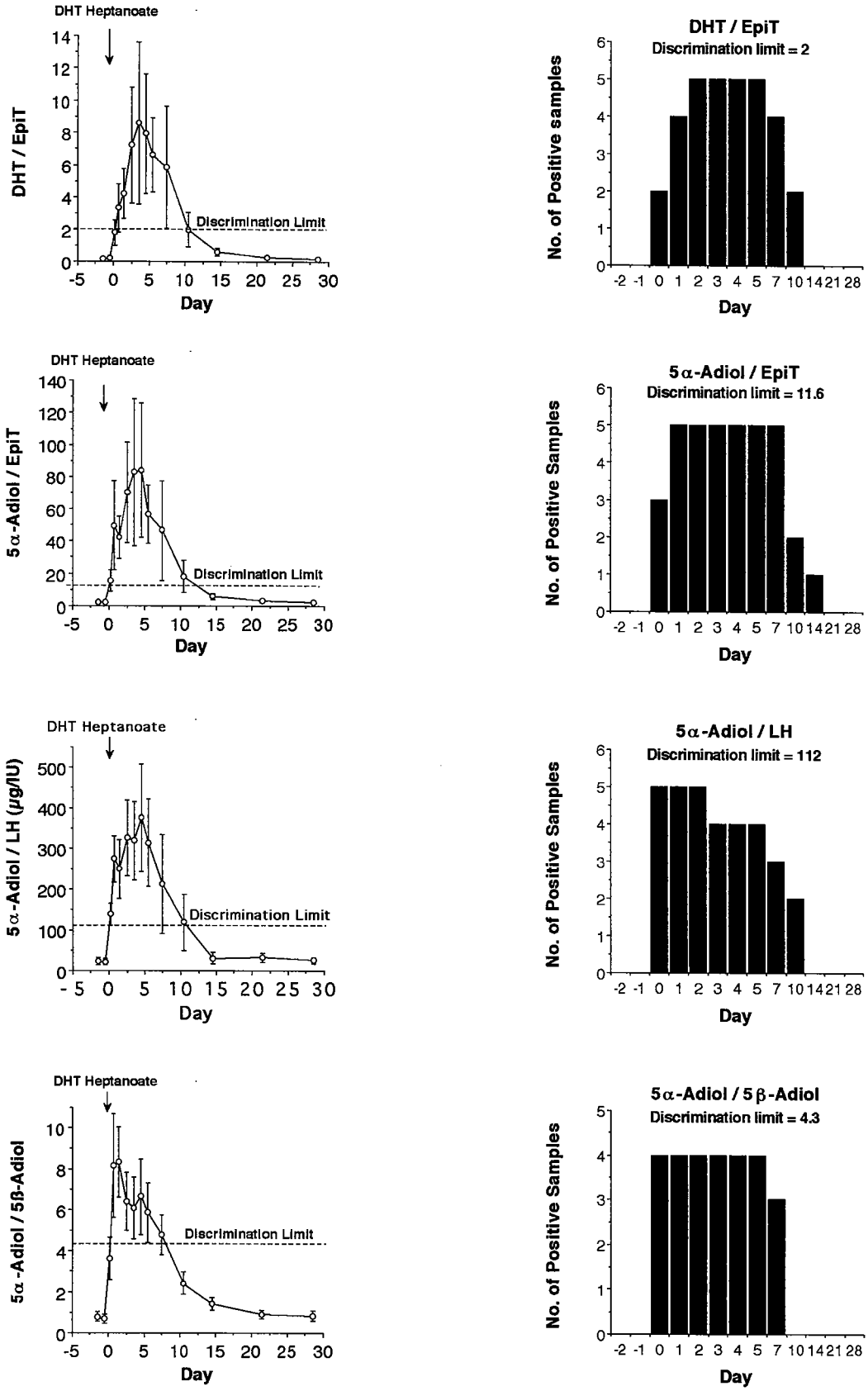

Fig. 1. Effect of i.m. administration of DHT heptanoate (250 mg) on the mean urinary concentration ratios DHT/EpiT, 5α-Adiol/EpiT, 5α-Adiol/LH and 5α-Adiol/5β-Adiol in six healthy men (error bars represent SEM; n = 6).

The large error bars on the graphs reflect a variability amongst the volunteers that comes as a result of both the wide range of basal values observed and the large variation in the degree of response. Examples are displayed in Table 1: the basal sample from subject 4 has smaller values of DHT/EpiT and 5α–Adiol/EpiT than those from other volunteers, resulting from a larger basal EpiT concentration; samples from subject 2, compared to the group, showed a much greater increase in ratios following administration.

Table 1. Day 3 concentration ratios compared with basal to show variation in response

Subject	DHT / EpiT		5α-Adiol / EpiT		5α-Adiol/ LH		5α- / 5β-Adiol	
	basal	day 3	basal	day 3	basal	day 3	basal	day 3
1	0.3	5.2	2.2	48.8	52.8	563.1	0.2	1.4
2	0.3	33.5	3.4	310.0	9.9	436.6	0.7	10.1
3	0.2	5.1	4.0	61.8	22.1	138.4	1.1	5.2
4	0.01	0.75	0.3	10.8	6.3	88.5	1.5	11.0
5	0.3	2.9	1.5	33.4	39.0	592.5	0.3	3.7
6	0.2	4.2	1.5	31.6	6.2	95.0	0.4	5.1

Although the overall degree of response is of interest, what is of greater importance is the ability of the test to determine positive from negative samples. The histograms show how many of the samples exceeded the discrimination limits on each day. In all but the ratio of 5α–Adiol/5β–Adiol, five of the six volunteers gave samples whose ratios exceeded the discrimination limits. The one exception was due to the samples collected from the individual who had a larger average basal EpiT concentration compared to the other volunteers (158 & 90 μg/L on days -2 and -1 respectively, compared with a mean for the rest of the group of 37 & 41 μg/L). With 5α–Adiol/5β–Adiol, two out of the six volunteers had samples whose ratios did not exceed the discrimination limit; samples from these volunteers were characterised by having large concentrations of 5β–Adiol compared to the rest of the group.

The peak height ratio of A/E was also determined on selected days (Fig. 2) as this is a marker chosen by some International Olympic Committee (IOC) accredited laboratories. The A/E ratio was found to increase, approximately doubling in the first 24-48 hours, and then slowly returned to basal.

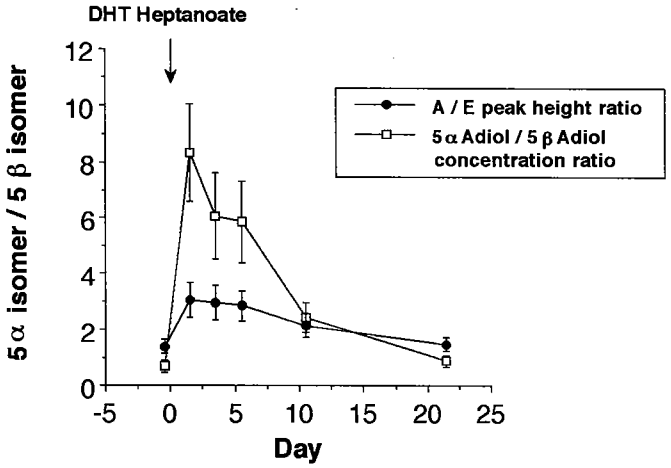

Fig. 2. Comparison of the effect of i.m. administration of DHT heptanoate (250 mg) on the urinary peak height ratio of A/E with the concentration ratio of 5α–Adiol/5β–Adiol in six healthy men (mean ± SEM; n = 6)

Discussion

We have shown previously (1) that 125 mg DHT administered percutaneously twice daily for 3 days caused increases in the hormone concentration ratios DHT/EpiT, 5α–Adiol/EpiT, 5α–Adiol/LH and 5α–Adiol/5β–Adiol. With the exception of 5α–Adiol/5β–Adiol, all ratios for the group exceeded the discrimination limits of 2, 11.6, 112.4 and 4.3 respectively. These chosen markers again proved useful as evidence of i.m. injection. In comparison to percutaneous administration the responses were generally more marked, a fact we attribute to the larger dose and different route of administration. Concentration ratios for the group clearly exceeded the discrimination limits of all four markers and indeed even by day 10 were still elevated above these

limits in all cases except 5α–Adiol/5β–Adiol. A return to basal levels did not happen until around day 28.

Following i.m. injection, samples from five out of the six volunteers had concentration ratios that for several days exceeded the discrimination limits for DHT/EpiT and 5α–Adiol to EpiT and LH. This compares with respective numbers of six, seven and five volunteers out of the ten from the percutaneous study, whose ratios exceeded the discrimination limits on day 3 of administration, the day in which ratios for the group were most augmented. Therefore under our proposed confirmatory procedure, five of the six individuals administered intramuscularly with DHT heptanoate would be considered positive. The fact that one volunteer would have escaped detection, despite the dose administered and its direct route of delivery, might suggest that our discrimination limits are too large, and thus favours investigating the development of a discrimination function incorporating several hormone concentration ratios.

The peak height ratio of A/E was not shown to be a sensitive marker of percutaneous DHT administration as although increases were seen they were small due to the fact that steroids of adrenal origin contribute largely to the formation of glucuronide conjugates of A and E. The A/E ratio is however reported to be a good marker of oral administration (2, 3) due to extensive first pass metabolism, and Geyer *et al* showed sublingual administration also to have a considerable effect. In this study i.m. injection caused an approximate doubling of the peak height ratio of A/E. However, compared with the concentration ratio of 5α–Adiol/5β–Adiol (Fig. 2), remembering this to be the least sensitive of our chosen markers, the increase in the ratio of A/E itself is relatively small. Hence for i.m. administration of modest doses of DHT, as with for percutaneous application, we see little change in the ratio of A/E and therefore do not favour it as a marker of doping by these routes of administration.

In conclusion, we had previously developed a confirmatory procedure proposing the hormone concentration ratios DHT/E, 5α–Adiol/E, 5α–Adiol/LH and 5α–Adiol/5β–Adiol as suitable markers for detecting DHT doping in male athletes. These markers were found to be sensitive for percutaneous administration but investigation of alternative routes of administration was

recommended. With i.m. administration, the larger dose and different route of administration augmented the responses seen previously, demonstrating the ability of our chosen markers to detect the i.m. administration of DHT heptanoate. Further evidence is therefore provided of the suitability of these hormone concentration ratios as a method of confirming doping with DHT.

Acknowledgements

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