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EVALUATION OF INFLUENTIAL FACTORS ON THE T/E RATIO AS DETERMINED IN DOPING ANALYSIS

ABSTRACT

The ratio of the concentration of testosterone glucuronide to the concentration of epitestosterone glucuronide (T/E ratio) as determined in urine is the most frequently used method to prove testosterone abuse by athletes. A T/E ratio higher than 6 has been considered as proof for abuse in the past; however, cases of naturally occurring higher T/E ratios have been described.

Since the introduction of the T/E ratio in doping analysis, the parameters that may or may not influence the T/E ratio, possibly leading to false-positive results, have been debated. To achieve more insight on the influencing circumstances, an overview is given to obtain an objective view on the merits of the urinary T/E ratio.

Relevant analytical aspects of the T/E ratio, potential parameters of endogenous and exogenous origins, as well as some alternative methods to determine testosterone abuse, such as the urinary testosterone/luteinizing hormone ratio, gas chromatography/combustion/isotope ratio mass spectrometry, hair analysis and high-performance liquid chromatography/mass spectrometry, are discussed.

INTRODUCTION

During the Moscow Olympic Games of 1980, a high frequency of testosterone (T) abuse was suspected. By that time, analytical methods to detect the administration of synthetic anabolic steroids by gas-chromatographic/mass spectrometric (GC-MS) screening procedures had improved. Therefore, athletes switched therefore to endogenous steroids like T.

Quantitation of T as a way to detect T abuse was inadequate because of its high metabolic turnover rate, circadian rhythm of T excretion, and an interindividual excretion variability. Donike *et al.* [1] introduced the ratio of urinary testosterone glucuronide (TG) to epitestosterone glucuronide (EG) concentration, the T/E ratio, as an indicator of T abuse. It was reported [1] that after oral, rectal or intramuscular T administration, the excretion of TG increased more than other T metabolites. Epitestosterone (E) was found not to be a metabolite of T because deuterium labeled T administration did not result in significant deuterium labeled EG excretion [2]. Production and metabolism of T and E are shown in Figure 1. The origin of E is still discussed. Although Dehennin [3] showed that half of total E production is of testicular origin, the remaining 50% is still debated. Administration of Adrenocorticotrophic Hormone (ACTH) results in an increased EG production, indicating an adrenal origin [4-5]. Also adrenal insufficiency as observed in Addison's disease correlates to significantly deceased T and E excretion rates [6]. Also peripheral production is possible [4,7-8]. Androst-5-ene-3β,17α-diol (Δ5-17α-AEDIOL) is suggested to be a potential precursor of E (Figure 1), taking into account the 3β-hydroxy-steroid dehydrogenase/4,5-isomerase activity and Δ5-17α-AEDIOL production in testicular tissue [3].

The mean T/E ratio of urine samples of Caucasian males and females in the first population study of Donike *et al.* [1] was 1-2. The values showed a logarithmic normal distribution with an upper limit value lower than 6 [9-10]. Using these data, the Medical Commission of the International Olympic Committee (IOC) banned the use of T in 1982 and stated that a T/E ratio above 6 was sufficient proof for T abuse. When applying this criterion in research and routine analyses, cases of naturally occurring T/E ratios above 6 appeared [11-12]. Dehennin *et al.* [12] administered testosterone enanthate in several doses intramuscularly to healthy men over a period of 6 months. They found via linear interpolation between doses that the T/E ratio exceeded the cutoff point of 6 when natural production (around 45 mg/week) was doubled by weekly administration of a comparable dose of exogenous T. Nowadays, the IOC states that a follow up investigation is needed for T/E ratios above 6. In the follow up, possible elevated T/E ratios due to physiological or pathological circumstances should be proven. This proof may be supplied by review

of previous tests, endocrinological investigations [13], or unannounced testing over several months.

The aim of multiple tests is the establishment of an individual reference range of an athlete, depending on the intraindividual T/E variability [14]. A single T/E ratio that is higher than the upper limit of the individual reference range (mean + 3 * standard deviation) indicates T abuse [15-17]. Since its introduction, critics have put forward several cases in which the T/E ratio was up for discussion because of the assumed risk of false-positive or false-negative results. Despite this criticism, the T/E ratio has been the most frequently applied method to detect T abuse. A lot of research has been done to investigate the factors that could influence the outcome of a T/E ratio analysis. To get insight into the validity of the T/E ratio, this article gives an overview on the research that has been done on the presently known influencing parameters.

ANALYTICAL ASPECTS

Methodology

A lot of basic research has been performed for the application of endogenous steroid analysis [25-29]. In doping screening analyses T and E are usually determined by GC-MS [30], although radioimmunoassay techniques also have been reported [31]. Several, but basically similar sample cleanup procedures are applied in doping laboratories (Figure 2). For most accurate determination of the T/E ratio, deuterium labeled T and E are required as the internal standards. Procedures applying of d_3--testosterone and d_3-epitestosterone have been developed [32-34].

Solid-phase extraction (SPE) is applied to remove the inorganic material from the urinary matrix. Usually XAD-2 resin or C_{18} columns are used for the extraction. This step was introduced at the time that only *Helix pomatia* was used for enzymatic hydrolysis in anabolic steroid screening procedures. SPE was needed to remove inorganic substances that inhibit β-glucuronidase arylsulfatase hydrolysis by *Helix pomatia* [35]. Nowadays, SPE is often still applied, but has not been proven necessary for β-glucuronidase hydrolysis by *Escherichia coli* and is therefore omitted by several laboratories from the screening procedures.

As the analytical procedure for the T/E ratio is aimed at TG and EG, removal of non-conjugated T and E is applied prior to hydrolysis. Therefore, a liquid-liquid extraction with a solvent like diethyl ether or *t*-butyl methyl ether is usually applied. Because the excretion of non-conjugated T and E is below 1% from total excreted T and E, omitting this step does not result in a significant change in the outcome of the T/E

ratio. Also, this extraction is often excluded in screening procedures for time efficiency and is then only applied in confirmation procedures.

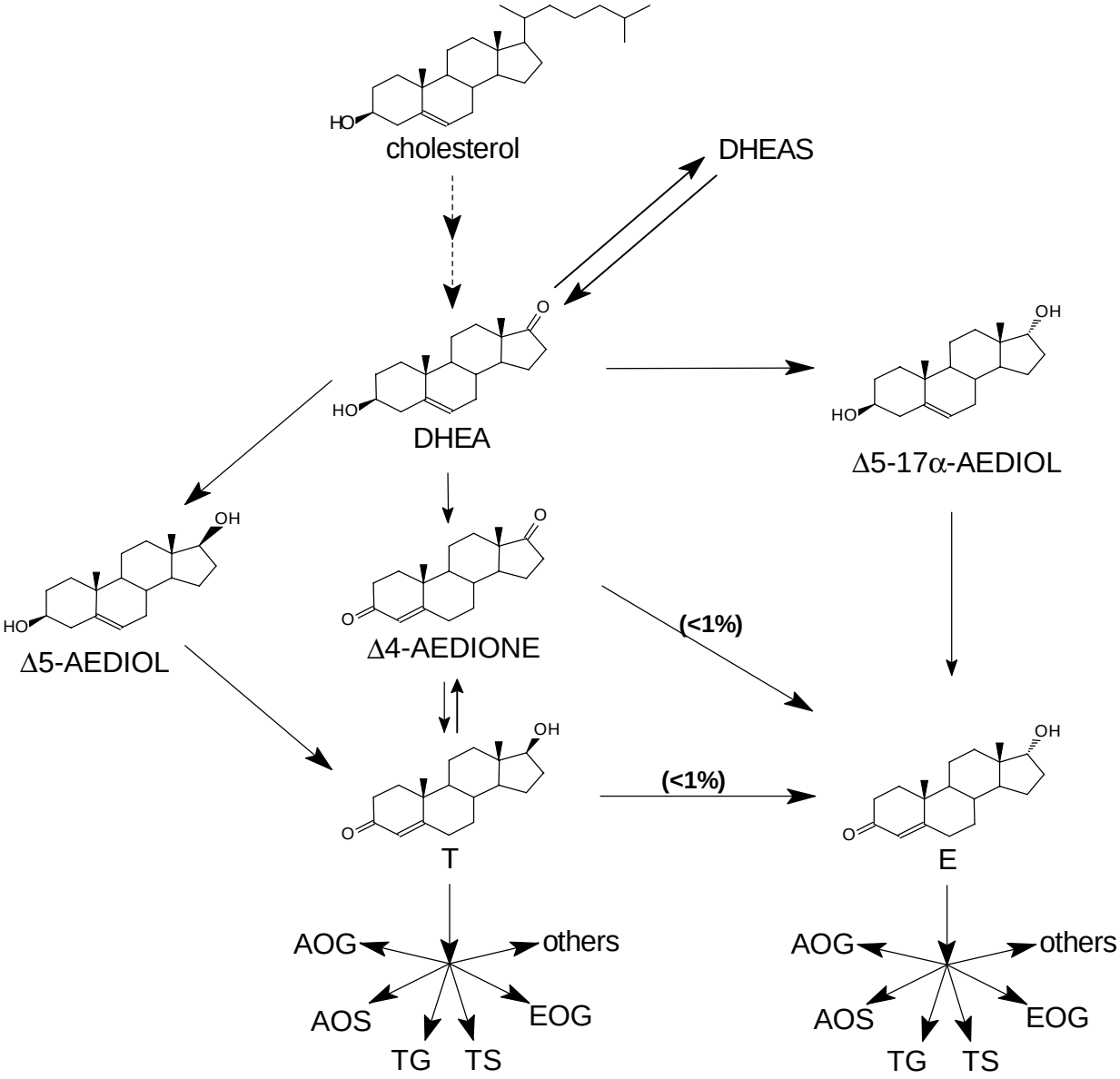

Figure 1. Steroid metabolism producing and eliminating T and E [18-24]. Cholesterol is the first precursor of all steroid hormones. Relevant precursors are dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEAS), androst-4-ene-3,17-dione (Δ4-AEDIONE), androst-5-ene-3β,17α-diol (Δ5-17α-AEDIOL) and androst-5-ene-3β,17β-diol (Δ5-AEDIOL). Some relevant metabolites are androsterone glucuronide (AOG), androsterone sulfate (AOS), testosterone glucuronide (TG), testosterone sulfate (TS) and etiocholanolone glucuronide (EOG). Conversion of Δ4-AEDIONE or T to E is less than 1% of alternative routes.

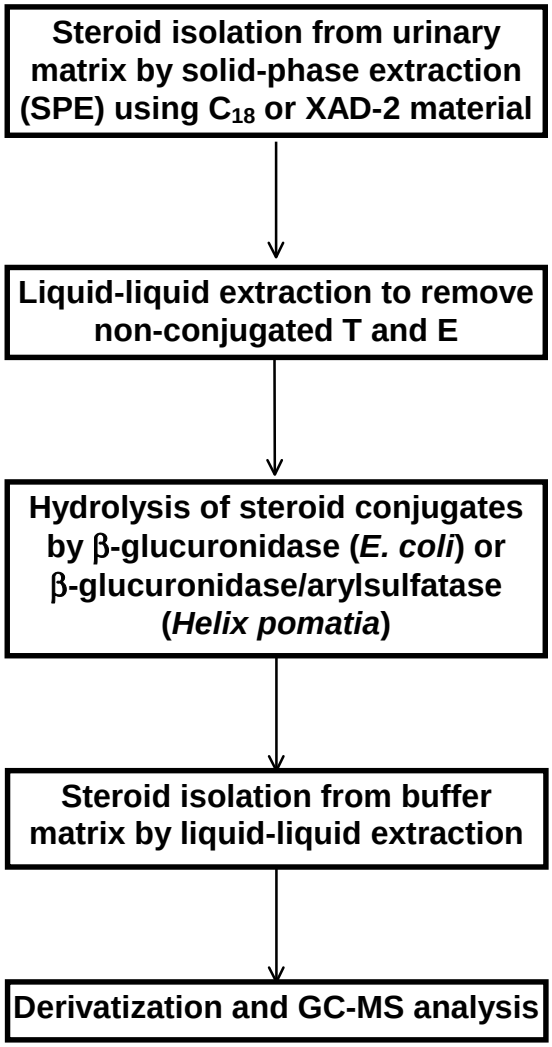

Figure 2. Basic procedure for steroid profiling.

Before isolation, the remaining steroids are deconjugated by an enzymatic hydrolysis. Several hydrolysis methods are available for application in steroid analysis [36]. A digestive juice from *Helix pomatia* was used in the past for its β-glucuronidase and arylsulfatase activity. However, because a 3β-hydroxysteroid oxidoreductase and a 3-oxosteroid-5,4-isomerase activity have been observed in this formulation, use of *Helix pomatia* is usually avoided in doping screening procedures [37-40]. Bacterial (*E. coli*) preparations with a pure β-glucuronidase activity and no arylsulfatase activity are now generally accepted for hydrolysis. Therefore, the T/E ratio is usually based on TG and EG. When hydrolysis is controlled by application of deuterium labeled androsterone glucuronide, hydrolysis can be performed directly in the urine matrix as has been outlined by Geyer *et al.* [41]. Following the suggested

method the conjugated and non-conjugated steroids are combined in a single fraction.

It is suspected that a naturally high TG/EG ratio is associated to a low excretion level of EG compared to a higher excretion level of epitestosterone sulfate (ES) due to stimulated sulfotransferase activity in the testes. As a result, Dehennin [42] therefore suggested the TG/(EG+ES) ratio and the EG/ES ratio in order to collect more relevant information in subjects with high T/E ratios. It was shown that a physiologically high TG/EG ratio is associated with an EG/ES ratio below 1. For this purpose, methanolysis has been suggested as an alternative chemical hydrolyzation method [43].

After hydrolysis the steroids are extracted into an apolar phase like diethyl ether or *t*-butyl methyl ether. Steroids in the dried extract are then derivatized to trimethylsilylenol-trimethylsilyl (TMSenol-TMS) ethers and injected into a GC-MS [25]. Applying selected ion monitoring (SIM), T and E are quantified using molecular ion at *m/z* 432.

Inter- and intra-laboratory variation

A collaborate study has been done by six international doping laboratories to describe the within- and between-laboratory variances for determination of the T/E ratio [44]. Some of the experimental factors that could possibly influence the outcome were left uncontrolled. Every laboratory analyzed the same 4 urine samples with different T/E ratios in triplicate. The within-laboratory variation was lower than the between-laboratory variation (maximum of 8.3% and 11.7% respectively). The estimate of the urinary T/E ratios differed significantly between laboratories. This study showed it to be necessary to standardize methods between the laboratories as much as possible to improve the T/E ratio as a parameter. As described before, full standardization of methods has still not been achieved between doping laboratories. Clearly, more attention should be paid to this aspect. In this study boldenone (1-dehydrotestosterone) and 17α-methyltestosterone were used as internal standard. Better results have been obtained with application of deuterium labeled T and E [45].

Matrix problems

Linnet [46] described the effect of the urinary matrix on the T/E ratio. Calibration curves of T and E, which were taken from methanol without extraction from a urinary matrix, appeared to be non-linear, caused by an increasing relative molar response of T compared to the internal standard 17α-methyltestosterone with increasing concentration levels. T and E added to blank urine and taken through an extraction

procedure, without previous enzymatic hydrolysis, was needed to stabilize the relative molar response over a wide concentration range. The suggestion was made to base the calibration curves on T and E added to blank urine. Geyer *et al.* [47] suggested the use of so-called artificial urine. Artificial urine samples have been produced by addition of 1-(*N*,*N*-diisopropylamino)-alkanes (DIPAs 14-23, Figure 3) to calibration standards that elute in the same range as the calibrated steroids. Paraffin was suggested as another possibility, but it has not been investigated. Use of artificial urine samples results in higher molar response of the calibrated steroids and a more linear calibration curve. Another possible method is the use of urine of postmenopausal women or children to prepare calibration standards.

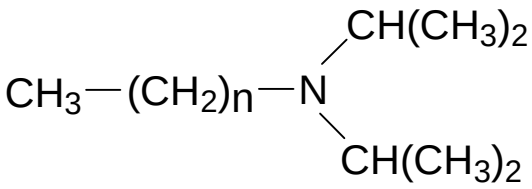

Figure 3. 1-(*N*,*N*-diisopropylamino)-alkane (DIPAs) with n=13 for DIPA 14 and n=22 for DIPA 23

Microbial degradation

Bacterial activities in urine may cause significant changes in measured steroid profiles, as was brought forward in the Diane Modahl case in 1994 [48-49]. Because urine samples are collected under unsterile conditions, bacteria have the chance to grow when samples are stored too long at a too high temperature. To minimize bacterial contamination, urine samples should be stored at -20° C. Bacterial species from strains as *Staphylococcus* and *Enterococcus* are expected to grow in urine during unfavorable storage conditions [50]. These microorganisms cause urinary alteration by oxidoreduction reactions of endogenous steroids. The primary reaction that occurs is the deconjugation of glucuronides and sulfates [51]. Loss of T and E from the fraction of glucuronides to the fraction of non-conjugated steroids will lower the measured concentration in the screening procedure. Geyer *et al.* [52] reported an increased T/E from 5.3 to 9.8, determined in a combined fraction of conjugated and non-conjugated steroids. This increase was caused by an increased concentration of T in the fraction of non-conjugated steroids compared to E [17,52-53]. The suggested mechanism for the T production is the bacterial hydrolysis of the sulfate conjugate of Δ5-AEDIOL followed by 3α- and 3β-hydroxy-Δ^5-steroid-dehydrogenase and steroid-Δ4,5-isomerase activity [54]. In a study by de la Torre *et al.* [55] no

production of T and E was observed in several selected bacterial species that are expected to proliferate in urine as a result of adulteration. So far, no TG/EG alteration due to bacterial growth has been proven. However, as uncontrolled hydrolysis and oxidoreduction reactions occur in the urine, an increased T/E ratio due to bad sample storing can not be excluded.

To prevent false-positive outcomes due to bacterial activity, the analysis of markers is suggested to prove urine degradation. Markers of bacterial urine degradation are the accumulation of 5α- and 5β-androstan-3,17-dione [53,56], which are the product of deconjugation followed by oxidation of androsterone (AO) and etiocholanolone (EO). Ayotte *et al.* [57] described 5α-androstan-3,17-dione coeluting with E, both showing a TMSenol-TMS derivative molecular ion at *m/z* 432 with significant abundance. An inaccurate determination of the T/E ratio in a regular screening procedure from the sum of deconjugated and non-conjugated steroids can be the result of microbial activities when 5α-androstan-3,17-dione and E coelute. This has however not appeared to be a problem of general concern because 100% dimethylpolysiloxane film columns should generally provide a baseline separation. Other specific ions of 5α- and 5β-androstan-3,17-dione at *m/z* values 275 and 290 can be implemented in the SIM procedure to detect more specifically urine contamination by microorganisms. Another sign of bacterial activity could be an increased urinary pH. However, signs of degradation were reported [56] reported to appear in only 50% of the urine samples, which had been stored at 37° C for several days, and showed a pH increased to a value above 8. Moreover, several bacterial species that have previously been identified in urine, are able to proliferate without alkalizing the medium [55]. Proving urine sample deterioration is therefore done on the basis of accumulation of 5α- and 5β-androstan-3,17-dione, visible microbial growth, the fraction of nonconjugated steroids, and if possible, a high pH value.

FACTORS OF ENDOGENOUS ORIGIN

Age and development

In several kinds of sports, doping control takes place on athletes from adolescent or pre-adolescent age groups. In order to apply the T/E ratio as conclusive evidence on youngsters, knowledge is needed about the endocrinology from those age groups. Dehennin *et al.* [58] studied the steroid excretion rates from 140 males, aging between 13 and 20, divided into 5 pubertal stages according to the criteria of Tanner [59]. They concluded that the excretion rates of T and E as glucuronides or sulfates increased during pubertal development with no significant differences in the T/E

ratio. This means a stable T/E ratio during pubertal development in boys. Raynaud *et al.* [60] reported a significant difference in the correlation slopes of T and E excretion versus chronological age in boys; T versus age rose approximately twice as fast. Because of a wide variability of individual values, there was also no significant difference in the mean T/E ratio between the successive pubertal stages. Comparable results have been obtained in older studies applying different sample cleanup protocols and nonselective flame ionization detection techniques [61-62]. Schweizer *et al.* [63] reported that the changes in T/E between different stages were insignificant, but with a higher T/E instability at the prepubertal stages, where early signs of pubertal development can be observed. Lapcik *et al.* [64] reported a decrease in the plasma ratio of non-conjugated E to T in males, as determined by radioimmunoassay. Only non-conjugated T and E were determined. Broad age classes were used for correlation study, and sexual development was not regarded. Therefore, no relevant conclusions can be extracted from this study on the development of the endocrinology in relation to T and E during adolescence.

A study performed with a large number of adolescent girls [65] showed a larger increase of E excretion at higher Tanner stages. This resulted in a decreasing T/E ratio during adolescent development. However, a comparable decreasing T/E variability was detected at higher stages of development in girls as compared to boys. No significant difference was observed between a population of extensively exercising girls and the control group.

Endocrinological diseases

For endocrinological diseases in general, the number of recent scientific reports is very limited. From a series of endocrinological diseases, hirsutism is best described in relation with the T/E ratio. Hirsutism increases the excretion of T and E, of which the magnitude correlates to the occurrence of symptoms. France and Knox [66] reported increased excretion rates of T and E in case of hirsutism with irregular menses, hirsutism with virilization, and in most cases of hirsutism with regular menses. Although different sample cleanup procedures and unselective GC detection were used, from the data can be concluded that there was no influence of hirsutism on the urinary T/E ratio. Pal [67] studied a group of 90 females with iodiopathic hirsutism compared to 90 healthy females aged between 16 and 46 years. In subjects with the most prominent hair growth (30%), the excretion of T and E was above the reference range. No conclusions can be drawn about the T/E ratio. These results appear to contradict with the results from de Nicola *et al.* [68], who reported a much larger increase in E excretion resulting in an approximate 10-fold decreased T/E ratio in 14 female subjects, using comparable analytical techniques.

Other endocrine disorders (adrenal adenoma, adrenal carcinoma, adrenal hyperplasia, adrenal tumor, congenital adrenal hyperplasia, virilizing adrenal tumor and virilizing ovarian tumor) were reported to increase T and E excretion. However, glucuronides and sulfates were measured and a limited number of subjects were used. No specific data regarding the T/E ratio was presented [69]. Hypogonadism is reported to reduce the excretion rate of T and E in males to an average same extent, so no major changes in T/E are expected [70], except for an increased analytical variation in the laboratory because of the lower values.

Ethnic origin

Asian sub-populations excrete T at a lower level than Caucasians, which results in a relatively low T/E ratio. During the 10th Asian Games in Seoul a mean T/E ratio of 0.76 was observed [71]. This is significantly lower than the mean value of Caucasians. A T/E distribution from a multiethnic reference population consisting of two different lognormal distributions has been reported [56,72]. The first with a mode of 0.16 involving a 95% reference range between 0.07 and 0.25 and the second with a mode of 1.0 and a 95% reference range between 0.5 and 3.5. Low T excretion in Asian sub-populations increases the risk of false-negative results after T administration [73]. The IOC criterion of 6 is therefore questionable when subjects of Asian origin are concerned. In IOC regulations, however, no ethnic differences are taken into account. The IOC criteria have been determined using subjects from the Caucasian population, which is only a minor group of the world population.

Differentiation in enzymatic metabolic activities between Asian and Caucasian populations, has been extensively studied in case of polymorphism of alcohol dehydrogenase [74-75] and human cytochrome P450 enzymes such as CYP2D6 and CYP2C19 [76-77]. Polymorfism of metabolic enzymes in relation to doping analysis is a subject that has not been studied in detail. In addition, no specific data on other races have been reported.

Menstrual Cycle

Contradictory results on the influence of the menstrual cycle on the urinary T/E ratio have been obtained. Some reports concluded there were no clear T/E pattern during the menstrual cycle of a few observed subjects [78-79]. However Catlin *et al.* [80] reported a T/E peak during menses in three subjects throughout a total of five menstrual cycles. However, no quantitative results were reported in this study. Clearly, more specific research is needed on this subject involving a larger number of subjects.

Circadian rhythm

T is produced with a diurnal variation of 20-40% [81-82]. A maximum concentration in blood is observed around 6.00 to 8.00 a.m. and a minimum around 8.00 to 10.00 p.m., assuming a regular sleeping pattern. The diurnal rhythm is observed with a high interindividual variation. The circadian rhythm in T production is reflected in a circadian rhythm in urinary T excretion. A diurnal rhythm is also observed for E [83]. The variation in T/E ratio is expected to be less than 30% [84] in males. The observed variation in females will be more significant [78] because the higher analytical variation due to lower urinary concentrations of T and E is superimposed on the circadian variation.

Pregnancy

T production increases with progressing pregnancy, and after delivery T levels promptly return to basal concentration [85-86]. The excretion of T and E is elevated only in the third trimester of the pregnancy [87-88], and the T/E ratio is not influenced significantly during pregnancy. After delivery, excretion levels drop to basal levels within a week. One week after delivery, a large shift in T/E due to a faster decrease in E than T to basal levels has been reported [6]. This effect could be explained by the increasing sex hormone binding globulin (SHBG) concentration during pregnancy and the lack of E binding to SHBG.

Exercise

The influence of exercise on the T/E ratio is still unclear. De Boer *et al.* [89] reported no significant change in mean T/E ratio that could be attributed to exercise, which was done by five well-trained volunteers. The exercise was performed on a bicycle ergometer with a programmed workload to simulate a race course of the Tour the France. These results were supported by a study performed on 13 cyclists participating in the Tour de Suisse and the Tour de France in 1992 [90]. Samples were taken every day before and after the exercise, and no influence on the T/E was found. Maynar *et al.* [91] reported decreased urinary concentrations of T and E during training periods and increased concentrations during competition. These results were obtained from urine samples of 16 professional racing cyclists. However, only urinary concentrations were measured instead of excretion rates; therefore, these results should be confirmed. It was concluded that the T/E ratio decreased significantly during training and competition. Increasing androgen levels were suggested to be due to a decreased SHBG level as result of extensive exercise. Yap *et al.* [92] reported insignificant decreases of T excretion during different intensities of physical exercise-stress. The experiment was conducted on 4

groups of 7 to 12 male athletes. Also psychological stress during competition has been investigated in a pentathlon pistol shooting experiment [93]. Plasma testosterone concentrations increased while luteinizing hormone (LH) levels were constant. The effect was more significant in older subjects. No effect was observed on the urinary T/E ratio.

These contradictory results should be a challenge for further investigation, especially because the control of athletes often takes place after a match. A lot of parameters should be taken into account, for example, exercise duration, intensity and repetition, the type of exercise, gender, general condition and ethnic background of volunteers.

FACTORS OF EXOGENOUS ORIGIN

Oral contraceptives

Only one preliminary study of Mareck-Engelke [94] with not more than four volunteers is available, and it showed an elevation of the T/E ratio due to the application of oral contraceptives as the result of suppressed E excretion. In this study, morning urine samples were taken through a menstrual cycle, before and some time after stopping oral contraceptive administration. Administration of oral contraceptives also resulted in an unstable T/E ratio. In one of the volunteers, the ratio approached the level of 6 twice in one cycle. The mean T/E increased 2-3 times due to administration of a single- phase or three-phase contraceptive. Further research with more volunteers is needed to estimate a risk of false-positive results.

Ketoconazole

In 1992 Oftebro [11] mentioned the application of the ketoconazole test to discriminate between a “naturally” high T/E ratio and a high T/E ratio due to administration of T. Originally ketoconazole was developed as an antifungal agent, but it also appeared to inhibit T biosynthesis [95-96]. Santen *et al.* [97] observed that plasma levels of androst-4-ene-3,17-dione decreased after ketoconazole administration, whereas its precursor 17α-hydroxyprogesterone increased. It was concluded that this was the result of 17,20-lyase inhibition. Chemically related antifungal agents like isoconazole, miconazole, econazole and clotrimazole also inhibit T synthesis *in vitro* [98], in contrast to the inactive triazole antifungal agent fluconazole [99-100]. Kicman *et al.* [101] described the application of ketoconazole in a test to differentiate between athletes using T and athletes with a naturally high T/E ratio. Besides the T excretion rate, the E excretion rate was also lowered due to

ketoconazole administration, but to a lesser extent. This resulted in a decreased T/E ratio during a few hours. Kicman *et al.* [101] reported a mean decrease in T/E of 57% (range 49-66%) 7 hours after a single oral administration of 400 mg ketoconazol in six male subjects. When supraphysiological dosages of T have been administered prior to the ketoconazole test, little effect is expected for the T excretion. The E excretion will decrease similar to the natural situation, resulting in an increased T/E ratio during a few hours. Usually the protocol for a ketoconazole test describes a dose of 400 mg ketoconazole or 800 mg as suggested by Oftebro *et al.* [102]. The ketoconazole test is used on a confirmatory basis and is performed with voluntarily permission from the involved athlete.

Ethanol

In 1988, Falk *et al.* [103] reported the increase of the T/E ratio due to the consumption of ethanol. The increase was observed at dosages higher than 1 g/kg bodyweight. A dose of 2 g/kg bodyweight resulted in an increase of 30-90%, which was, however, insufficient to reach the IOC criterion of 6. Only four male subjects were used, and urine sampling was done only once every 8 hours. After publication of this study, some cases appeared of athletes who had an occasional T/E above 6, which were claimed to be due to ethanol consumption [104-105]. The increase of T/E is more apparent in females compared to males [104,106-107], which is explained by the different routes and quantities of T production [107]. Male production of T is mainly of gonadal origin, whereas peripheral conversion from androst-4-ene-3,17-dione accounts for 50% of female production of T.

The mechanism for the increased T/E ratio due to ethanol administration is still unknown. Acute oral ethanol administration produces a suppression of plasma T, whereas LH levels do not change significantly [108-109]. This supposes a direct action from ethanol on the steroid production. Several hypotheses have been put forward to explain the observed changes. Falk *et al.* [103] suggested the dependency of the T/E ratio on the actual NADH/NAD^+ ratio. It has been proven that ethanol metabolism increases the ratio of NADH over NAD^+, which results in a suppressed steroid oxidation [110]. This is supported by previous studies, which have demonstrated increased ratios of urinary 17β-hydroxysteroid and 17-ketosteroid sulfates [111-113]. Karila *et al.* [107] suggested two additional mechanisms. One is that ethanol administration causes an endocrine response to stress, leading to a stimulation of the hypothalamic-pituitary-adrenal axis. This hypothesis is supported by the increase of plasma concentrations of adrenal steroids as cortisol, dehydroepiandrosterone sulfate (DHEAS), dehydroepiandrosterone (DHEA) and androst-4-ene-3,17-dione (Δ4-AEDIONE) in females after intake of 2 g/kg ethanol [114-115]. In alcoholics, cases have been described of alcohol-induced

overproduction of cortisol, causing pseudo-Cushing's syndrome. However, the underlying mechanism is unclear [116]. The second possible mechanism is the direct inhibition of T metabolism by ethanol, which is supported by the reduced excretion rate of the main metabolites of T: AO and EO [115].

Anabolic Androgenic Steroids

DHEA, androst-4-ene-3,17-dione and androst-4-ene-3β,17β-diol

During the last few years, T precursors like DHEA, Δ4-AEDIONE and androst-4-ene-3β,17β-diol (Δ4-AEDIOL) are increasingly used by athletes to improve their performance. The occurrence of these compounds as ergogenic substances in sports is mainly based on their manipulation of the endocrine system to increase T production. Although they are registered in the United States as food additives and therefore are legal over-the-counter drugs, the IOC has put them on the list of forbidden substances [117]. The true benefit for the athlete's performance is, however, doubted [118]. DHEA, particularly as its sulfate DHEAS, is the most abundant circulating steroid hormone. Like Δ4-AEDIONE and Δ4-AEDIOL it has no anabolic and only weak androgenic properties. Natural DHEA production is maximal at the age of 20-30 years (around 4 mg/day as DHEA and 25 mg/day as DHEAS [119-120]). After the third decade of life the production declines gradually to 10-20% at the age of 70-80 [121].

Oral administration of 50-200 mg usually is applied to boost up the T level in plasma for a few hours. The absorption level is low due to the first pass effect. Therefore, doses used are quite large compared to other exogenous anabolic steroids. Clearance of orally administered T precursors is fast, so chromatographic analysis of the urinary concentration can only be performed in the first hours after administration, depending on the dose. A urinary DHEA glucuronide concentration of 300 ng/ml has been proposed by Dehennin *et al.* [122] as a threshold for screening of DHEA. Bowers reported a high occurrence of false-negatives when this threshold would be applied [123].

Due to the increased overall T production resulting from DHEA administration, unlike E production, the T/E ratio is affected. In a case of one male subject, oral administration of 50 mg DHEA resulted in an increase of the T/E ratio from 0.5 to 3.1 at 3 hours after administration. The T/E had returned to basal value after 20 hours [124]. However, other studies [2,123,125] also reported cases in which there was a minimal effect on the T/E ratio. In the study performed by Bowers [123], only one of four male subjects showed a T/E increase, from 2.4 to 8.1 in a 24 h pooled urine sample after administration of 50 mg DHEA. The T/E ratio of the other three subjects stayed far below the threshold of 6 after administration of 50 or 100 mg DHEA. In

conclusion, there is a risk of a T/E>6 during the first day when 50 mg or more DHEA is taken. The direct conversion of DHEA via T to metabolites as AO and EO prevents in many cases an extensive release of T into the circulation, so the T/E ratio will often remain below 6.

Nowadays, DHEA creams are also available on the market. A possible advantage could be the administration of DHEA to the muscles and avoiding first passage through the liver. In this way, DHEA can possibly intracrinologically be metabolized to potent androgens like T and 5α-dihydrotestosterone (5α-DHT) without getting them into the circulation [126]. In such a way, a risk of high T/E ratios could be avoided, but no scientific data is available on this subject. The rapid uptake of steroids after dermal application is demonstrated by Kapelrud *et al* [127]. They measured a rapid T/E increase after topical application of ointments containing T and triamcinolone. In this formulation, the triamcinolone increased the dermal uptake of T.

A few preliminary studies have been published on Δ4-AEDIONE and Δ4-AEDIOL on a limited number of subjects [128-130]. Comparable T/E elevations have been observed on a few subjects after administration of Δ4-AEDIONE or Δ4-AEDIOL [128]. The maximum increase was much higher when the baseline T/E was above average. The effect of increasing T excretion compared to E was not observed in one subject of Asian origin. In this subject, T levels were insignificantly affected as E excretion showed a large increase that lasted around 5 hours after 50 mg Δ4-AEDIONE or 100 mg Δ4-AEDIOL administration. This striking difference illustrates the ethnic differences in metabolic enzyme activities as has already been discussed in this paper. Uralets *et al.* [128] also reported the presence of the 17α-epimere androst-4-ene-3β,17α-diol in the commercial formulation of Δ4-AEDIOL (Androdiol), resulting in a lower T/E ratio as expected on the basis of the Δ4-AEDIONE excretion study.

Testosterone metabolite 5α-dihydrotestosterone (5α-DHT)

Metabolites which are abused by athletes are limited to 5α-DHT. Reports [131-132] studying the determination of 5α-DHT abuse by profiling endogenous steroids stated that 5α-DHT administration had no significant effect on the urinary TG/EG ratio. 5α-DHT was administered in these studies intramuscularly as heptanoate (150 mg and 250 mg once) [131,133] and percutaneously (125 mg twice daily) [132].

"Exogenous" anabolic steroids

The abuse of anabolic steroids of synthetic origin have an extensive effect on general endocrinology. Long-term anabolic steroid use results in a suppression of gonadal androgen production due to testicular or ovarian atrophy, which can be detected in a urinary steroid profile even after cessation of anabolic steroid use [134]. This hypogonadal state results in a decline of steroid hormone excretion as is clearly demonstrated for AO and EO [135-136]. The lowered urinary concentration of T and E creates a higher T/E variability than in normal circumstances. Small analytical variations will have a greater impact on the outcome of the T/E ratio. De la Torre *et al.* [137] showed that administration of metenolone (1-methyl-5α-androst-1-en-17β-ol-3-one) decreased the T/E ratio. On the other hand when metenolone was simultaneously administered with stanozolol (17α-methyl-17β-hydroxy-5α-androst-2-eno[3,2-*c*]pyrazole), the T/E ratio increased. In both cases, the T excretion decreased to the same extent. Whereas in case of metenolone administration, the E excretion remained constant and in case of administration of metenolone and stanozolol the E excretion declined extensively. These differences can be explained by the lack of binding of stanazolol to the androgen receptor and to SHBG, resulting in a general suppression of endogenous steroids by inhibition of metabolizing enzymes. The strong binding of metenolone to the androgen receptor would result in a more direct action in target tissues [138]. Increased T/E values have also been found after metandienone administration. This increase was due to a decreased E excretion [53].

Masking agents

Evidently, E administration could potentially be used to decrease the T/E ratio. However, E is not available as a pharmaceutical formulation, it can still be possible for athletes to obtain this steroid in other ways. Because approximately 1% of T and 30% of E is excreted directly or after glucuronidation, administration of T and E in a ratio of approximately 30 to 1 does not result in change of T/E [31]. Therefore, E has been added to the IOC list of forbidden substances as a masking agent for T abuse. As a consequence a maximum E concentration of 200 ng/ml in urine has been set as a criterion for E abuse. However, ratios of other excreted androgen metabolites can also be used to detect the combined abuse of T and E. The ratios of TG/Δ5-17α-AEDIOLG and EG/Δ5-17α-AEDIOLG have been introduced with respective threshold values of 2.5 and 1.5 to determine simultaneous administration of T and E [42]. Administration of 40 mg T undecanoate (equivalent to 20.36 mg T) and 1 mg E or 1.57 mg E undecanoate (equivalent to 1 mg E) resulted in a TG/Δ5-17α-AEDIOLG

and EG/Δ5-17α-AEDIOLG exceeding these threshold values, whereas TG/EG remained below 6 [139].

Two other masking agents are important to mention, probenecid and bromantan. Probenecid is an uricosuric agent that is used in the treatment of gout. It decreases the excretion of endogenous and exogenous conjugated steroids by competitive inhibition of active transport mechanisms in the proximal tubulus. The excretions of all steroid conjugates are suppressed in the same extent. A decline in excretion to 20% has been reported after administration of two doses of 1 gram probenecid to four male volunteers [140]. No change of the T/E ratio was observed. However, because of the lowered excretion and an additional weak diuretic effect, the T/E ratio might have a higher variance because of analytical variation. The masking agent bromantan does not show an effect on the T/E ratio. Its main mono hydroxy-metabolite can, however, coelute with E and therefore interfere with the analysis of the T/E ratio. The fact that the main bromantan metabolite and E coelute creates an analytical challenge. It is questionable that bromantan is regarded as a masking agent, because coelution of compounds appearing on the IOC list of forbidden substances with other substances is a problem which should be solved by the IOC-accredited laboratories and should not be a concern of the athlete. Contradictory, trimethoprim and sulfamethoxazol, of which the metabolites are reported to interfere with the analysis of T and E, are not placed in the IOC list [52,141]. Extraction with *n*-pentane is recommended to prevent this analytical problem.

Gonadotrophins human chorionic gonadotrophin (hCG) and LH

Gonadotrophins like human hCG, follicle stimulating hormone (FSH), and LH increase gonadal steroid synthesis. hCG is of special interest to athletes because of its relatively long plasma half-life. T and E production are both increased by hCG [142]. hCG is administered intramuscularly by athletes to reduce hypogonadotrophic hypogonadism after anabolic steroid use [143]. Another interesting aspect of hCG is that it decreases a high T/E due to T administration [144]. Because the T/E ratio can be decreased to a value far below 6 in this way, hCG can be regarded as a masking agent for T. Administration of hCG can not be detected by measuring the effects on the steroid profile, so direct hCG detection is necessary. The possibility of false hCG-positive cases due to early-stage pregnancy limits this test to male athletes.

ALTERNATIVE METHODS FOR THE T/E RATIO

Alternative ratios involving T

To determine T administration, other endocrinological parameters have been introduced besides the T/E ratio. Because T administration suppresses the pituitary secretion of gonadotrophins Brooks *et al.* [145] introduced the ratio between urinary T (non-conjugated plus glucuronide) and LH in 1979 as a parameter, and Kicman *et al.* [31] and Perry *et al.* [146] proved its sensitivity as a marker for T abuse. The T/LH will increase as a result of T administration. The advantage of this method is the possibility of detecting the use of combined T and E. In the past, a disadvantage has been the possibility of cross-reaction in the LH-assay by hCG, resulting in lowered T/LH values after hCG administration. Research regarding T/LH is restricted to males. Application to females is not possible due to the midcycle LH peak and the possible use of oral contraceptives that suppress LH excretion. Another restriction of the T/LH ratio is the extensively decreased values at lower Tanner classes [58].

Carlström *et al.* [147-148] introduced the serum T/17α-hydroxyprogesterone (17OHP) ratio as a parameter. 17OHP is a major testicular precursor of T and is suppressed after T administration. Its applicability is limited to confirmation procedures, as the IOC does still not allow the use of blood samples in doping control screenings, except for hematocrit analyses as applied in health control procedures. As a confirmation procedure, it has appeared to be a valuable parameter [149]. Other parameters that are used for confirmation of high T/E ratios are the less specific AO/T ratio and the T concentration [17].

GC-combustion-isotope ratio MS (GC-C-IRMS)

In recent years, GC-C-IRMS has been developed as an alternative technique for T detection. This technique made it possible to discriminate exogenous T from endogenous T by measuring the $^{13}C/^{12}C$ ratio of the steroid. Eluted compounds from the GC are combusted in a catalytic furnace to N_2 and CO_2. For the carbon isotope ratio determination, masses 44 and 45 are determined with great precision and accuracy [150]. The measured carbon isotope ratio ($^{13}C/^{12}C_{sample}$) is related to an international fossil carbonate standard “Pee Dee belemnite” or “PDB” ($^{13}C/^{12}C_{PDB}$):

$$\delta^{13}C‰ = \left(\frac{^{13}C/^{12}C_{sample} - ^{13}C/^{12}C_{PDB}}{^{13}C/^{12}C_{PDB}} \right) \times 10^3$$

In a study of Becchi *et al.* [151] the $\delta^{13}C‰$ from urinary endogenous T was not greater than -27‰ whereas $\delta^{13}C‰$ in 9 urine samples from T excretion studies was greater than -27‰. This difference is explained by the different origin of natural and

synthetic testosterone. Plant species have different ^{13}C levels [152]. Endogenous T originates from cholesterol from plant material and meat in the human diet. The $\delta^{13}C‰$ of endogenous testosterone is therefore a reflection of the average ^{13}C content in the human diet. Synthetically derived T is mostly derived from soy, which has a relatively low ^{13}C content. The described carbon isotope ratio method has been shown to be a powerful way to detect T abuse associated with a T/E>30 [153-154]. However, the detection of exogenous T by GC-C-IRMS after the T/E had returned to below 6 after oral administration of testosterone undecanoate has been reported [155]. Determination of exogenous T with GC-C-IRMS was possible for more than twice as long as with application of T/E. The cases of a naturally high T/E have not yet been investigated. A method which measured the carbon isotope ratio of T metabolites 5α-androstane-3α,17β-diol and 5β-androstane-3α,17β-diol was able to detect T administration in cases in which the T/E remained below 6 [156]. It must be said that the five volunteers used were of Chinese ethnicity. Therefore, the cut-off limit of T/E=6 is questionable for the used population. Use of volunteers from the Caucasian race would have been better to compare the isotope ratio technology with the use of T/E. Confirmation of T administration in the five Chinese volunteers was possible for eight days. A ratio of $\delta^{13}C‰$ for androstanediols to pregnanediol was proposed in this study as a discriminating parameter, applying a cutoff limit of 1.1 for T abuse. The disadvantage of using T metabolites as parameter is that DHEA and Δ4-AEDIONE also metabolize to androstanediols. One would therefore still need the steroid profile to confirm the abused steroid or apply the isotope ratio technology to other specific metabolites [157].

Since the 1998 Olympic Winter games in Nagano, the use of GC-C-IRMS has been widely accepted as a confirmation method of T abuse. However, a relatively large volume of urine (reported respectively minimum of 25 ml [156] and 2-20 ml [155,158]) must be available for the analysis and a time-consuming cleanup procedure is needed to obtain the necessary highly purified steroid fractions. With the current state of the art, GC-C-IRMS is therefore only applied as a confirmation method in IOC-accredited laboratories. The T/E is still the method of choice for screening purposes. Because IRMS was only introduced in doping analysis a few years ago, insufficient research has been done on the influence of important parameters as ethnic origin and related food consumption on the isotope ratio of T and its metabolites. Although Shackleton *et al.* [156] suggested the lack of racial influence on the isotope ratio of T metabolites by studying 15 individuals from 11 different nationalities, it is apparent that more research is needed on this subject. Also, more data should be collected to study the analytical between-laboratory variation of GC-C-IRMS. Recent studies to study the application of GC-C-IRMS technology for T screening were performed [159-161]. Although the use of isotope ratio mass spectrometry for T confirmation purposes is increasing, its application is

limited to a few IOC accredited laboratories, mainly because of the expense of its implementation. The T/E ratio is therefore still the most popular method and will remain to be very important in the near future of doping analysis.

Hair analysis for T

Hair analysis was developed in the forensic sciences to detect drugs of abuse [162]. Recently, high-resolution mass spectrometry (HRMS) was also applied to detect anabolic steroids post-mortem in hair of a bodybuilder [161]. An interesting new development is the measurement of T in hair [164]. A clear distinction could be made between T in child hair, female hair (both <10 pmol/g) and male hair (10-80 pmol/g). For these analyses 50 mg hair samples were used. Although hair analysis will not be suitable for screening, it could be a useful technique for confirmation purposes to obtain extra information besides the T/E ratio.

High-performance liquid chromatography-mass spectrometry (HPLC-MS)

A technique that has recently been introduced in doping control is electrospray HPLC-MS or HPLC-MS-MS. The great advantage of HPLC-MS over GC-MS is that derivatization [165] or deconjugation [166] of steroids is not essential, so T/E ratio analyses can be performed cheaper and faster. In addition, analytical interpretation is simplified because of absent incomplete hydrolysis or derivatization. T/E ratio measurements can be performed in a more robust and therefore in a more accurate way. Glucuronides and sulfates can be measured in one analysis, which makes this technique very promising for routine and also confirmation analysis. Bowers *et al.* [167] studied the analysis of steroids as TG, TS, EG and ES with electrospray HPLC-MS. A full separation of all steroids without derivatization was possible with detection limits of 3-25 pg on-column with a capillary packed column, which is very sensitive compared to conventional quadrupole GC-MS analysis. Qualitatively, best results were obtained in the positive ion mode. Because, as Dehennin *et al.* [42-43] already suggested, naturally high T/E ratios are mostly caused by a relatively low EG level, whereas ES is relatively high, HPLC-MS is a convenient method to determine glucuronidated and sulfated T and E in one analysis [168]. In the future electrospray HPLC-MS will gain importance as a more robust method to determine the T/E ratio.

CONCLUDING REMARKS

Because the T/E ratio is influenced by many of the discussed parameters, positive cases of T abuse cause a lot of scientific and legal debate. A lot of research has been done to improve the analysis and to find alternative methods to replace the T/E ratio analysis. This resulted in several promising confirmatory methods, such as T/LH and T/17OHP, the ketoconazole test and especially GC-C-IRMS. In the future more impact can be expected of HPLC-MS and hair analysis for confirmation. For screening purposes HPLC-MS is the most promising technique to determine the T/E ratio because GC-C-IRMS is still rather time consuming and expensive. The T/E ratio is therefore still the most efficient screening method and will remain to be so in the near future.

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