Potential Use of Ketoconazole in a Dynamic Endocrine Test to Differentiate between Biological Outliers and Testosterone Use by Athletes

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Determination of the ratio of testosterone to epitestosterone (T/E) in urine is used to detect testosterone administration in athletes, with a ratio >6 considered as evidence of an offense. We show that administration of ketoconazole, which inhibits testosterone biosynthesis, may be useful for differentiating between an athlete who is using testosterone and one who naturally gives a ratio >6. In a control subject pretreated with testosterone, ketoconazole caused the ratio to increase; conversely, it caused a decrease in the ratio in an athlete under investigation. Repeated administration of ketoconazole to two normal men caused a decrease in the ratio due to a large decrease in the urine excretion rate of testosterone relative to epitestosterone. Stimulation with human chorionic gonadotropin exacerbated the differences in excretion rates. A single administration of ketoconazole to six normal men caused the T/E ratios to decrease significantly within 8 h, a suitable time scale for use in a dynamic test.

Indexing Terms: sports medicine · epitestosterone · androgens · anabolic steroids

The misuse of anabolic agents in sport is well recognized and such substances continue to comprise the largest percentage of positive samples detected by the International Olympic Committee (IOC) accredited laboratories. The IOC states that the banned class of anabolic steroids includes substances that are related in structure and activity to testosterone. Currently, only untimed urine samples are collected from athletes to determine whether a banned drug has been administered. For detection of foreign steroids, all that is required is unequivocal identification in the urine sample of the parent compound or a diagnostic metabolite. Results of analysis from all of the IOC-accredited laboratories in 1991 suggest that the majority of athletes who misuse steroids appear now to have switched to the naturally occurring androgen testosterone to evade detection.

Methods for detecting the administration of androgens such as testosterone, which are also naturally secreted, depend on the consequent distortion of the normal hormone profile. Normal men produce 30-fold more testosterone endogenously than its inactive epimer, epitestosterone (17α-hydroxyandrost-4-en-3-one), but excrete only ~1% of testosterone unchanged compared with ~30% of epitestosterone. Consequently, the ratio of the concentration of testosterone to epitestosterone (T/E) in urine is ~1 (1). This ratio is independent of the degree of dilution of urine but is distorted by the administration of testosterone, which causes an increase in the excretion rate of testosterone and a decrease in the excretion rate of epitestosterone as a result of suppression of the hypothalamic–pituitary–gonadal axis (2). Conversely, stimulation of testicular steroidogenesis by administration of human chorionic gonadotropin (hCG) results in a concomitant increase in the urinary excretion rate of testosterone and epitestosterone and hence no significant change in the ratio (3). Little, if any, epitestosterone is derived from the peripheral metabolism of testosterone, 4-androstene-3,17-dione, or dehydroepiandrosterone (1, 4, 5); its biosynthetic steroid precursor is not known.

In 1983 the IOC adopted the T/E ratio as the sole test for testosterone administration, with action usually being taken against an athlete if the urinary T/E concentration ratio exceeds 6. However, some male athletes who denied using testosterone have reportedly produced T/E ratios between 6 and 9 (6). Indeed, the case has been put forward for using at least two indices, such as the T/E and testosterone to luteinizing hormone ratios, as criteria for detecting testosterone administration (7), not least to overcome the possible lack of specificity in measuring T/E alone. In addition, for such cases, a simple dynamic test may be of value in differentiating between athletes using exogenous testosterone and those unusual individuals who naturally have a T/E ratio >6 and wish to prove their innocence.

Ketoconazole is a pharmaceutically licensed antifungal agent that also inhibits testosterone biosynthesis in men (8, 9) and therefore may be useful for such differential diagnosis. This drug in the appropriate dose inhibits both the 17α-hydroxylase and 17,20-lyase activity of cytochrome P450c17 (10), the enzyme that converts pregnenolone to dehydroepiandrosterone and progesterone to androstenedione in the human testes, ovaries, and adrenal glands (11, 12). The other enzymes necessary for the biosynthesis of testosterone, 3β-hydroxysteroid dehydrogenase isomerase and 17β-hydroxysteroid dehydrogenase (also known as 17β-hydroxysteroid oxidoreductase), are cytochrome P450-independent enzymes and are not inhibited by ketoconazole (13).
The administration of ketoconazole to an athlete who has taken supraphysiological doses of testosterone would be expected to have little effect on the serum concentration of testosterone, whereas the opposite would be expected for individuals with naturally large urinary T/E ratios. In testing this hypothesis, ketoconazole was administered, as part of a clinical investigation (14), to an athlete who presented himself after having failed a drug test because of a T/E ratio > 6. The changes in the urinary T/E ratios, when compared in our study with a normal male control who had previously received testosterone, indicated that measurement of this ratio before and after ketoconazole administration is a useful tool for differential diagnosis.

To investigate further the effect of ketoconazole on the T/E ratio, we administered ketoconazole twice daily to two normal male volunteers, to maintain the 17α-hydroxylase-17,20-lyase block. During the period of ketoconazole administration, hCG was also given to exacerbate any resulting difference between the urinary excretion rates of epitestosterone and testosterone. These two volunteers had previously been shown to respond to hCG stimulation with a large but comparable increase in their urinary excretion rates of both testosterone and epitestosterone, with hence little change in their urinary T/E ratios (3).

Finally, we undertook a third experiment, to examine whether ketoconazole would also cause a significant decrease in the T/E ratios of six normal men between 6 and 8 h after administration; such a time scale would be suitable for the introduction of a dynamic test.

Materials and Methods

The procedures followed were in accordance with the ethical standards of our institutions, and informed consent was obtained from all the subjects who volunteered for this study.

Experiment 1

Effect of repeated ketoconazole administration on an athlete with an above-normal T/E ratio, on a control, and on the same control after testosterone administration. A urine sample collected from a Norwegian male athlete (23 years old) of Olympic standard was analyzed by the IOC-accredited laboratory at The Aker Hospital, Oslo, Norway. Extraction and gas chromatography–mass spectrometry analysis (GC-MS) of anabolic steroids by a validated method (15) showed that the abundance of the bis-trimethylsilyl ether (TMS) derivatives of the molecular ions of testosterone and epitestosterone gave a T/E ratio of 9.4. The athlete, in pleading his innocence, presented himself for clinical investigation to The Aker Hospital. The athlete consented that during the clinical investigation he would be taken at any time without forewarning to another clinical establishment and once there he would be quarantined for up to 2 weeks. Quarantining of the athlete, which was at Sunnaas Hospital in Norway, was deemed necessary to ensure that tests could take place under controlled conditions. Before departure, the athlete was body-searched and all his belongings were inspected by a qualified sampling officer. The athlete was not informed where he was to be quarantined and had no access to send messages for outside communication during the quarantine period. Importantly, he was in the constant presence of at least one sampling officer throughout that period. All food brought in was ordered and checked by the sampling officers, who also ensured that all the athlete’s urine was properly collected as described in the IOC standard operating procedures.

During the quarantine period (days –2 to 5 of the study) ketoconazole (Nizoral®; 400 mg orally) was administered at midnight on days 0, 1, and 2. Throughout the period, the athlete was instructed to void his bladder at 0600; subsequent urine together with a venous blood sample was collected for analysis at 0800. The results were compared with those for a normal adult male volunteer (39 years old) who was given the same regime of ketoconazole administration. Nine months later, the treatment regime was repeated on the same volunteer, 7 days after administration of testosterone heptanoate (Primoteston depot®; 250 mg intramuscularly).

The collected serum and urine samples were stored frozen at −20 °C until analysis. Serum testosterone was determined with a commercially supplied immunoassay kit (ICN Biomedicals, Costa Mesa, CA). The immunoassay was performed in duplicate and the between-assay coefficient of variation (CV) was <7% for concentrations between 2 and 30 nmol/L. The urinary T/E ratios were determined by a validated method (15), except that the steroids were extracted with SepPak C18 cartridges (Waters Chromatography Div./Millipore Corp., Milford, MA) instead of Amberlite resin (XAD-2).

Experiment 2

Effect of repeated ketoconazole with hCG administration on the urinary excretion rates of testosterone and epitestosterone in two normal men. Ketoconazole (300 mg) was administered orally at 1000 and 2200, on days 0 to 6, to two healthy male volunteers, ages 28 (subject 1) and 33 years (subject 2). At 1200 noon on day 2 a single intramuscular dose of hCG (5000 IU; Profasi®) was given.

All urine samples were collected from each subject from 1000 to 2200 and from 2200 to 1000 on days –2 to 10 (i.e., two pooled samples apiece each day) and the pooled urine volumes recorded. All the urine samples were divided into polypropylene containers, frozen rapidly in liquid nitrogen, and stored at −70 °C before analysis.

The hydrolysis and extraction of the steroid glucuronide conjugates was by a method previously described (3). The β-glucuronidase enzyme used in the method was prepared from Helix pomatia extract (no. 22876; Serva Feinbiochemica, Heidelberg, Germany). H. pomatia extracts, besides having some sulfatase activity also have some 3β-hydroxysteroid dehydrogenase/isomerase activity; the degree of conversion can depend on the manufacturer’s batch, the conditions of incubation and, if lyophilized, whether the enzyme is used immediately...
after dissolving or not (16, 17). To characterize the reliability of our method, we prepared 2 mg/L steroid standards of androst-5-ene-3β,17α-diol (kindly supplied by Prof. Kirk, Steroid Reference Collection, Queen Mary College, London, UK) and androst-5-ene-3β,17β-diol (Sigma Chemical Co. Ltd., Dorset, UK) in acetate buffer (0.1 mol/L, pH 5.2) and incubated this overnight at 37 °C with the enzyme. The steroids, together with the corresponding 4-ene-3-oxo steroids epitestosterone and testosterone, were analyzed by GC-MS (3) to monitor any potential enzymatic conversion. The transformation of the 3β-hydroxy-5-ene steroids to 4-ene-3-oxo products by the method described was insignificant.

The steroids in the hydrolyzed fractions were converted to their bis-TMS derivatives and analyzed by GC-MS as described elsewhere (3). The ratios of the abundance of the molecular ions at a mass-to-charge ratio (m/z) for the bis-TMS derivatives of testosterone (m/z 432) and epitestosterone (m/z 432) to the abundance at m/z 435 for the bis-TMS derivative of trideuterated testosterone (internal standard) for each calibration standard were calculated and the respective calibration curves constructed. The abundance ratios were then calculated for each sample and the steroid concentrations were interpolated from the respective curves. Between-assay CVs for quality-control samples (n = 24) for the analysis of urinary total testosterone were 4.8% (x̄ = 69 nmol/L) and 10.6% (x̄ = 159 nmol/L) and for total epitestosterone were 9.8% (x̄ = 10.5 nmol/L) and 13.2% (x̄ = 216 nmol/L).

Experiment 3

Effect of ketoconazole administration on the urinary T/E ratios in six normal men. Six healthy male volunteers (ages 22–40 years, mean 29 years) received ketoconazole (400 mg) orally at 1000. Urine samples were collected just before the dose (basal, t = 0 h) and between 0 and 4, 4, and 6, and 6 and 8 h after the dose; these were designated as time 0 h, 2 h, 5 h, and 7 h, respectively. The total concentrations of testosterone and epitestosterone in the urine were measured by GC-MS by a method previously described (3).

Results

Experiment 1. From days –16 to –1, the athlete’s concentration of serum testosterone ranged from 15.4 to 22.6 nmol/L (x̄ ± SD, 19.2 ± 2.3 nmol/L) and his urinary T/E ratios from 6.0 to 8.9 (x̄ ± SD, 7.2 ± 1.0). After ketoconazole administration the serum concentration of total testosterone decreased to very small values in both the athlete and the control subject (Figure 1). In contrast, only minor changes were observed in the same control after previous administration of testosterone heptanoate on day –7. The changes in urinary T/E ratios after ketoconazole administration are given in Figure 2. There were pronounced decreases in the ratios for the athlete and control during ketoconazole administration. Conversely, after testosterone administration, the augmented T/E ratio achieved in the control was further increased after ketoconazole administration.
Experiment 2. The urinary T/E ratios followed a similar pattern in both subjects (Figure 3), decreasing within the first 12 h of ketoconazole administration, remaining suppressed despite hCG stimulation, and rising above basal values upon discontinuation of ketoconazole. Compared with testosterone, the relative decrease in the urinary excretion rates of epitestosterone was much less for both subjects 2 days after ketoconazole administration (Figure 4). After hCG stimulation on day 2, the excretion rate of epitestosterone increased above basal values for subject 1 and returned to basal values for subject 2, whereas the excretion rate of testosterone remained below basal values for both subjects throughout the administration period.

Experiment 3. The decreases in urinary T/E ratios after ketoconazole administration, expressed as a percentage of basal values (T/E ratio range = 0.21 to 2.74), are shown in Figure 5. The T/E ratios decreased to a mean of 57% (range 49–66%) of basal values between 6 and 8 h (t = 7 h) after administration.

Discussion

The decrease in the serum concentrations of testosterone in the athlete and the control subject in response to ketoconazole was typical of that observed in other studies (8–10). Comparison with the control’s response after testosterone heptanoate administration indicated that this athlete had a naturally large urinary T/E ratio and that he was not taking exogenous testosterone. Indeed, further functional tests with dexamethasone and gonadotropin-releasing hormone supported this finding (14), and similar cases of biological outliers have recently been reported in one adult and two adolescents (18). The decrease in the T/E ratio for the athlete after ketoconazole was unexpected, because the relative change in the rate of excretion of epitestosterone usually matches that of testosterone in normal men, even after hCG stimulation (2, 3).

The second experiment, albeit involving only two subjects, showed that the decrease in the T/E ratios after ketoconazole administration was due to large and rapid decreases in the rate of excretion of urinary testosterone in comparison with proportionately much smaller decreases in epitestosterone. The large decreases in testosterone excretion were caused by the inhibition of 17α-hydroxylase/17,20-lyase activity by ketoconazole. In both subjects, the urinary excretion of testosterone was still considerably less than basal values throughout the ketoconazole administration period, whereas epitestosterone excretion returned to basal values and greater after hCG stimulation. All in all, this suggests that epitestosterone may be formed, at least in part, via a
different steroidal pathway from that of testosterone.

In the final experiment, ketoconazole administration to six normal men confirmed that the decrease in the T/E ratio between 6 and 8 h after administration was significant (P < 0.001). Nevertheless, further investigations should be done to select an optimal dose of ketoconazole that would cause a maximal decrease in the T/E ratio within a given time. Our results demonstrate that ketoconazole administration may be a useful tool in distinguishing athletes who have a T/E ratio >6 because of low production of epitestosterone from those who have a large ratio because of testosterone administration. For the control who had received testosterone, the resulting increase in the T/E ratio was further increased by the action of ketoconazole because of the additional suppression of epitestosterone production. Of course, repeated administration of testosterone would have a cumulative effect, resulting in further suppression of the hypothalamic-pituitary-testicular axis and epitestosterone production; in such cases ketoconazole would be expected to cause little change in an already high ratio. Nonetheless, such results would contrast greatly with the decrease in the T/E ratio of an athlete whose ratio was abnormally large from natural causes.

Some have suggested monitoring athletes whose T/E ratio is between 6 and 10 by repeated collection of urine samples over a sustained period, e.g., every sporting competition combined with out-of-competition periods. Such a strategy would differentiate between innocent subjects, whose T/E ratios would remain abnormally large, and cheaters who took testosterone, whose ratios would fall to <6 after withdrawal of testosterone administration. Although the IOC List of Doping Classes and Methods for 1992 advises that individuals who give urine samples with T/E ratios between 6 and 10 should be further investigated, constant monitoring is expensive and there are no details for the interpretation of such data. Little is currently known about these unusual cases of individuals with normal testosterone but abnormally small epitestosterone excretion rates, some of whom may have T/E ratios >10. Of course, all such athletes would still be expected to have ratios for urinary testosterone to luteinizing hormone within normal limits. Nonetheless, athletes could have the option of having access to this rapid test, which could prove whether their T/E ratio above the cutoff limit was due to natural causes. However, ketoconazole should be administered with care, given the recent report of its interaction with certain antihistamines, e.g., terfenadine and astemizole, which can cause serious cardiac arrhythmias (19). In addition, ketoconazole partially inhibits the gluco- and mineralocorticoid biosynthetic pathways (20) and causes a transient blunting of the cortisol response in response to tetracosactrin stimulation in humans (21). Nevertheless, recovery from the partial steroidal blockade occurs between 4 and 16 h after a single 400-mg dose, and symptoms suggestive of some adrenal insufficiency have been described in only a few cases of prolonged ketoconazole therapy (20). Of course, the ethics of giving a drug to an otherwise healthy individual should be given due consideration before a dynamic test is instituted.

In conclusion, our results demonstrate that ketoconazole causes differential suppression in the urinary excretion rates of testosterone and epitestosterone. The administration of ketoconazole to an athlete with a naturally above-normal T/E ratio results in a decrease in the ratio but causes the ratio to increase in normal men who have received exogenous testosterone. This difference in responses indicates that ketoconazole could be used to distinguish between male athletes with naturally large T/E ratios and those who have large ratios due to testosterone administration. The substantial decrease in the urinary T/E ratios in normal men 6–8 h after a single administration of ketoconazole (400 mg, orally) means that the time scale involved would allow the ketoconazole test to be carried out within a designated room in the presence of a sampling officer without too much inconvenience. Moreover, the fact that only urine samples would be required adds to the simplicity of the test as well as the analysis. Investigations are currently underway to develop the ketoconazole test.

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