STEROID PROFILING
IN
DOPING ANALYSIS

Financial support by:

NOTOX BV, 's-Hertogenbosch
Sopachem, Lunteren

CIP-GEGEVENS KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Van de Kerkhof, Daniël Henri

Steroid profiling in doping analysis / Daan van de Kerkhof
Utrecht: Universiteit Utrecht, Faculteit Farmacie
Proefschrift Universiteit Utrecht – Met samenvatting in het Nederlands
ISBN 90-393-2918-4

Printing: FEBODRUK BV, Enschede
Cover design: Renée van de Kerkhof

STEROID PROFILING

IN

DOPING ANALYSIS

PROFILERING VAN STEROIDEN IN DOPING ANALYSE
(met een samenvatting in het Nederlands)

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
AAN DE UNIVERSITEIT UTRECHT
OP GEZAG VAN RECTOR MAGNIFICUS, PROF. DR. W.H. GISPEN
INGEVOLGE HET BESLUIT VAN HET COLLEGE VAN PROMOTIES
IN HET OPENBAAR TE VERDEDIGEN
OP DONDERDAG 13 DECEMBER 2001 DES NAMIDDAGS TE 12:45 UUR

DOOR

DANIËL HENRI VAN DE KERKHOF

geboren op 16 april 1970
te Helmond

Promotores Prof. Dr. R.A.A. Maes
Prof. Dr. J.H.H. Thijssen
(Faculty of Pharmacy, Utrecht University, Utrecht, The Netherlands)

Co-promotor Dr. D. de Boer
(Instituto Nacional do Desporto, Laboratório de Análises de Dopagem e Bioquímica, Av. Prof. Egas Moniz (Estádio Universitário), 1600-190 Lisboa, Portugal)

Content

PREFACE

Steroid profiling is one of the established techniques in doping analysis for detecting the abuse of steroids that are identical to steroids of endogenous origin. It is based on the analysis of several steroids that can originate from the endocrine system, in urine samples. This analysis is mostly aiming at metabolites and/or precursors of testosterone.

Since the introduction of testosterone as a widely abused doping agent, the concentration ratio of testosterone versus epitestosterone (T/E ratio) has been considered as the most sensitive and robust parameter to consider for detection of testosterone abuse. Despite the fact that the T/E ratio is accepted as analytical method in doping analysis, it has often played a controversial role in doping cases. This is mainly caused by insufficient published data that is available about the parameters that could possibly affect the "natural" T/E ratio, resulting in a significant chance of false positive cases. Nevertheless, the T/E ratio has been applied since the early eighties and can still not be displaced by the more recently developed isotope ratio mass spectrometry for the detection of testosterone.

This thesis discusses several items that are related to the analytical chemistry of steroid profiling. In **part I** the main focus is placed on the T/E ratio. Chapter 1 discusses the possible limitations of the T/E ratio by reviewing potential influential factors on this parameter. One of those factors is the consumption of alcohol. In Chapter 2 an experiment is described in which the relation between the metabolism of alcohol and the increase of the T/E ratio is discussed. For this purpose male and female subjects consumed an average dose of alcohol.

Steroid profiling techniques used today have mostly been developed in the time that testosterone was one of the few abused compounds identical to endogenous steroids. T/E ratios higher than the cutoff criterion of six were regarded as specific evidence of testosterone abuse, unless a naturally high T/E ratio could be proven. However, in the present time the abuse of substances identical to endogenous steroids has become much more complicated by the introduction of precursors in the biosynthesis of testosterone as "food supplements". An increased T/E ratio as a result of oral administration of steroids as dehydroepiandrosterone (DHEA) and androst-4-ene-3,17-dione has been described several times in literature. Therefore,

the T/E ratio should not be considered as a specific parameter for detection of testosterone abuse, but more as an indication that manipulation of the endocrine system has occurred.

Part II of this thesis is focussed on the development of steroid profiling techniques that provide better possibilities for identification of the steroids administered. Criteria of sensitivity (defined as the response of a parameter after administration) and specificity (the response of a parameter after administration of one particular steroid, compared to the response for other steroids) are used to evaluate new parameters in steroid profiling.

In Chapter 3 is described that detection of oxygenated metabolites could possibly provide information of equal sensitivity but higher specificity than non-oxygenated metabolites. In Chapters 4 to 7, aspects of sensitivity and specificity are investigated by performing excretion studies in male subjects with DHEA and androst-4-ene-3,17-dione used as model steroids. Additionally, an excretion study with 3-acetyl-7-keto-DHEA is evaluated in a case study design in an appendix.

In **part III** several analytical procedures of steroid profiling are discussed. As gas chromatography coupled to mass spectrometry (MS of MS/MS) is still considered as the method of choice for steroid analysis, derivatization is an essential sample preparation step to improve chromatographic behavior and to increase mass spectrometric sensitivity. Derivatization is often a bottleneck in the achieved analytical performance. In Chapter 8, trimethylsilylation reactions upon 3-keto-4-ene steroids were scaled up from an analytical (μg) to a preparative (mg) scale to gain fundamental insight into observed differences in product formation. The thermodynamically versus kinetically controlled formation of 3,5-dienolTMS and 2,4-dienolTMS derivatives is discussed.

Chapter 9 describes the formation of ethyl thio adducts with steroids upon derivatization with MSTFA/NH_4I/ethanethiol. These analytical artifacts could lead to interpretation problems in sample analysis.

In the final general discussion (Chapter 10) the perspectives of steroid profiling have been reviewed.

PART I:

steroid profiling
and
the T/E ratio

1

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.

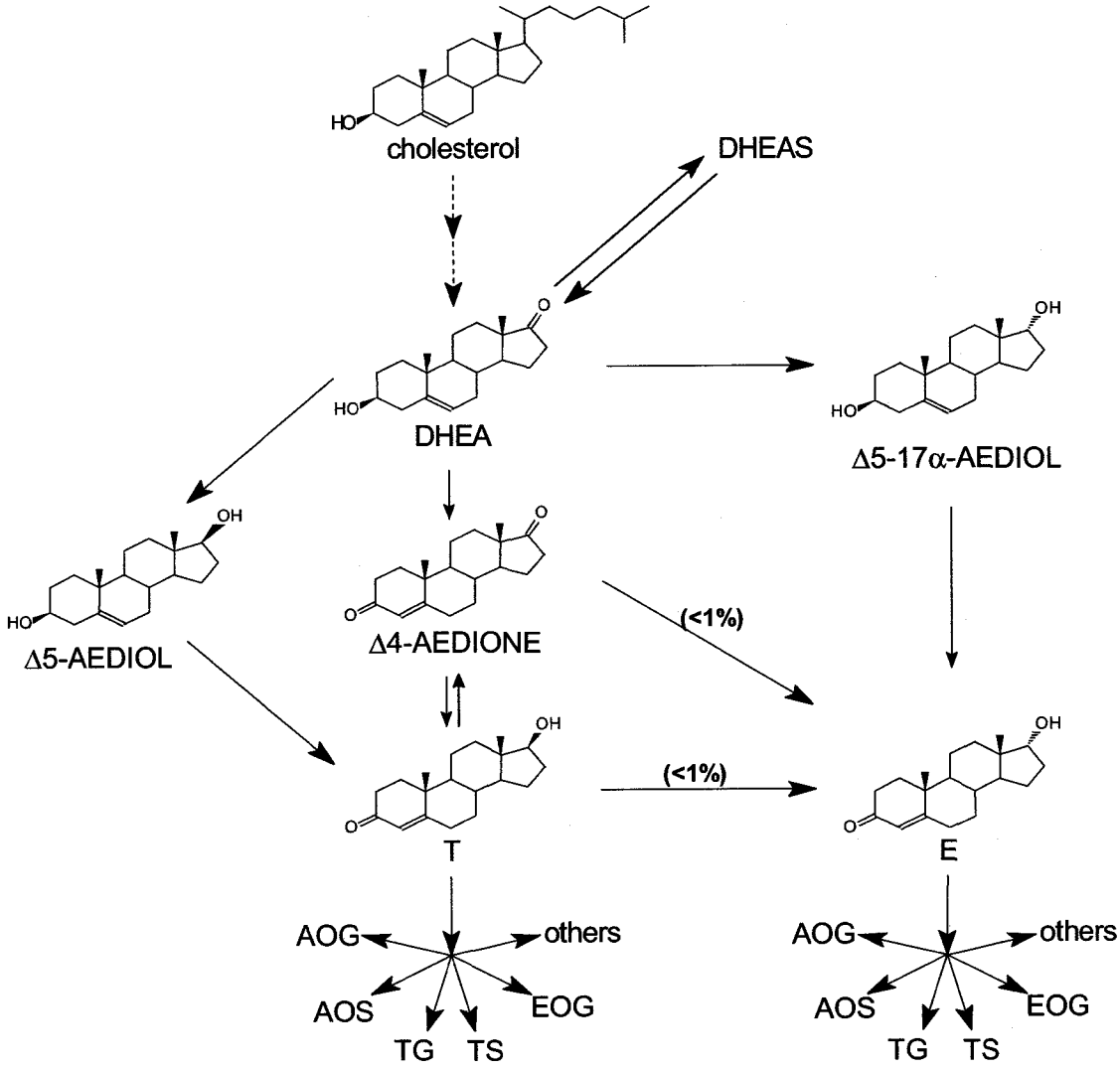


Figure 1. Steroid metabolism producing and eliminating T and E [18-24]. Cholesterol is the first precursor of all steroid hormones. Relevant precursors are dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEAS), androst-4-ene-3,17-dione (Δ4-AEDIONE), androst-5-ene-3β,17α-diol (Δ5-17α-AEDIOL) 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.

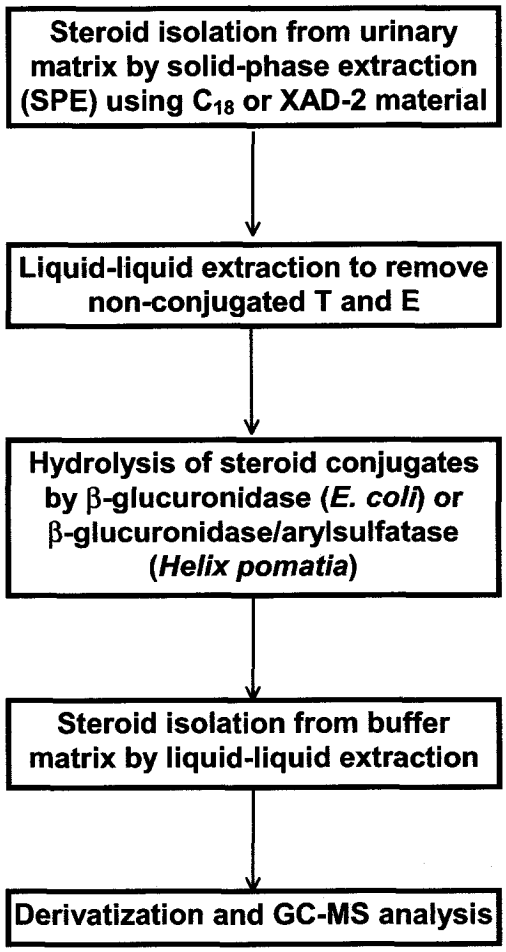


Figure 2. Basic procedure for steroid profiling.

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.

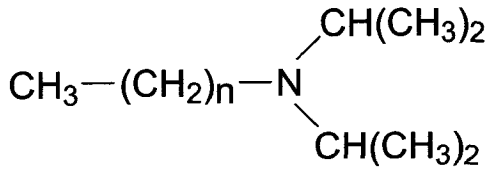


Figure 3. 1-(*N,N*-diisopropylamino)-alkane (DIPAs) with n=13 for DIPA 14 and n=22 for DIPA 23

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.

REFERENCES

1. M. Donike, K.-R. Bärwald, K. Klostermann, W. Schänzer and J. Zimmermann. Nachweis von exogenem Testosteron. In *Sport: Leistung und Gesundheit*, H. Heck, W. Hollmann, H. Liesen, R. Rost, Eds. Deutscher Ärzte Verlag, Köln, 1983, pp 293-298.
2. J. Zimmermann. *Untersuchungen zum Nachweis von exogenen Gaben von Testosteron*. Hartung-Gorre Verlag Konstanz, Köln, 1986.
3. L. Dehennin. Secretion by the human testis of epitestosterone, with its sulfoconjugate and precursor androgen 5-androstene-3β,17α-diol. *J. Steroid Biochem. Molec. Biol.* **44:** 171-177 (1993).
4. H. Wilson and M.B. Lipsett. Metabolism of epitestosterone. *J. Clin. Endocr. Metab.* **26:** 902-914 (1966).
5. J. Tamm, M. Apostolakis and K.D. Voigt. The effects of ACTH and hCG on the urinary excretion of testosterone in male patients with various endocrine disorders. *Acta Endocr. (Copenh)* **53:** 61-72 (1966).
6. T.J.A. Waasdorp, D. de Boer, L. van Schaik, H. Gerretsen, P. Schoone and J.H.H. Thijssen. Testosterone and epitestosterone under certain physiological and pathological conditions. In *Recent advances in doping analysis*, M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke and S. Rauth, Eds. Sport und Buch Strauss, Köln, 1994, pp 53-59.
7. R.V. Brooks and G. Giuliani. Epitestosterone: Isolation from human urine and experiments on possible precursors. *Steroids* **4:** 101-116 (1964).
8. J. Blaquier, R.I. Dorfman and E. Forchielli. Formation of epitestosterone in male patients with various endocrine disorders. *Acta Endocr. (Copenh)* **54:** 208-214 (1967).

9. M. Donike, S. Rauth and A. Wolansky. Reference ranges of urinary endogenous steroids determined by gas chromatography/mass spectrometry. In *Proceedings of the 10th Cologne workshop on dope analysis*, M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke and S. Rauth, Eds. Sport und Buch Strauss, Köln, 1993, pp 69-86.

10. S. Rauth. *Referenzbereiche von urinären Steroidkonzentrationen und Steroidquotienten - Ein Beitrag zur Interpretation des Steroidprofils in der Routinedopinganalytik*. Thesis Deutsche Sporthochschule Köln, 1994.

11. H. Oftebro. Evaluating an abnormal urinary steroid profile. *Lancet* **339:** 941-942 (1992).

12. L. Dehennin and A.M. Matsumoto. Long-term administration of testosterone enanthate to normal men: Alterations of the urinary profile of androgen metabolites potentially useful for detection of testosterone misuse in sport. *J. Steroid Biochem. Molec. Biol.* **44:** 179-189 (1993).

13. P. Hemmersbach, K.I. Birkeland, B. Bjerke, T. Sand and N. Norman. The use of endocrine parameters in the evaluation of doping analysis results. In *Proceedings of the 10th Cologne workshop on dope analysis*, M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke and S. Rauth, Eds. Sport und Buch Strauss, Köln, 1993, pp 91-96.

14. J. Baenziger and L. Bowers. Variability of T/E ratios in athletes. In *Recent advances in doping analysis*, M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke and S. Rauth, Eds. Sport und Buch Strauss, Köln, 1994, pp 41-51.

15. M. Donike, S. Rauth, U. Mareck-Engelke, H. Geyer and R. Nitschke. Evaluation of longitudinal studies, the determination of subject based reference ranges of the testosterone/epitestosterone ratio. In *Recent advances in doping analysis*, M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke and S. Rauth, Eds. Sport und Buch Strauss, Köln, 1994, pp 33 39.

16. M. Garle, R. Ocka, E. Palonek and I. Björkhem. Increased urinary testosterone/epitestosterone ratios found in Swedish athletes in connection with a national control program. Evaluation of 28 cases. *J. Chromatogr. B* **687:** 55-59 (1996).

17. H. Geyer, U. Mareck-Engelke, W. Schänzer and M. Donike. The Cologne protocol to follow up high testosterone/epitestosterone ratios. In *Recent advances in doping analysis (4)*, W. Schänzer, H. Geyer, A. Gotzmann and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, 1997, pp 107-126.

18. E.-E. Baulieu and P. Mauvais-Jarvis. Studies on testosterone metabolism. I. Conversion of testosterone-17α-^{3}H to 5α- and 5β-androstane-3α,17β-diol-17α-^{3}H: A new "17β-hydroxyl pathway". *J. Biol. Chem.* **239:** 1569-1577 (1964).

19. E.-E. Baulieu and P. Mauvais-Jarvis. Studies on testosterone metabolism. II. Metabolism of testosterone-4-^{14}C and androst-4-ene-3,17-dione-1,2-^{3}H. *J. Biol. Chem.* **239:** 1578-1584 (1964).

20. P. Robel, R. Emiliozzi and E.-E. Baulieu. Studies on testosterone metabolism. III. The selective "5β-metabolism" of testosterone glucuronide. *J. Biol. Chem.* **241:** 20-29 (1966).

21. P. Mauvais-Jarvis and E.-E. Baulieu. Studies on testosterone metabolism. IV. Urinary 5α- and 5β-androstanediols and testosterone glucuronide from testosterone and dehydroisoandrosterone sulfate in normal people and hirsute women. *J. Clin. Endocrinol. Metab.* **25:** 1167-1178 (1965).

22. P. Robel, R. Emiliozzi and E.-E. Baulieu. Studies on testosterone metabolism. V. Testosterone-^{3}H 17-glucuronide-^{14}C to 5β-androstane-3α,17β-diol-^{3}H 17-glucuronide-^{14}C, the "direct" 5β-metabolism of testosterone glucuronide.. *J. Biol. Chem.* **241:** 5879-5885 (1966).

23. P. Mauvais-Jarvis, H. Floch, I. Jung, P. Robel and E.-E. Baulieu. Studies on testosterone metabolism. VI. Precursors of urinary androstanediols. *Steroids* **11:** 207-224 (1968).

24. R.V. Brooks. Androgens. *Clin. Endocrinol. Metab.* **4:** 503-520 (1975).

25. M. Donike and J. Zimmermann. Zur Darstellung von Trimethylsilyl-, Triethylsilyl- und *tert.*-Butyldimethylsilyl-enoläthern von Ketosteroiden für gas-chromatographische und massenspektrometrische Untersuchungen. *J. Chromatogr.* **202:** 483-486 (1980).

26. P. Pfeifer and G. Spiteller. Steroid profiles of healthy individuals. *J. Chromatogr. B* **223:** 21-32 (1981).

27. G. Spiteller. Steroid profiling by GC/MS. *Spectra* (Finnigan MAT) **10:** 15-20 (1985).

28. C.H.L. Shackleton. Profiling steroid hormones and urinary steroids. *J. Chromatogr. B* **379:** 91-156 (1986).

29. J.W. Honour. Steroid profiling. *Ann. Clin. Biochem.* **34:** 32-44 (1997).

30. M. Donike, J. Zimmermann, K.-R. Bärwald, W. Schänzer, V. Christ, K. Klostermann and G. Opfermann. Routinebestimmung von Anabolika im Harn. *Deutsche Zeitschr. Sportmed.* **1:** 14-23 (1984).

31. A.T. Kicman, R.V. Brooks, S.C. Collyer, D.A. Cowan, M.N. Nanjee, G.J. Southan and M.J. Wheeler. Criteria to indicate testosterone administration. *Br. J. Sp. Med.* **24:** 253-264 (1990).

32. W. Schänzer and M. Donike. Synthesis of deuterated steroids for GC/MS quantification of endogenous steroids. In *Recent advances in doping analysis (2)*, M. Donike, H. Geyer, A. Gotzmann, and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, 1995, pp 93-112.

33. E. Nolteernsting, H. Geyer, U. Mareck-Engelke, W. Schänzer and M. Donike. Standardization of the T/E determination by deuterated internal standards. In *Recent advances in doping analysis (2)*, M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke and S. Rauth, Eds. Sport und Buch Strauss, Köln, 1995, pp 113-112.

34. E. Nolteersting, W. Schänzer and M. Donike. Experiences with d3 testosterone/d3 epitestosterone as internal standards for screening and confirmation of T/E ratios. In *Recent advances in doping analysis (3)*, M. Donike, H. Geyer, A. Gotzmann and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, 1996, pp 191-200.

35. V. Graef, E. Furuya, and O. Nishikaze. Hydrolysis of steroid glucuronides with β-glucuronidase preparations from bovine liver, Helix pomatia and E. coli. *Clin. Chem.* **23:** 532-535 (1977).

36. P. Vestergaard. The hydrolysis of conjugated neutral steroids in urine. *Acta Endocrinol.* **suppl 217:** 96-120 (1978).

37. G. Messeri, G. Cugnetto, G. Moneti and M. Serio. Helix pomatia induced conversion of some 3β-hydroxysteroids. *J. Steroid Biochem.* **20:** 793-796 (1984).

38. E. Vanluchene, W. Eechaute and D. Vandekerckhove. Conversion of free 3β-hydroxy-5-ene-steroids by incubation with helix pomatia. *J. Steroid Biochem.* **16:** 701-703 (1982).

39. C. Clausnitzer and J. Grosse. Analytische Probleme bei der Ermittlung und Interpretation des Testosteron-Epitestosteron-Quotienten im rahmen von Dopingkontrollen. *Med. u. Sport* **25:** 212-215 (1985).

40. E. Houghton, L. Grainger, M.C. Dumasia and P. Teale. Application of gas chromatography/mass spectrometry to steroid analysis in equine sports: Problems with enzyme hydrolysis. *Org. Mass Spectrom.* **27:** 1061-1070 (1992).

41. H. Geyer, W. Schänzer, U. Mareck-Engelke, E. Nolteernsting and G. Opfermann. Screening procedure for anabolic steroids - The control of hydrolysis with deuterated androsterone glucuronide and studies with direct hydrolysis. In *Recent advances in doping analysis (5)*, W. Schänzer, H. Geyer, A. Gotzmann and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, 1997, pp 99-102.

42. L. Dehennin. On the origin of physiologically high ratios of urinary testosterone to epitestosterone: Consequences for reliable detection of testosterone administration by male athletes. *J. Endocrinol.* **142:** 353-360 (1994).

43. L. Dehennin, P. Lafarge, Ph. Dailly, D. Bailloux and J.-P. Lafarge. Combined profile of androgen glucuro- and sulfoconjugates in post-competition urine of sportsmen: a simple screening procedure using gas chromatography-mass spectrometry. *J. Chromatogr. B* **687:** 85-91 (1996).

44. D.H. Catlin, D.A. Cowan, R. de la Torre, M. Donike, D. Fraisse, H. Oftebro, C.K. Hatton, B. Starcevic, M. Becchi, X. de la Torre, H. Norli, H. Geyer and C.J. Walker. Urinary testosterone (T) to epitestosterone (E) ratios by GC/MS. I. Initial comparison of uncorrected T/E in six international laboratories. *J. Mass Spectrom.* **31:** 397-402 (1996).

45. M. Donike. Steroid profiling in Cologne. In *Proceedings of the 10th Cologne workshop on dope analysis*, M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke and S. Rauth, Eds. Sport und Buch Strauss, Köln, 1993, pp 47-68.

46. K. Linnet. Effect of biological matrix on the urinary testosterone/epitestosterone ratio measured by gas chromatography/mass spectrometry in doping analysis. *Biol. Mass Spectrom.* **22:** 412-418 (1993).

47. H. Geyer, U. Mareck-Engelke, E. Nolteernsting, G. Opfermann and M. Donike. The matrix problem in calibration of the GC/MS for endogenous steroids. The search for an artificial urine. In *Recent advances in doping analysis (2)*, M. Donike, H. Geyer, A. Gotzmann and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, 1995, pp 199-209.

48. J.W. Honour. Testing urine for drug abuse. *Lancet* **348L:** 41-43 (1996).

49. J.W. Honour. Misuse of natural hormones in sport. *Lancet* **349:** 1786 (1997).

50. P. Hemmersbach, K.I. Birkeland, H.R. Norli and S.H. Ringertz. Urine storage conditions and steroid profile analysis. In *Recent advances in doping analysis (4)*, W. Schänzer, H. Geyer, A. Gotzmann and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, 1997, pp 99-106.

51. J.M. Kjeld, C.M. Puah and G.F. Joplin. Rise of unconjugated sex steroid hormones in human urine on storage. *Clin. Chim. Acta* **80:** 285-91 (1977).

52. H. Geyer, W. Schänzer, U. Mareck-Engelke and M. Donike. Factors influencing the steroid profile. In *Recent advances in doping analysis (3)*, M. Donike, H. Geyer, A. Gotzmann and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, 1996, pp 95-113.

53. H. Geyer. *Die gas-chromatographisch/massenspektrometrische Bestimmung von Steroidprofilen im Urin von Atleten*. Köln, Germany: Deutsche Sporthochschule Köln, 1990.

54. R.F. Bilton. Microbial production of testosterone. *Lancet* **345:** 1186-1187 (1995).

55. R. de la Torre, X. de la Torre, J. Segura, M.T. Smeyers, R. Ventura, J.M. Torres. C. Alia and T. Baró. Urine contamination by micro-organisms and alterations in the endogenous steroid profile. A prospective study. In *Recent advances in doping analysis (6)*, W. Schänzer, H. Geyer, A. Gotzmann and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, 1999, pp 223-235.

56. C. Ayotte, D. Goudreault and A. Charlebois. Testing for natural and synthetic anabolic agents in human urine. *J. Chromatogr. B* **687:** 3-25 (1996).

57. C. Ayotte, A. Charlebois, S. Lapointe, D. Barriault and M. Sylvestre. Validity of urine samples: microbial degradation. In *Recent advances in doping analysis (4)*, W. Schänzer, H. Geyer, A. Gotzmann and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, 1997, pp 127-137.

58. L. Dehennin, A. Delgado and G. Pérès. Urinary profile of androgen metabolites at different stages of pubertal development in a population of sporting male subjects. *Eur. J. Endocrinol.* **130:** 53-9 (1994).

59. J.M. Tanner. *Growth at adolescence*, 2nd ed. Blackwell, Oxford, 1962.

60. E. Raynaud, M. Audran, J.Ch. Pagès, C. Fédou, J.F. Brun, J.L. Chanal and A. Orsetti. Determination of urinary testosterone and epitestosterone during pubertal development: a cross-sectional study in 141 normal male subjects. *Clin. Endocrinol.* **38:** 353-9 (1993).

61. D. Gupta and H. Butler. Excretion of testosterone and epitestosterone glucuronides in pre-school, pre-adolescent and adolescent children. *Steroids* **14:** 343-67 (1969).

62. D.P. Dalzell and T.M.A. Elatter. Gas chromatographic determination of urinary excretion of testosterone, epitestosterone and androstenedione in pre-adolescent and adolescent children. *J. Clin. Endocrinol. Met.* **36:** 1237-1243 (1973).

63. C. Schweizer, C. Cardis, M. Cauderay, L. Rivier and M. Saugy. T/E ratio variations through puberty in male adolescents. In *Recent advances in doping analysis (4)*, W, Schänzer, H. Geyer, A. Gotzmann and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, 1997, pp 159-171.

64. O. Lapcik, R. Hampl, M. Hill and L. Stárka. Plasma levels of epitestosterone from prepuberty to adult life. *J. Steroid Biochem. Molec. Biol.* **55:** 405-408 (1995).

65. C. Schweizer, C. Cardis, M. Cauderay, L. Rivier and M. Saugy. Profile variations through puberty in young adolescent girls. In *Recent advances in doping analysis (6)*, W. Schänzer, H. Geyer, A. Gotzmann and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, 1999, pp 205-221.

66. J.T. France and B.S. Knox. Urinary excretion of testosterone and epitestosterone in hirsutism. *Acta Endocrinol.* **56:** 177-87 (1967).

67. S.B. Pal. Urinary testosterone and epitestosterone excretions in women and iodiopathic hirsutism. *Endokrinologie* **73:** S296-S300 (1979).

68. A.F. de Nicola, R.I. Dorfman and E. Forchielli. Urinary excretion of epitestosterone and testosterone in normal individuals and hirsute and virilized females. *Steroids* **7:** 351-366 (1966).

69. S.B. Pal. Urinary excretion of testosterone and epitestosterone in men, women and children, in health and disease. *Clin Chim Acta* **33:** 215-227 (1971).

70. W. von Hubl, W. Siebert, M. Büchner and H. Hering. Die Ausscheidung von Testosteron, Epitestosteron und Androstenedion vor und nach HCG-applikation bei normalen Männern und Patienten mit primärem und sekundärem Hypogonadismus. *Endokrinologie* **58:** S62-S74 (1971).

71. J. Park, S. Park, D. Lho, H.P. Choo, B. Chung, C. Yoon, H. Min and M.J. Choi. Drug testing at the 10th Asian Games and 24th Seoul Olympic Games. *J. Anal. Toxicol.* **14:** 66-72 (1990).

72. U. Flenker, E. Nolteernsting, W. Schänzer and M. Donike. Detection and classification of different male steroid profiles by means of multivariate statistics. In *Recent advances in doping analysis (4)*, W. Schänzer, H. Geyer, A. Gotzmann and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, 1997, pp 203-210.

73. X. de la Torre, J. Segura, Z. Yang, Y. Li and M. Wu. Testosterone detection in different ethnic groups. In *Recent advances in doping analysis (4)*, W. Schänzer, H. Geyer, A. Gotzmann and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, 1997, pp 71-89.

74. D.P. Agarwal and H.W. Goedde. Pharmacogenetics of alcohol metabolism and alcoholism. *Pharmacogenetics* **2:** 48-62 (1992).

75. S.J. Yin. Alcohol dehydrogenase: enzymology and metabolism. *Alcohol Alcohol Suppl* **2:** 113-119 (1994).

76. L. Bertilsson. Geographical/interracial differences in polymorphic drug oxidation. *Clin. Pharmacokinet.* **29:** 192-209 (1995).

77. D.J. Touw and D.D. Breimer. Het cytochroom P450-enzymsysteem. Deel 1. *Pharm. Weekbl.* **132:** 948-963 (1997).

78. U. Mareck-Engelke, H. Geyer and M. Donike. Stability of steroid profiles (4): The circadian rhythm of urinary ratios and excretion rates of endogenous steroids in female and its menstrual dependency. In *Recent advances in doping analysis (2)*, M. Donike, H. Geyer, A. Gotzmann and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, 1995, pp 135-155.

79. U. Mareck-Engelke, U. Flenker and M. Donike. Stability of steroid profiles (5): The annual rhythm of urinary ratios and excretion rates of endogenous steroids in female and its menstrual dependency. In *Recent advances in doping analysis (3)*, M. Donike, H. Geyer, A. Gotzmann and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, 1996, pp 177-189.

80. D.H. Catlin, C.K. Hatton and S.H. Starcevic. Issues in detecting abuse of xenobiotic anabolic steroids and testosterone by analysis of athletes' urine. *Clin. Chem.* **43:** 1280-1288 (1997).

81. E. Nieschlag. Circadian rhythm of plasma testosterone. In *Chronobiological aspects of endocrinology*, J. Aschoff, F. Ceresa and F. Halberg, Eds. Symposia medica Hoechst 9, Schattauer, Stuttgart, 1974, pp 117-128.

82. G.A. Lincoln, P.H. Rowe and R.A. Racey. The circadian rhythm in plasma testosterone concentration in man. In *Chronobiological aspects of endocrinology*, J. Aschoff, F. Ceresa and F. Halberg, Eds. Symposia medica Hoechst 9, Schattauer, Stuttgart, 1974 pp 137-149.

83. M. Lagoguey, F. Dray, J.-M. Chauffournier and A. Reinberg. Circadian and circannual rhythms of urine testosterone and epitestosterone glucuronides in healthy adult men. *Int. J. Chronobiol.* **1:** 91-93 (1973).

84. U. Mareck-Engelke, H. Geyer and M. Donike. Stability of steroid profiles (3): The circadian rhythm of urinary ratios and excretion rates of endogenous steroids in male. In *Recent advances in doping analysis (2)*, M. Donike, H. Geyer, A. Gotzmann and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, 1995, pp 121-133.

85. P. O'Leary, P. Boyne, P. Flett, J. Beilby and I. James. Longitidinal assessment of changes in reproductive hormones during normal pregnancy. *Clin. Chem.* **37:** 667-672 (1991).

86. K. Demisch, J.K. Grant and W. Black. Plasma testosterone in women in late pregnancy and after delivery. *J. Endocrinol.* **42:** 477-81 (1968).

87. K. Schollberg, E. Seiler and J. Holtorff. Die Ausscheidung von Testosteron und Epitestosteron bei Frauen am ende der Schwangerschaft. *Acta Endocrinol.* **60:** 579-585 (1969).

88. N. Longhino, M. Tajic, A. Drazancic and P. Drobnjak. Studies of urinary androgen excretion in normal pregnancy. *Am. J. Obstet. Gynec.* **108:** 364-368 (1970).

89. D. De Boer. *Profiling anabolic androgens and corticosteroids in doping analysis.* Utrecht, 1992, pp 136-52.

90. R. Nitschke. *Steroidprofile und Veränderungen biochemischer Parameter von Hochleistungsradrennfahrern unter extremen Ausdauerbelastungen während zwei Rundfahrten.* Köln, 1997.

91. M. Maynar, M.J. Caballero, P. Mena, C. Rodríguez, R. Cortés and J.I. Maynar. Urine excretion of androgen hormones in professional racing cyclists. *Eur. J. Appl. Physiol.* **68:** 200-204 (1994).

92. B.K. Yap, R. Kazlauskas, K. Elghazi, G.A.R. Johnston and R.P. Weatherby. Profiling of urinary testosterone and luteinizing hormone in exercise-stressed male athletes, using gas chromatography/mass spectrometry and enzyme immunoassay techniques. *J. Chromatogr. B* **687:** 117-125 (1996).

93. C.Y. Guezennec, J.P. Lafarge, V.A. Bricout, D. Merino and B. Serrurier. Effect of competition stress on tests used to assess testosterone administration in athletes. *Int. J. Sports Med.* **16:** 368-372 (1995).

94. U. Mareck-Engelke, U. Flenker and W. Schänzer. Stability of steroid profiles (6): The influence of oral contraceptives on steroid profiles. In *Recent advances in doping analysis (4)*, W. Schänzer, H. Geyer, A. Gotzmann and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, 1996, pp 139-157.

95. A. Pont, P.L. Williams, S. Azhar, R.E. Reitz, C. Bochra, E.R. Smith and D.A. Stevens. Ketoconazole blocks testosterone synthesis. *Arch. Intern. Med.* **142:** 2137-2140 (1982).

96. C.S. Suresh, R.S. Swerdloff and J. Rajfer. In vitro inhibition of testosterone biosynthesis by ketokonazole. *Endocrinol.* **116:** 1920-1925 (1985).

97. R.J. Santen, H. van den Bossche, J. Symoens, J. Brugmans and R. deCoster. Site of action of low dose ketoconazole on androgen biosynthesis in men. *J. Clin. Endocrinol. Metab.* **57:** 732-736 (1983).

98. Th. Schürmeyer and E. Nieschlag. Effect of ketoconazole and other imidazole fungicides on testosterone biosynthesis. *Act. Endocrinol.* **105:** 275-280 (1984).

99. M.A. Touchette, P.H. Chandrasekar, M.A. Milad and D.J. Edwards. Contrasting effects of fluconazole and ketoconazole on phenytoin and testosterone disposition in man. *Br. J. Clin. Pharmac.* **34:** 75-78 (1992).

100. D.P. Hanger, S. Jevons and J.T.B. Shaw. Fluconazole and testosterone: in vivo and in vitro studies. *Antimicrob. Agents Chemother.* **32:** 646-648 (1988).

101. A.T. Kicman, H. Oftebro, C. Walker, N. Norman and D.A. Cowan. Potential use of ketoconazole in a dynamic endocrine test to differentiate between biological outliers and testosterone use by athletes. *Clin. Chem.* **39:** 1798-1803 (1993).

102. H. Oftebro, J. Jensen, P. Mowinckel and H.R. Norli. Establishing a ketoconazole suppression test for verifying testosterone administration in the doping controle of athletes. *J. Clin. Endocrinol. Met.* **78:** 973-977 (1994).

103. O. Falk, E. Palonek and I. Björkhem. Effect of ethanol on the ratio between testosterone and epitestosterone in urine. *Clin. Chem.* **34:** 1462-1464 (1988).

104. T.J.A. Seppenwoolde-Waasdorp, D. de Boer, H.M.J. van Engelen, A.D. Vrijdag and R.A.A. Maes. Evaluation of endogenous steroid profiles in urine (2). Effects of ethanol intake reinvestigated. In *Recent advances in doping analysis (3)*, M. Donike, H. Geyer, A. Gotzmann and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, 1996, pp 157-65.

105. A. Leinonen, T. Karila and T. Seppälä. An increased testosterone to epitestosterone ratio due to high doses of ethanol - a case report on a female powerlifter. In *Recent advances in doping analysis (3)*, M. Donike, H. Geyer, A. Gotzmann and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, 1996, pp 167-176.

106. U. Mareck-Engelke, H. Geyer, U. Schindler, U. Flenker, R. Iffland and M. Donike. Influence of ethanol on steroid profile parameters. In *Recent advances in doping analysis (3)*, M. Donike, H. Geyer, A. Gotzmann and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, 1996, pp 143-155.

107. T. Karila, V. Kosunen, A. Leinonen, R. Tähtelä and T. Seppälä. High doses of alcohol increase urinary testosterone-to-epitestosterone ratio in females. *J. Chromatogr. B* **687:** 109-116 (1996).

108. J.H. Mendelson, N.K. Mello and J. Ellingboe. Effects of acute alcohol intake on pituitary-gonadal hormones in normal human males. *J. Pharmacol. Exp. Ther.* **202:** 767-782 (1977).

109. R. Ylikahri, M. Huttunen, M. Harkonen and H. Adlercreutz. Hangover and testosterone. *Br. Med. J.* **2:** 445 (1974).

110. A.A.-B. Badawy. The metabolism of alcohol. *Clin. Endocrinol. Metab.* **7:** 247-271 (1978).

111. T. Cronholm and J. Sjövall. Effect of ethanol on the concentrations of solvolyzable plasma steroids. *Biochim. Biophys. Acta* **152:** 233-236 (1968).

112. T. Cronholm and J. Sjövall. Effect of ethanol metabolism on redox state of steroid sulfates in man. *Eur. J. Biochem.* **13:** 124-131 (1970).

113. M. Axelson, T. Cronholm, B.-L. Sahlberg and J. Sjövall. Changes in the metabolic profile of steroids in urine during ethanol metabolism in man. *J. Steroid Biochem.* **14:** 155-159 (1981).

114. C. Gagliardi. In *Current obstetric and gynecologic diagnosis and treatment*, Appleton and Lange, East Nordwalk, 1991, pp 1046.

115. C.F. Cobb, D.H. van Thiel, M.F. Ennis, J.S. Gavaler and R. Lester. Is acetaldehyde an adrenal stimulant? *Curr. Surg.* **36:** 431-434 (1979).

116. R. Groote Veldman and A.E. Meinders. On the mechanism of alcohol-induced pseudo-Cushing's syndrome. *Endocr. Rev.* **17:** 262-268 (1996).

117. IOC List of Classes of Prohibited Substances and Methods of Doping, 1999.

118. R. Johnson. Abnormal testosterone:epitestosterone ratios after dehydroepiandrosterone supplementation. *Clin. Chem.* **45:** 163-164 (1999).

119. P. Diamond, L. Cusan, J.-L. Gomez, A. Bélanger and F. Labrie. Metabolic effects of 12-month percutaneous dehydroepiandrosterone replacement therapy in postmenopausal women. *J. Endocrinol.* **150:** S43-S50 (1996).

120. E.-E. Baulieu. Dehydroepiandrosterone (DHEA): A fountain of youth? *J. Clin. Endocrinol. Metab.* **81:** 3147-3151 (1996).

121. A.J. Morales, J.J. Nolan, J.C. Nelson and S.S.C. Yen. Effects of replacement dose of dehydroepiandrosterone in men and women of advancing age. *J. Clin. Endocrin. Metab.* **78:** 1360-1367 (1994).

122. L. Dehennin, M. Ferry, P. Lafarge, G. Pérès and J.-P. Lafarge. Oral administration of dehydroepiandrosterone to healthy men: Alteration of the urinary androgen profile and consequences for the detection of abuse in sport by gas chromatography-mass spectrometry. *Steroids* **63:** 80-87 (1998).

123. L. Bowers. Oral dehydroepiandrosterone supplementation can increase the testosterone/epitestosterone ratio. *Clin. Chem.* **45:** 295-297 (1999).

124. R. Kazlauskas. Effects of dehydroepiandrosterone on urinary steroids. In *Recent advances in doping analysis (5)*, W. Schänzer, H. Geyer, A. Gotzmann and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, 1998, pp 83-90.

125. T.Z. Bosy, K.A. Moore and A. Poklis. The effect of oral dehydroepiandrosterone (DHEA) on the urinary testosterone/epitestosterone (T/E) ratio in human male volunteers. *J. Anal. Toxicol.* **22:** 455-459 (1998).

126. F. Labrie, A. Bélanger, L. Cusan and B. Candas. Physiological changes in dehydroepiandrosterone are not reflected by serum levels of active androgens and estrogens but of their metabolites: Intracrinology. *J. Clin. Endocrin. Metab.* **82:** 2403-2409 (1997).

127. H. Kapelrud, O. Johannesen and H. Oftebro. Case report: Testosterone/epitestosterone ratio in urine: A possible diagnostic tool in the disclosure of exogenous testosterone administration. *J. Int. Med.* **232:** 453-455 (1992).

128. V.P. Uralets and P.A. Gillette. Over-the-counter anabolic steroids 4-androsten-3,17-dione; 4-androsten-3β,17β-diol and 19-nor-4-androsten-3,17-dione: Excretion studies in men. In *Recent advances in doping analysis (6)*, W. Schänzer, H. Geyer, A. Gotzmann and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, 1999, pp 147-169.

129. P. van Eenoo, F.T. Delbeke, N. Desmet and P. De Backer. Excretion studies with 4-androstene-3,17-dione. In *Recent advances in doping analysis (6)*, W. Schänzer, H. Geyer, A. Gotzmann and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, 1999, pp 171-180.

130. M. Garle and E. Palonek. Androstenedione: Excretion studies frome single and multiple dose experiments. In *Recent advances in doping analysis (6)*, W. Schänzer, H. Geyer, A. Gotzmann and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, 1999, pp 181-184.

131. G.J. Southan, R.V. Brooks, D.A. Cowan, A.T. Kicman, N. Unnadkat and C.J. Walker. Possible indices for the detection of the administration of dihydrotestosterone to athletes. *J. Steroid Biochem. Molec. Biol.* **42:** 87-94 (1992).

132. A.T. Kicman, S.B. Coutts, C.J. Walker and D.A. Cowan. Proposed confirmatory procedure for detecting 5α-dihydrotestosterone doping in male athletes. *Clin. Chem.* **41:** 1617-1627 (1995).

133. S.B. Coutts, A.T. Kicman, D.T. Hurst and D.A. Cowan. Intramuscular administration of 5α-dihydrotestosterone heptanoate: Changes in urinary hormone profile. *Clin. Chem.* **43:** 2091-2098 (1997).

134. Donike M. Longterm influence of anabolic steroid misuse on the steroid profile. In *Official proceedings IInd International Athletic Foundation World Symposium on Doping in Sport,* P. Belotti, G. Benzi and A. Ljungqvist, Eds. International Athletic Foundation, Monte Carlo, 1990, pp 107-116.

135. P.V. Fennessey, R.W. Gotlin, D. Martin, S. Smith and L.M. Harrison. Anabolic steroids in body builders: Use, metabolic disposition and physiological effects. *J. Pharm. Biomed. Anal.* **6:** 999-1002 (1988).

136. L.M. Harrison, D. Martin, R.W. Gotlin and P.V. Fennessey. Effect of extended use of single anabolic steroids on urinary steroid excretion and metabolism. *J. Chromatogr. B* **489:** 121-126 (1989).

137. X. De la Torre, J. Ortuño, J. Segura, J. Cabrer and E. Vilardell. Stanozolol, metenolone and steroid profile. In *Recent advances in doping analysis*, M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke and S. Rauth, Eds. Sport und Buch Strauss, Köln, 1994, pp 69-83.

138. T. Saartok, E. Dahlberg and J.A. Gustafsson. Relative binding affinity of anabolic-androgenic steroids: Comparison of the binding to the androgen receptors in skeletal muscle and in prostate, as well as to sex hormone-binding globulin. *Endocrinol.* **114(6):** 2100-2106 (1984).

139. L. Dehennin. Detection of simultaneous self-administration of testosterone and epitestosterone in healthy men. *Clin. Chem.* **40:** 106-109 (1994).

140. H. Geyer, W. Schänzer and M. Donike. Probenecid as masking agent in dope control - Inhibition of the urinary excretion of steroid glucuronides. In *Proceedings of the 10th Cologne workshop on dope analysis*, M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke and S. Rauth, Eds. Sport und Buch Strauss, Köln, 1993, pp 141-51.

141. H. Geyer, U. Mareck-Engelke, W. Schänzer and M. Donike. Simple purification of urine samples for improved detection of anabolic and endogenous steroids. In *Recent advances in doping analysis*, M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke and S. Rauth, Eds. Sport und Buch Strauss, Köln, 1994, pp 97-104.

142. D.A. Cowan, A.T. Kicman, C.J. Walker and M.J. Wheeler. Effect of administration of human chorionic gonadotrophin on criteria used to asses testosterone administration in athletes. *J. Endocrinol.* **131:** 147-154 (1991).

143. H. Martikainen, M. Alén, P. Rahkila and R. Vihko. Testicular responsiveness to human chorionic gonadotrophin during transient hypogonadotrophic hypogonadism induced by androgenic/anabolic steroids in power athletes. *J. Steroid Biochem.* **25:** 109-112 (1986).

144. D. De Boer, E.G. De Jong and R.A.A. Maes. Doping control of testosterone and human chorionic gonadotrophin: A case study. *Int. J. Sports Med.* **12:** 46-51 (1991).

145. R.V. Brooks, G. Jeremiah, W.A. Webb and M. Wheeler. Detection of anabolic steroid administration to athletes. *J. Steroid Biochem.* **11:** 913-917 (1979).

146. P.J. Perry, J.H. MacIndoe, W.R. Yates, S.D. Scott and T.L. Holman. Detection of anabolic steroid administration: Ratio of urinary testosterone to epitestosterone vs. the ratio of urinary testosterone to luteinizing hormone. *Clin. Chem.* **43:** 731-735 (1997).

147. K. Carlström, E. Palonek, M. Garle, H. Oftebro, J. Stanghelle and I. Björkhem. Detection of testosterone administration by increased ratio between serum concentrations of testosterone and 17α-hydroxyprogesterone. *Clin. Chem.* **38:** 1779-1784 (1992).

148. K. Carlström, M. Garle, E. Palonek, H. Oftebro, J. Stanghelle, C. Gottlieb and I. Björkhem. Serum assays in the detection of anabolic/androgenic steroid doping. In *Blood samples in doping control,* P. Hemmerbach and K.I. Birkeland, Eds. Pensumtjeneste, Oslo, 1994, pp 93-100.

149. E. Palonek and M. Garle. Single injection of testosterone to 7 volunteers: Results from this study. In *Proceedings of the 10th Cologne workshop on dope analysis*, M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke and S. Rauth, Eds. Sport und Buch Strauss, Köln, 1993, pp 131-139.

150. D.E. Matthews and J.M. Hayes. Isotope-ratio-monitoring gas chromatography-mass spectrometry. *Anal. Chem.* **50:** 1465-1473 (1978).

151. M. Becchi, R. Aguilera, Y. Farizon, M.-M. Flament, H. Casabianca and P. James. Gas chromatography/combustion/isotope-ratio mass spectrometry analysis of urinary steroids to detect misuse of testosterone in sport. *Rap. Comm. Mass Spectrom.* **8:** 304-308 (1994).

152. B.N. Smith and S. Epstein. Two categories of $^{13}C/^{12}C$ ratios for higher plants. *Plant Physiol.* **47:** 380-384 (1971).

153. R. Aguilera, M. Becchi, H. Casabianca, C.K. Hatton, D.H. Catlin, B. Starcevic and H.G. Pope Jr. Improved method for detection of testosterone abuse by gas chromatography/combustion/isotope ratio mass spectrometry analysis of urinary steroids. *J. Mass Spectrom.* **31:** 169-176 (1996).

154. R. Aguilera, M. Becchi, C. Grenot, H. Casabianca and C.K. Hatton. Detection of testosterone misuse: Comparision of two chromatographic sample preparation methods for gas chromatographic-combustion/isotope ratio mass spectrometric analysis. *J. Chromatogr. B* **687:** 43-53 (1996).

155. S. Horning, H. Geyer, M. Machnik, W. Schänzer, A. Hilkert and J. Oeβelmann. Detection of exogenous testosterone by $^{13}C/^{12}C$ analysis. In *Recent advances in doping analysis (4)*, W. Schänzer, H. Geyer, A. Gotzmann and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, 1997, pp 275-284.

156. C.H.L. Shackleton, A. Phillips, T. Chang and Y. Li. Confirming testosterone administration by isotope ratio mass spectrometric analysis of urinary androstanediols. *Steroids* **62:** 379-387 (1997).

157. C.H.L. Shackleton, E. Roitman, A. Phillips and T. Chang. Androstanediol and 5-androstenediol profiling for detecting exogenously administered dihydrotestosterone, epitestosterone, and dehydroepiandrosterone: Potential use in gas chromatography isotope ratio mass spectrometry. *Steroids* **62:** 665-673 (1997).

158. S. Horning, H. Geyer, A. Gotzmann and W. Schänzer. Detection of exogenous steroids by $^{13}C/^{12}C$ analysis. In *Recent advances in doping analysis (5)*, W. Schänzer, H. Geyer, A. Gotzmann and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, 1998, pp 135-148.

159. R. Aguilera, D.H. Catlin, M. Becchi, A. Philips, C. Wang, R.S. Swerdloff, H.G. Pope and C.K. Hatton. Screening urine for exogenous testosterone by isotope ratio mass spectrometric analysis of one pregnanediol and two androstanediols. *J. Chromatogr. B* **727:** 95-105 (1999).

160. X. de la Torre, J.C. Gonzalez, S. Pichini, J.A. Pascual and J. Segura. Screening procedure of testosterone and androgen abuse by IRMS. To be published in *Recent advances in doping analysis (7)*, W. Schänzer, H. Geyer, A. Gotzmann and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, 2000.

161. U. Flenker and W. Schänzer. A screening procedure for synthetic testosterone and its precursors based on GC/C/IRMS. To be published in *Recent advances in doping analysis (7)*, W. Schänzer, H. Geyer, A. Gotzmann and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, 2000.

162. H. Sachs and P. Kintz. Testing for drugs in hair. Critical review of chromatographic procedures since 1992. *J. Chromatogr. B* **713:** 147-161 (1998).

163. D. Thieme, J. Grosse, H. Sachs and R.K. Mueller. Detection of several anabolic steroids of abuse in human hair. In *Recent advances in doping analysis (6)*, W. Schänzer, H. Geyer, A. Gotzmann and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, 1999, pp 9-29.

164. M.J. Wheeler, Y.-B. Zhong, A.T. Kicman and S.B. Coutts. The measurement of testosterone in hair. *J. Endocrinol.* **159:** R5-R8 (1998).

165. Y.-C. Ma and H.-Y. Kim. Determination of steroids by liquid chromatography/mass spectrometry. *J. Am. Soc. Mass Spectrom.* **8:** 1010-1020 (1997).

166. Sanaullah and L.D. Bowers. Direct measurement of testosterone and epitestosterone glucuronates and sulfates with HPLC/MS and HPLC/MS/MS. In *Recent advances in doping analysis (3)*, W. Schänzer, H. Geyer, A. Gotzmann and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, 1996, pp 271-275.

167. L.D. Bowers, Sanaullah. Direct measurement of steroid sulfate and glucuronide conjugates with high-performance liquid chromatography-mass spectrometry. *J. Chromatogr. B* **687:** 61-68 (1996).

168. L.D. Bowers. Analytical advances in detection of performance-enhancing compounds. *Clin. Chem.* **43:** 1299-1304 (1997).

2

THE INFLUENCE OF ETHANOL CONSUMPTION ON THE T/E RATIO

ABSTRACT

In doping analysis the ratio of testosterone (T) glucuronide versus epitestosterone (E) glucuronide (T/E ratio) is applied for the detection of T administration. It is well established that ethanol consumption also increases the T/E ratio. However, reported observations of such increased T/E ratios are mostly limited to the application of high dose levels of ethanol (~2 g/kg bodyweight).

In this experiment a dose of 1.2 g ethanol per kg bodyweight was administered to eight male and eight female subjects. Collected urine and plasma samples were analyzed for T, E and several precursors and metabolites of T. Two separate conjugate fractions were analyzed (I: sulfates, glucuronides and non-conjugated steroids; II: glucuronides and non-conjugated steroids).

Plasma concentrations of T decreased significantly in case of males, whereas sex hormone binding globulin increased. No significant effect was observed on plasma E and luteinizing hormone. Excretion rates of T and androstanediols increased, while those of androsterone and etiocholanolone decreased. Excretion rates of E, dehydroepiandrosterone and androst-5-ene-3β,17β-diol were relatively stable. Based on intra- and interindividual data, the T/E ratio significantly increased ($p<0.05$) in both males and females, in both studied fractions. The increase was observed during the first 10-12 hours after start of consumption. Female subjects showed a significantly larger effect than male subjects. The maximum detected T/E ratio after administration showed positive (inter-subject) correlation with the mean pre-administration T/E ratio. It was concluded that the chance of a T/E ratio increasing above the International Olympic Committee criterion of six is realistic and should be kept in mind for especially out-of-competition doping controls.

The cause of T/E increase could not be explained by one specific mechanism. The simultaneous use of cofactors for the enzymatic activity in steroid and ethanol metabolism seems to play a predominant role, but is not completely understood.

INTRODUCTION

Since its introduction by Donike *et al.* [1], the ratio of testosterone glucuronide vs. epitestosterone glucuronide (T/E ratio) as determined in urine is one of the established methods in doping analysis to detect testosterone (T) administration. A T/E ratio above six is considered as an indication of T abuse. In 1988 Falk *et al.* [2] reported an increase of the T/E ratio in male subjects due to the consumption of ethanol. Also, published cases have appeared of athletes who had an incidental T/E ratio larger than six, that was claimed to be caused by ethanol consumption [3,4].

So far, published studies show a larger effect of ethanol administration on the T/E ratio of females as compared to males [3,5-6]. It can be expected that this difference is related to the relatively large contribution of peripheral conversion of androst-4-ene-4,17-dione (Δ4-AEDIONE) to the total female T production [5].

Still limited knowledge is available about the relationship between ethanol and the T/E ratio. Several suggestions have been made, such as alteration of NAD^+ dependent steroid biosynthetic and metabolic pathways by competition with ethanol metabolism, ethanol inhibition of T metabolism and hypothalamic-pituitary-adrenal (HPA) axis stimulation [2,5]

The effects of ethanol consumption on the T/E ratio have mostly been observed applying relatively high dose levels of between 1.5 and 2.0 g/kg bodyweight [2,5,6]. Dose levels of around 1 g/kg were considered as too low to result in a significant increase [2,5]. This was, however, contradicted by Axelson *et al.* [7] who observed significant effects in steroid metabolism at a dose level of 0.3 g/kg. The lack of observed effect could have been caused by wrong timing of sample collection, as the first samples were taken at 8-10 hours after the administration period [2,5]. Seppenwoolde-Waasdorp *et al.* [3] showed that the maximum effect can be expected within 8 hours after ethanol administration. A significant effect was observed for both males and females in this study, applying a dose of 1 g/kg bodyweight. Additionally, Mareck-Engelke *et al.* [6] illustrated that the T/E ratio immediately increases after ethanol is detected in urine. Therefore, frequent urine sampling should be applied, starting before or immediately after administration of the first dose of ethanol [6].

In this study a dose of 1.2 g/kg was administered to 8 male and 8 female subjects in a controlled trial setting. A steroid profile of T and E and several precursors and metabolites were analyzed in urine and plasma. Emphasis was placed on the maximum effect that can be expected for the T/E ratio. Aspects of first and second phase metabolism were considered to obtain additional fundamental information about the endocrine disruption.

EXPERIMENTAL

Chemicals

5α-Androstan-3α-ol-17-one (androsterone, AO), 5β-androstan-3α-ol-17-one (etiocholanolone, EO), androst-4-ene-17α-ol-3-one (epitestosterone, E), 5α-androstane-3α,17β-diol (5α,3α-ADIOL), 5α-androstane-3β,17β-diol (5α,3β-ADIOL), 5β-androstane-3α,17β-diol (5β,5α-ADIOL), androst-5-ene-3β,17β-diol (Δ5-AEDIOL), androst-4-ene-3,17-dione (Δ4-AEDIONE), dehydroepiandrosterone-sulfate (DHEA-S), androsterone-sulfate (AO-S) and androsterone-glucuronide (AO-G) were purchased from Sigma (St. Louis, USA). Androst-4-ene-17β-ol-3-one (testosterone, T) was purchased from Organon (Oss, The Netherlands). Androst-5-ene-3β-ol-17one (dehydroepiandrosterone, DHEA) was purchased from Schering (Germany). The internal standard was a solution of [$^2H_4$]-testosterone (d_4-T), [$^2H_4$]-etiocholanolone (d_4-EO) and 17α-methyltestosterone (MeT) in methanol, and was kindly provided by M. Donike. β-Glucuronidase from *E. coli* (Type VIII-A Purified; 50% glycerol solution; 5.000-20.000 kIU/g) was purchased from Boehringer-Mannheim (Mannheim, Germany). Trimethylchlorosilane (TMCS, 99%), *N*-methyl-*N*-trimethylsilyltrifluoracetamide (MSTFA), isopropanol and ethanethiol (97%) were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Ammonium iodide (NH_4I) was purchased from Fluka Chemie (Buchs, Switzerland). Sodium carbonate, anhydrous phosphorus pentoxide (P_2O_5), potassium hydroxide (KOH), potassium dihydrogenphosphate (KH_2PO_4), sodium acetate trihydrate, acetic acid (96%), diethyl ether and methanol (analytical grade) were purchased from Merck (Darmstadt, Germany). All chemicals were of analytical grade unless indicated otherwise.

Columns for solid-phase extraction were IST Isolute octadecyl (C_{18}) columns (200 mg, non-endcapped) obtained from Sopachem, Lunteren, The Netherlands.

Study design and sample collection

In this study 16 healthy volunteers (8 males and 8 females), in the age of 18-25 years, participated. Informed consent was obtained, and a detailed study protocol was approved by the Medical Ethical Committee of the University of Utrecht. The volunteers were not allowed to use or recently have used medication. Smokers and female contraceptive users were excluded. The weight of the volunteers had to be within 15% of ideal body weight, as defined in the "Metropolitan Life Insurance Table". The average daily ethanol consumption was between one and three units, to limit ethanol habituation. No ethanol was consumed during at least 24 hours before start of the study. Diets were standardized during the trial.

The complete trial lasted 2.5 days, the first day being a control day. During this day no ethanol was consumed. During the second day, between 9 a.m. and 12 a.m., 1.2 g/kg body weight ethanol was ingested as a 20% mixture with orange juice. On the control day, the same amount of orange juice was used between 9 a.m. and 12 a.m. Urine samples were taken every 2 hours and overnight. Blood samples were collected every 4 hours on the control day and every 2 hours during the rest of the experiment. Time of sample collection and the urine void volume were recorded. Blood samples were taken in heparinized collection tubes and centrifuged at 1200 g during 10 minutes immediately after collection. The resulting plasma was collected in glass tubes and was stored at -20°C until time of analysis. Of each urine sample, 100 ml were collected and also stored at -20°C until time of analysis. During sample analysis, samples of one subject were always combined in one batch of analyses.

Sample preparation

Protocol I: Combined fraction of conjugated and non-conjugated steroids in urine

A C_{18} solid-phase extraction (SPE) column was conditioned by 2 times 2 ml of methanol and equilibrated with 2 ml of distilled water, taking care to ensure that the solid phase remained solvated. The density (d) of the urine samples was measured. To an aliquot urine (8 ml for $d<1.005$, 4 ml for $1.005 \leq d < 1.010$ and 2 ml for $d \geq 0.010$), 50 µl of the internal standard solution was added. After vortexing, each sample was introduced into an SPE cartridge. After washing the column with 2 ml of distilled water, the solid phase was dried by allowing air to flow through the cartridge for at least 2 minutes. The sample was then eluted with 2 ml of methanol and the extract was evaporated to dryness at 55°C under a stream of nitrogen.

After SPE the collected steroid conjugates were hydrolyzed in order to obtain the de-conjugated steroids. The dried extract was reconstituted in 1 ml of TMCS in methanol (1 M) and methanolysis was performed at 55°C for 1 hour. The applied methanolysis method was based on a method described by Dehennin *et al.* [8], in which deconjugation occurs under acid conditions in a water free medium. In the applied mixture of TMCS and methanol, hydrochloric acid (present as $[CH_3OH_2]^+Cl^-$) is produced that cleaves both glucuronides and sulfates from the steroid nucleus.

After incubation, the mixture was evaporated to dryness at 45°C under a stream of nitrogen and subsequently 1 ml of phosphate buffer (0.2 M, pH 7.0) was added to the residue.

Each buffer solution was extracted with 5 ml of diethyl ether by subsequent mixing for 5 minutes and centrifugation (4000 rpm, 5 min). The organic phase was collected and evaporated to dryness under a stream of nitrogen at 40°C. Any residual water was removed by further drying over P_2O_5/KOH in a vacuum exsiccator overnight.

The steroids were converted to trimethylsilylenol-trimethylsilyl (TMS-enolTMS) derivatives by incubation of the dried extract in 50 µl of a mixture of MSTFA/NH_4I/ethanethiol (1000:2:3; v/w/v) at 80°C for 30 min (see also Chapters 8 and 9). The derivatization mixture was transferred to glass vials and injected into the gas chromatography-mass spectrometry (GC-MS) system.

Protocol II: Fraction of glucuronides and non-conjugated steroids in urine

SPE was applied in the same way as Protocol I. To the dried extract, 1 ml of phosphate buffer (0.2 M, pH 7.0) and 50 µl of β-glucuronidase solution, that was 10 times diluted with the same buffer, were added and vortexed. Selective hydrolysis of glucuronides was performed for 30 min at 55°C. Extraction, drying and derivatization was the same as in Protocol I.

Protocol III: Analysis of hormone parameters in plasma

T was measured after diethyl ether extraction using an in house competitive radio-immunoassay employing a polyclonal antitestosteron-antibody (Dr. Pratt AZG 3290). [1α,2α-^{3}H(N)]-Testosteron (NET-387, DuPont NEN Nederland BV) was used as a tracer following chromatographic verification of its purity. The lower limit of detection was 0.24 nM and inter-assay variation was 7.6, 5.1, and 8.1% at 0.76, 2.26 and 13.2 nM, respectively (n=20).

Sex hormone binding globulin (SHBG) was measured using the immunometric assay SPECTRIA SHBG IRMA (68562, Orion Diagnostica, Espoo, Finland). The lower limit of detection was 5 nM and inter-assay variation was 3.4, 4.0 and 3.2% at 15, 41 and 100 nM respectively (n=20).

Luteinising Hormone (LH) was measured using the Enzymun Test LH (Roche Diagnostics Nederland BV, Almere, The Netherlands) using the ES-300 Analyser. Details about this method have been published previously [9].

Protocol IV: Analysis of ethanol in plasma samples

A volume of 1 µl plasma was directly injected (splitless) into a GC with flame ionization detection (FID), with isopropanol as internal standard (at a concentration of 1‰). The injector temperature was 250°C and the splitless time was 0.5 min. Helium was used as carrier gas with a pressure of 15 psi. The temperature of the column was at 60°C with a run-time of 4.5 min. A calibration curve of 5 data points was used for calibration. Calibration standards were spiked to blank plasma and included in the

analytical procedure. Sample analysis was performed within one week after collection.

Gas chromatography and mass spectrometry

For the analysis of the plasma samples a Varian Aerograph GC-FID was used with a 10% Halcomid M18 on Chromosorb WAW 80-100 mesh column (length 1.8 m, inner diameter 4.6 mm).

For the analysis of the steroids in urine samples a GC-MS system was used, that existed of a Hewlett Packard gas chromatograph (Model 5890, Agilent Technologies, Waldbronn, Germany) coupled to a Hewlett Packard quadrupole mass spectrometer (Model 5972A). The MS was used in selected ion monitoring mode (SIM) and electron ionization was applied at 70 eV. Gas chromatography was performed with a HP-1 Ultra column (length 18 m, inner diameter 0.20 mm, film thickness 0.11 μm). Via electronic pressure control the column flow (helium) was constant at 1 ml/min. Sample injection of 1 μl was performed in split mode (ratio 1/10). A Hewlett Packard autosampler (Model 7673, Agilent Technologies, Waldbronn, Germany) was used for auto-injection. The injector temperature was set to 250°C. The oven temperature program used was: initial temperature 180°C, 2°/min to 225°C, 30°/min up to 310°C, held for 5 min. The interface temperature was set to 280°C.

Calibration and quantification

Calibration samples were prepared by adding 100 μl of methanolic standard solutions to 4 ml demineralized water samples. These samples were included in the clean-up procedure with the urine samples. For quantification the following ions were monitored: AO and EO at m/z 434; T, E and DHEA at m/z 432; Δ4-AEDIONE at m/z 430; Δ5-AEDIOL at m/z 239; 5α,3α-ADIOL, 5α,3β-ADIOL and 5β,3α-ADIOL at m/z 241. As qualifier ions were monitored: AO and EO at m/z 419; T, E and DHEA at m/z 417; Δ4-AEDIONE at m/z 415; Δ5-AEDIOL at m/z 434; 5α,3α-ADIOL, 5α,3β-ADIOL and 5β,3α-ADIOL at m/z 256. Calibration curves contained six data-points, that covered the respective ranges: AO (100-6000 ng), EO (40-2400 ng), T (5-250 ng), E (5-250 ng), Δ4 AEDIONE (5-250 ng), DHEA (100-4400 ng), Δ5-AEDIOL (50-1500 ng), 5α,3α-ADIOL (25-1000 ng), 5α,3β-ADIOL (10-500 ng), 5β,3α-ADIOL (25-2500 ng). A check for possibly incomplete derivatization, caused by the presence of residual water in the derivatization mixture, was detected by qualitative monitoring of mono-TMS-derivatives of AO and EO at m/z 362 and 272. When significant mono-derivatives were detected as compared to fully derivatized AO and EO, the sample analysis was rejected.

A mixture of deuterium labeled T (d_4-T, detected at m/z 436), EO (d_4-EO, m/z 438) and non-labeled MeT (detected at m/z 301) in methanol was used as internal standard solution. Exact concentrations and location of the deuterium atoms were unknown. d_4-T was used for the quantification of T, E and Δ4-AEDIONE in Protocol I, whereas MeT was used for the same steroids in Protocol II. d_4-EO was used as internal standard for all other steroids in both protocols.

The area ratio of T/d_4-T was corrected, because the internal standard solution used was contaminated with small amounts of interfering non-deuterated T. No interference by the signal of d_4-EO on the area of EO was detected. However, as large quantities of EO are present in urine samples, there was some interference by the signal at m/z 438 of non-deuterated EO on the area of d_4-EO. As this was reproducible a correction was performed on the measured area ratio of EO/d_4-EO.

Validation experiments

Conversion of deconjugation was studied by hydrolysis of synthetic DHEA-sulfate (DHEA-S), AO-sulfate (AO-S) and AO-glucuronide (AO-G) with the methods of Protocol I and II. Therefore, 1 µg of the studied steroids was subjected to the respective hydrolysis procedures. Quantification was applied with calibration samples that were included in the same sample clean-up procedure.

The recovery of the extraction procedure was determined by processing spiked water samples of 2 ml at low and high concentration levels with Protocol II (excluding hydrolysis procedure). Recovery was calculated by comparison of the area response in the studied samples with the response of standards that were directly derivatized.

Stability of the studied steroid derivatives in the reagent at room temperature was assessed by repeated analysis of a 2 ml urine sample that had been processed by Protocol I.

Precision and accuracy were based on quality control (QC) samples in water at low, medium and high concentration levels of the calibration curve. For precision also a batch of blank urine was used as QC sample.

Statistical analysis

Student's one-tailed paired t-test was used for evaluation of interindividual mean plasma results, differences in mean excretion rates and mean steroid ratios with $p<0.05$ considered as significant. Mean values were calculated over the period 9 a.m. - 11 p.m. Differences between male and female T/E ratios and intraindividual

T/E ratios [10-12] were tested with Student's one-tailed non-paired t-test, with $p<0.05$ considered as significant.

RESULTS

Validation experiments

β-Glucuronidase hydrolysis for 30 min at 55°C resulted in around 90% conversion of AO-G and no conversion of DHEA-S and AO-S. Methanolysis resulted in an approximate 90% conversion of AO-G and 100% conversion of DHEA-S and AO-S. The extraction recovery determined was > 80% for all steroids studied.

After 20 hours a gradual decrease of area response occurred for all steroids and internal standards to around 80% at 60 hours after derivatization. This did however not significantly affect the area ratio of the steroids vs. the respective internal standards.

The intra- and inter-day precision (both n=5) as determined from water and blank urine samples was < 15% except for Δ4-AEDIONE (< 30%) in case of Protocol I and II. The accuracy was between 80 and 120%, except for AO, EO, Δ4-AEDIOL and Δ4-AEDIONE (between 70 and 130%). The relatively low accuracy of AO and EO can be explained by column overloading. Due to efficiency reasons, no re-analysis of AO and EO was performed after dilution. Results of Δ4-AEDIONE should be considered as indicative, as the accuracy and precision were limited and concentrations in urine samples were always low and often subjected to chromatographic interference.

Plasma analysis

The results of the plasma analyses are summarized in Table 1. Mean plasma concentrations did not significantly change except for a decrease in plasma T and increase in SHBG for males. No significant changes in females were established.

Urine analysis

The excretion data are summarized in Tables 2-4. In general, several changes in the steroid profile were observed after administration of ethanol. T excretion increased while E remained stable or even decreased. This lead to an increased mean T/E ratio. The increase of T excretion was only statistically significant in case of females

in the total and glucuronide fraction. The increase of the mean T/E ratio was significant for both males and females.

Table 1: *Mean plasma concentrations (± SEM) of endocrinological parameters*

Hormone in plasma	Unit	Day 1	Day 2
Males			
Testosterone	nmol/l	19.05 ± 6.65	17.03 ± 5.77 *
Epitestosterone	nmol/l	1.54 ± 0.66	1.27 ± 0.42
LH	IU/l	5.91 ± 1.94	5.75 ± 1.79
SHBG	nmol/l	23.86 ± 6.41	26.57 ± 7.98 **
Females			
Testosterone	nmol/l	1.60 ± 0.61	1.86 ± 0.91
Epitestosterone	nmol/l	0.77 ± 0.42	0.75 ± 0.33
LH	IU/l	4.78 ± 1.42 [1]	4.93 ± 1.98 [1]
SHBG	nmol/l	53.63 ± 29.79	53.13 ± 30.56

* Significant decrease at day 2 ($p<0.05$)
** Significant increase at day 2 ($p<0.05$)
[1] n=6, as two female subjects were rejected because of a mid-cycle increased LH concentration.

AO and EO excretion decreased in males and females, while AO/EO remained constant. The decreased excretion was only statistically significant in case of males. The excretion of 5α,3α-ADIOL increased for both males and females in the glucuronide and total fraction. The increase in excretion rate of 5β,3α-ADIOL was only significant in case of females for both fractions. The 5α,3α-ADIOL/5β,3α-ADIOL ratio did not change significantly. 5α,3β-ADIOL could only be determined in the total fraction and in low concentration in the glucuronide fraction of males. No significant change in excretion rate was established.

Statistical evaluation of the mean excretion rate differences as determined for the Δ5-steroids DHEA and Δ5-AEDIOL in Protocol I was aggravated by the high inter-subject variation (50-100%). DHEA excretion decreased in case of females in the total fraction. In the glucuronide fraction the excretion rate of DHEA remained stable. The Δ5-AEDIOL/DHEA and T/Δ4-AEDIONE ratios could only be determined in the total fraction, due to the low concentration of Δ5-AEDIOL and Δ4-AEDIONE in the glucuronide fraction. Both Δ5-AEDIOL/DHEA and T/Δ4-AEDIONE increased significantly in case of males and females. In both studied fractions AO/5α,3α-ADIOL and EO/5β,3α-ADIOL significantly decreased for both sexes.

In Figures 1-4 and Table 4 the individual data are presented for the T/E ratio in the studied fractions. The data of maximum plasma ethanol concentrations are also presented in Table 4. The ethanol concentrations achieved were reproducibly around 1-2‰. In general, the T/E ratio was increased for around 10-12 hours after the first administration. As presented in Table 4, the difference between the mean T/E ratio of day 1 and the maximum T/E ratio of day 2 was statistically significant for both males and females. The effect of ethanol consumption was significantly higher for females than for males ($p=0.024$ for the total fraction and $p=0.007$ for the glucuronide fraction).

In Figure 5, the inter-subject correlation between the mean T/E ratio during the pre-administration day (day 1) and the maximum T/E ratio during the administration day (day 2) is shown for both fractions and sexes. A positive correlation is illustrated, for males in particular.

Table 2: *Mean excretion data (μg/hour) of the total fraction of non-conjugated steroids, sulfates and glucuronides. Mean (±SEM) values were calculated from 9 a.m. – 23 p.m.*

	males day 1	males day 2	females day 1	females day 2	
T	1.99 ± 1.5	2.42 ± 1.48	0.34 ± 0.20	0.50 ± 0.26	F
E	2.03 ± 0.29	2.01 ± 0.49	0.86 ± 0.44	0.71 ± 0.35	
AO	224.89 ± 66.51	180.60 ± 72.49	138.24 ± 92.21	102.56 ± 48.36	
EO	137.60 ± 37.70	106.15 ± 33.23	96.24 ± 49.16	76.77 ± 39.58	M
5α,3α-ADIOL	5.94 ± 2.12	10.51 ± 4.63	2.42 ± 1.93	4.08 ± 2.95	M,F
5α,3β-ADIOL	3.78 ± 2.20	4.02 ± 1.11	3.94 ± 3.10	6.09 ± 3.35	
5β,3α-ADIOL	11.88 ± 7.91	15.34 ± 5.53	1.69 ± 0.95	1.75 ± 1.14	F
DHEA	68.15 ± 57.37	80.68 ± 80.18	13.66 ± 6.72	11.32 ± 5.90	F
Δ5-AEDIOL	29.27 ± 13.58	45.36 ± 30.79	14.23 ± 9.93	18.46 ± 16.29	
Δ4-AEDIONE	0.97 ± 0.33	1.08 ± 0.58	0.83 ± 0.42	0.87 ± 0.34	
T/E	1.01 ± 0.73	1.32 ± 0.86	0.43 ± 0.24	0.79 ± 0.37	M,F
AO/EO	1.71 ± 0.50	1.74 ± 0.53	1.70 ± 1.59	1.72 ± 1.55	
5α/5β,3α-ADIOL	0.65 ± 0.30	0.72 ± 0.23	0.86 ± 0.86	0.86 ± 0.87	
AO/T	215.80 ± 210.01	130.79 ± 128.30	533.00 ± 521.27	276.49 ± 264.45	M,F
EO/T	162.19 ± 204.52	89.22 ± 107.07	427.85 ± 536.80	234.74 ± 289.34	
AO/E	113.22 ± 40.37	92.33 ± 35.94	151.96 ± 41.83	147.47 ± 27.69	M
EO/E	68.16 ± 18.57	54.30 ± 19.25	124.57 ± 66.89	120.41 ± 56.37	M
AO/5α,3α-ADIOL	38.76 ± 4.51	17.45 ± 3.28	58.20 ± 14.00	27.30 ± 6.19	M*,F*
EO/5β,3α-ADIOL	17.10 ± 12.17	7.79 ± 4.07	28.93 ± 13.15	13.14 ± 4.19	M,F*
Δ5-AEDIOL/DHEA	0.86 ± 0.76	1.11 ± 0.89	1.00 ± 0.46	1.51 ± 0.67	M,F
T/Δ4-AEDIONE	2.08 ± 1.33	2.39 ± 1.42	0.41 ± 0.16	0.60 ± 0.25	M,F

M = Statistical significant difference ($p<0.05$) in case of males
F = Statistical significant difference ($p<0.05$) in case of females
* $p<0.01$

Table 3: *Mean excretion data (µg/hour) of the fraction of non-conjugated steroids and glucuronides. Mean (±SEM) values were calculated from 9 a.m. – 23 p.m.*

	males day 1	males day 2	females day 1	females day 2	
T	2.27 ± 3.01	2.64 ± 2.27	0.46 ± 0.38	0.69 ± 0.48	F**
E	1.49 ± 0.77	1.15 ± 0.60	0.64 ± 0.30	0.53 ± 0.21	M
AO	169.97 ± 50.16	112.18 ± 33.05	98.56 ± 76.17	70.12 ± 34.48	M*
EO	137.31 ± 40.47	88.67 ± 22.88	94.67 ± 70.19	71.81 ± 38.57	M*
5α,3α-ADIOL	5.76 ± 2.20	8.24 ± 2.41	1.92 ± 1.81	3.58 ± 2.33	M,F
5α,3β-ADIOL	1.85 ± 2.48	1.68 ± 2.24	-	-	
5β,3α-ADIOL	13.13 ± 8.51	14.43 ± 6.37	3.43 ± 4.39	6.42 ± 5.72	F
DHEA	4.16 ± 1.21	3.90 ± 0.85	3.61 ± 2.11	3.76 ± 2.01	
Δ5-AEDIOL	-	-	-	-	
Δ4-AEDIONE	-	-	-	-	
T/E	2.13 ± 1.80	2.73 ± 2.31	0.78 ± 0.51	1.44 ± 0.95	M,F***
AO/EO	1.26 ± 0.24	1.27 ± 0.29	1.32 ± 1.32	1.32 ± 1.27	
5α/5β,3α-ADIOL	0.61 ± 0.35	0.65 ± 0.25	0.81 ± 0.84	0.78 ± 0.74	
AO/T	164.30 ± 195.52	126.59 ± 157.60	441.40 ± 674.82	245.45 ± 412.77	
EO/T	142.07 ± 181.40	96.94 ± 120.20	488.08 ± 916.80	290.15 ± 563.55	
AO/E	131.10 ± 58.29	119.16 ± 70.60	149.97 ± 74.45	133.15 ± 49.48	
EO/E	102.92 ± 37.71	92.69 ± 48.04	161.30 ± 104.68	140.81 ± 79.02	
AO/5α,3α-ADIOL	31.20 ± 3.08	14.34 ± 4.32	62.13 ± 41.06	21.64 ± 7.69	M*,F
EO/5β,3α-ADIOL	16.76 ± 13.39	7.55 ± 4.29	40.58 ± 37.05	13.03 ± 5.40	M,F
Δ5-AEDIOL/DHEA	NA	NA	NA	NA	
T/Δ4-AEDIONE	NA	NA	NA	NA	

M = Statistical significant difference ($p<0.05$) in case of males
F = Statistical significant difference ($p<0.05$) in case of females
\- = Concentration too low for quantification
NA = Not available
* $p<0.01$
** $p=0.016$
*** $p=0.010$ for females and $p=0.035$ for males

Table 4: *Mean T/E ratios for the total and glucuronide fraction in females (AM to SM) and males (BH to TT). Maximum T/E ratios were statistically significant ($p<0.05$) for all subjects as compared to the intraindividual T/E ratio data during day 1.*

female and male subjects	Maximum concentration ethanol [‰]	T/E mean day 1 total fraction	T/E max day 2 total fraction	Normalized difference total [%][1,2]	T/E mean day 1 gluconides	T/E max day 2 glucuronides	Normalized difference glucuronides [%][3,4]
AM	1.7	0.38 ± 0.09	1.40	268	0.75 ± 0.10	3.21	328
BS	1.3	0.09 ± 0.02	0.22	144	0.07 ± 0.02	0.30	329
GH	2.0	0.29 ± 0.03	0.84	190	0.51 ± 0.10	1.64	222
JD	1.1	0.41 ± 0.04	0.88	115	0.78 ± 0.16	1.07	37
JJ	1.0	0.70 ± 0.28	3.20	357	1.47 ± 0.52	3.81	159
SA	1.5	0.58 ± 0.12	1.32	128	0.74 ± 0.23	1.92	159
SG	1.3	0.76 ± 0.10	1.83	141	1.66 ± 0.16	3.25	96
SM	1.7	0.42 ± 0.11	1.30	210	0.53 ± 0.08	1.32	149
BH	1.1	0.95 ± 0.08	1.53	61	1.71 ± 0.20	2.30	35
BV	1.1	0.13 ± 0.01	0.21	62	0.12 ± 0.01	0.23	92
BZ	1.4	0.16 ± 0.03	0.32	100	0.27 ± 0.02	0.45	67
EO	1.3	1.70 ± 0.22	3.29	94	4.50 ± 1.75	8.86	97
MR	0.9	2.33 ± 0.17	2.94	26	6.71 ± 0.87	9.88	47
PH	1.1	0.82 ± 0.10	1.53	87	2.07 ± 0.70	3.83	85
RT	1.3	1.04 ± 0.11	1.73	66	1.98 ± 0.44	3.35	69
TT	1.0	0.97 ± 0.10	1.52	57	1.61 ± 0.21	2.55	58

1 p=0.008 for the difference between the mean T/E ratio during day 1 and the maximum T/E ratio of day 2 in case of males (total fraction)
2 p=0.006 for the difference in case of males (total fraction)
3 p=0.004 for the difference in case of females (glucuronide fraction)
4 p=0.022 for the difference in case of males (glucuronide fraction)

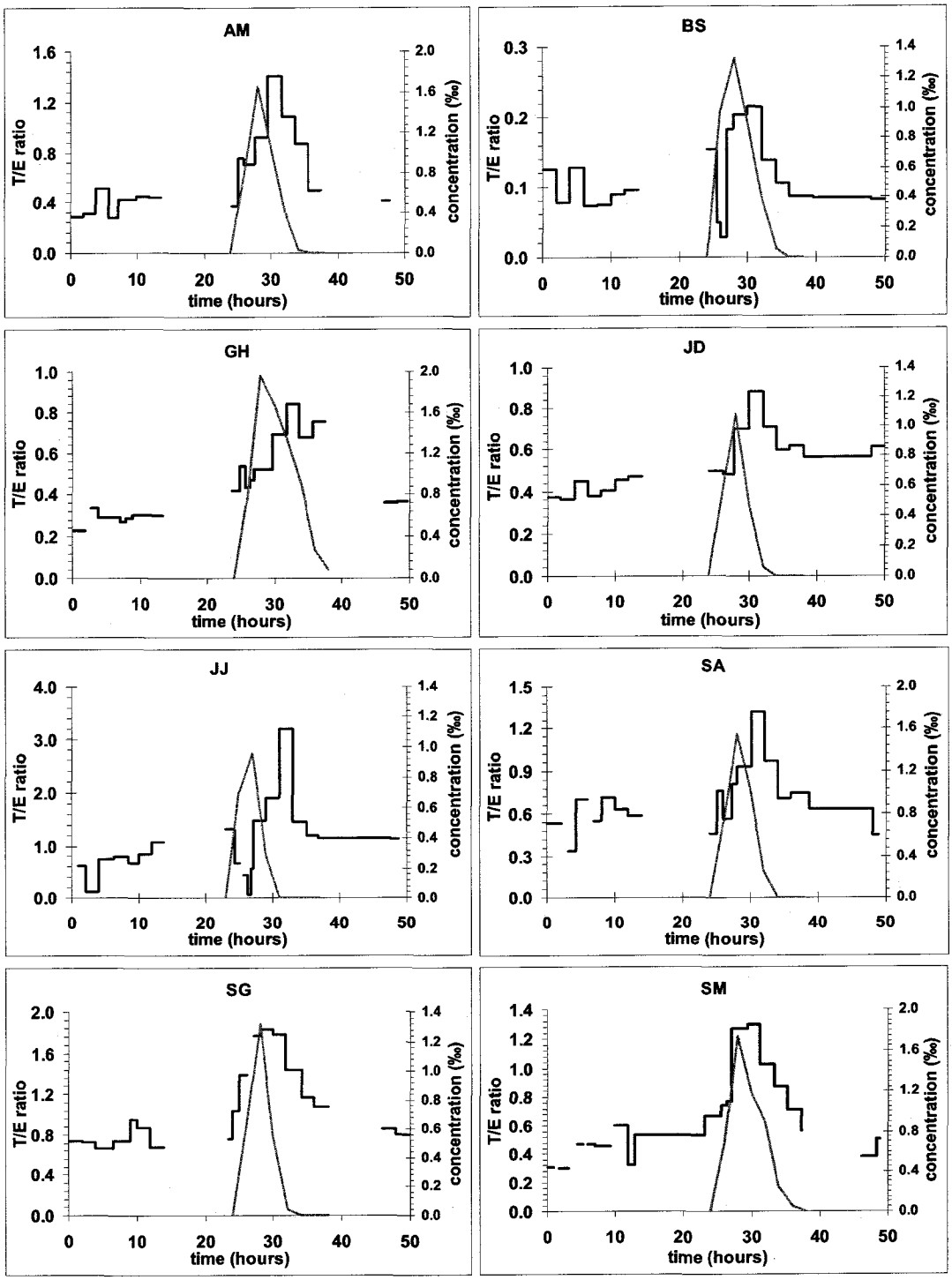


Figure 1: Total fraction of sulfates/glucuronides/non-conjugated steroids: T/E ratio in urine samples and plasma ethanol concentration in female subjects. Ethanol administration occurred between 24 and 27 hours.

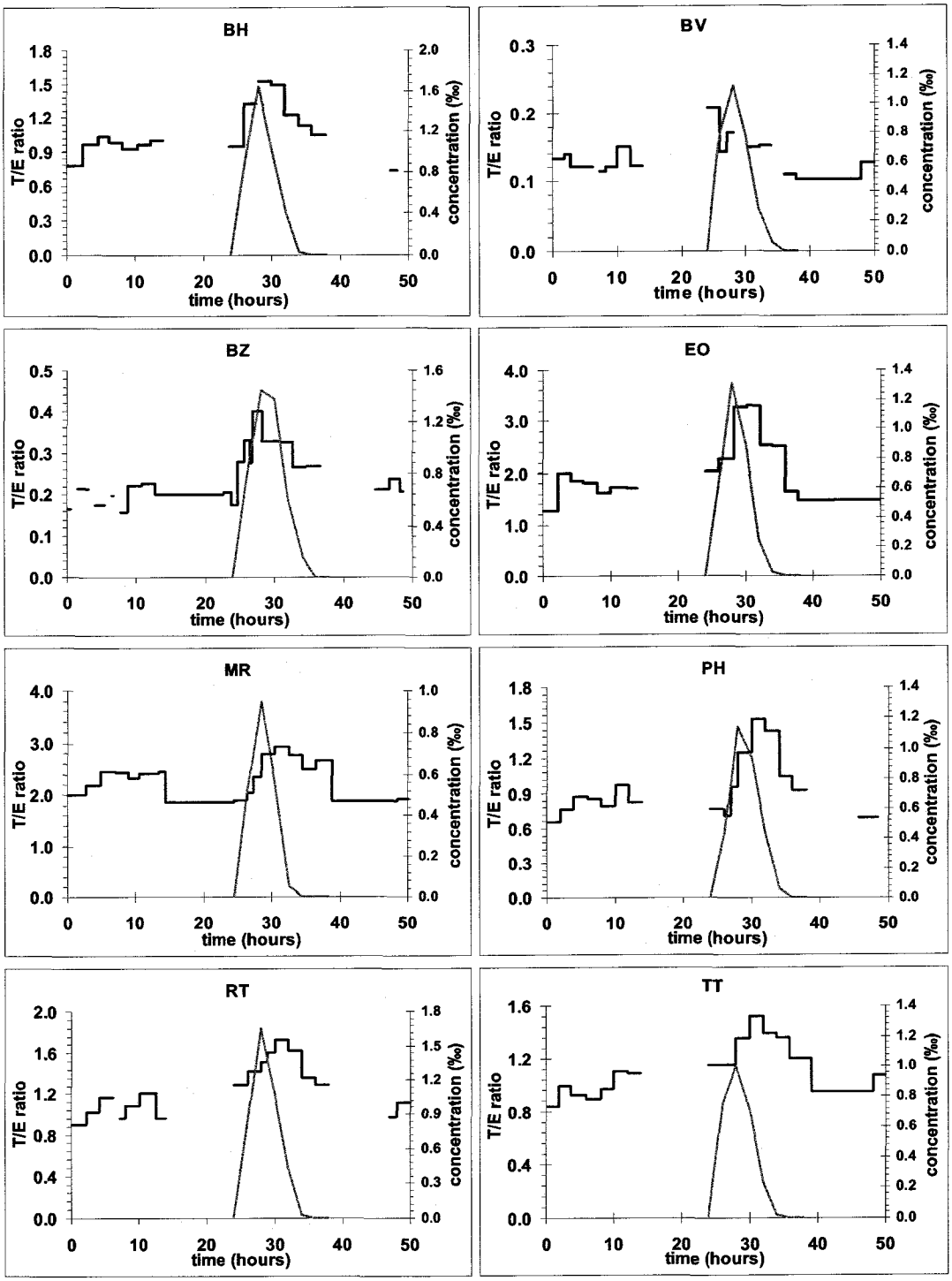


Figure 2: Total fraction of sulfates/glucuronides/non-conjugated steroids: T/E ratio in urine samples and plasma ethanol concentration in male subjects. Ethanol administration occurred between 24 and 27 hours.

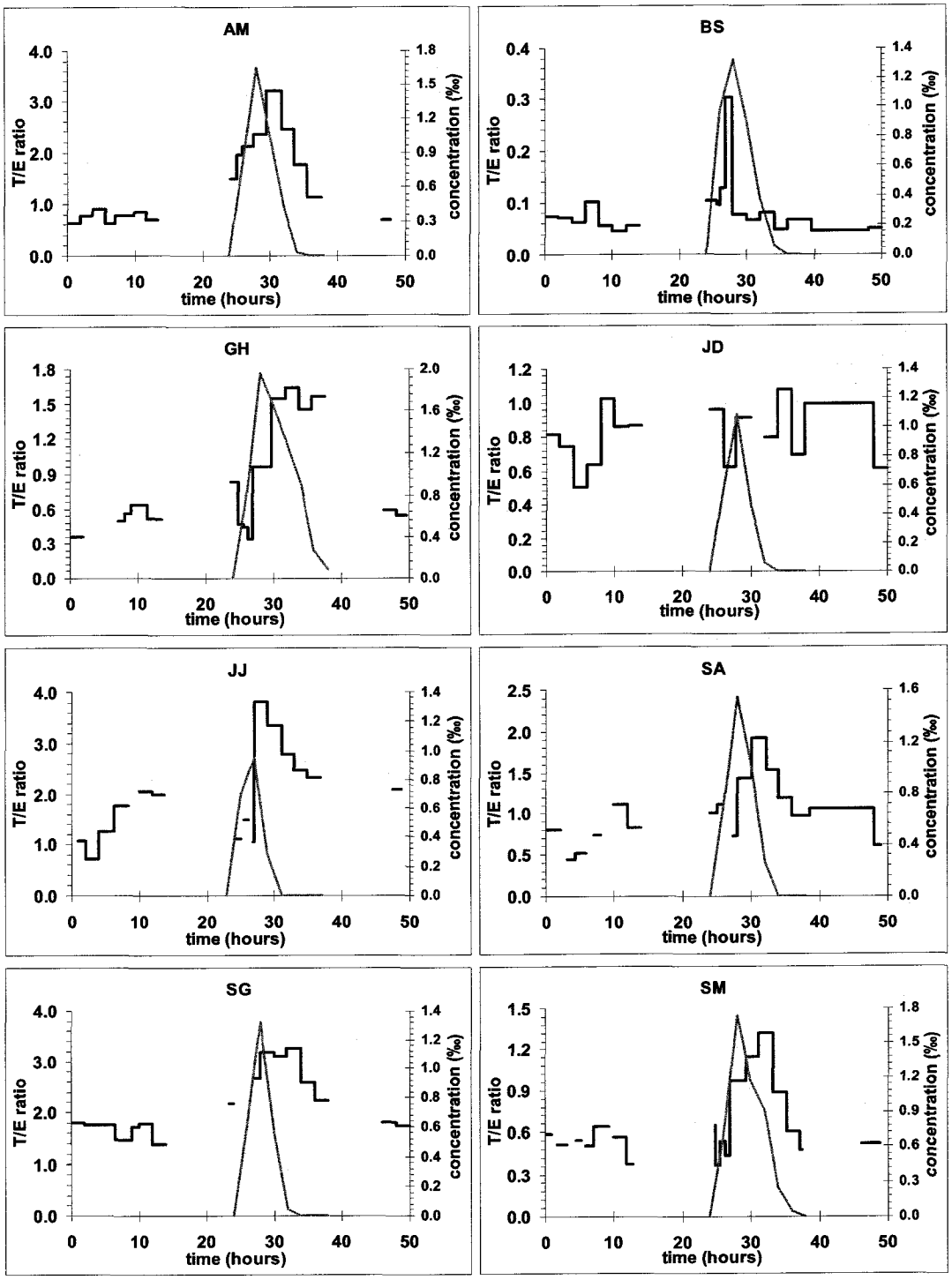


Figure 3: Fraction of glucuronides/non-conjugated steroids: T/E ratio in urine samples and plasma ethanol concentration in female subjects. Ethanol administration occurred between 24 and 27 hours.

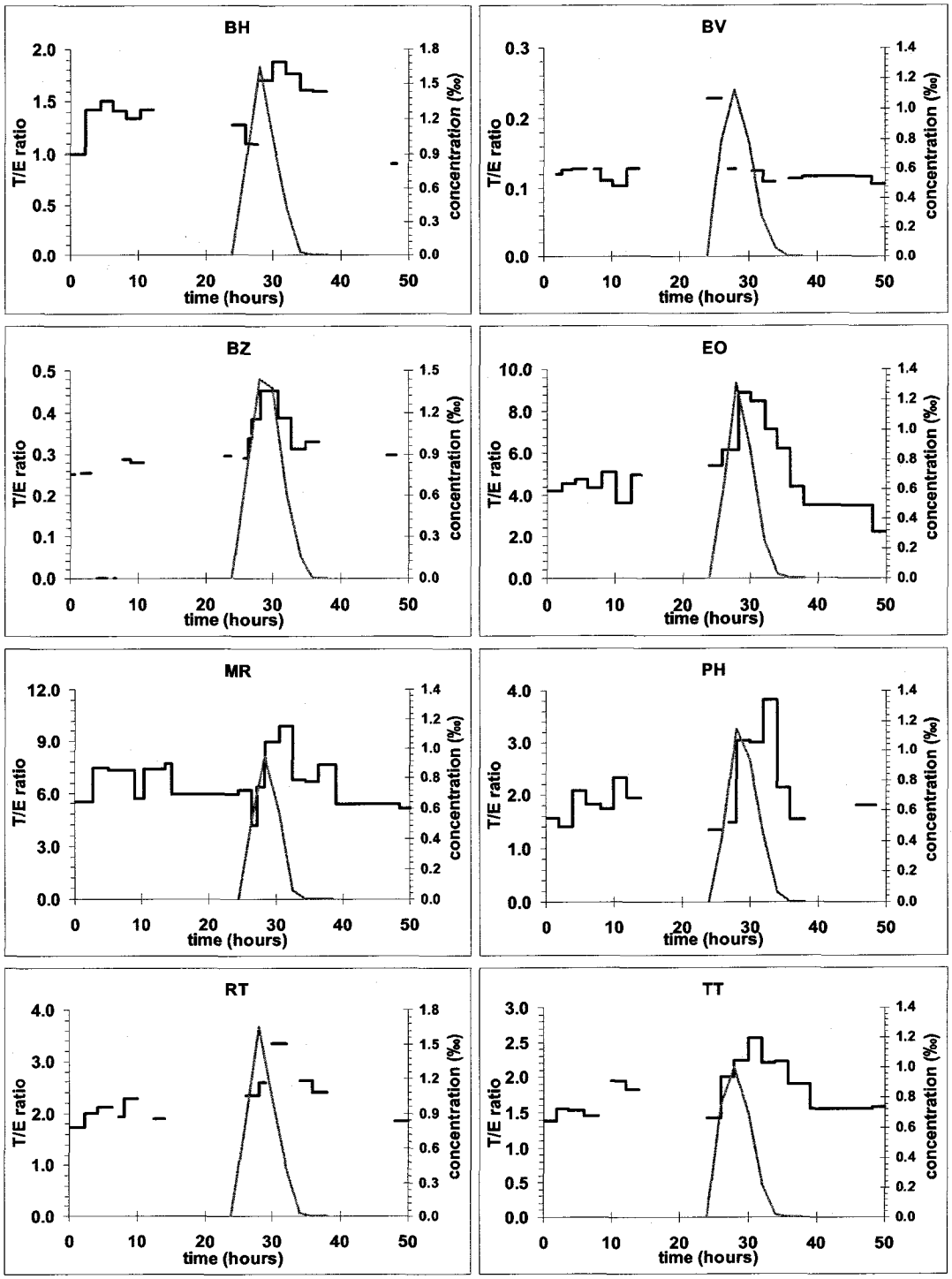


Figure 4: Fraction of glucuronides/non-conjugated steroids: T/E ratio in urine samples and plasma ethanol concentration in male subjects. Ethanol administration occurred between 24 and 27 hours.

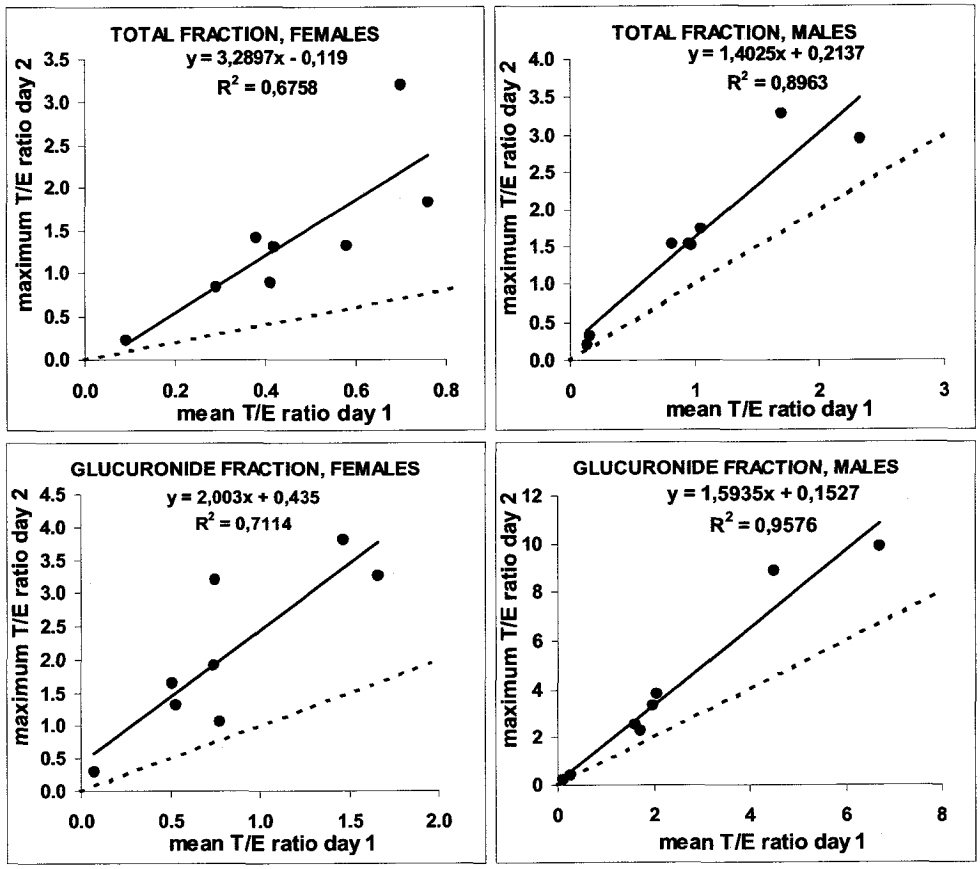


Figure 5: The inter-subject correlation between the mean T/E ratio during the pre-administration day (day 1) and the maximum T/E ratio detected during the administration day (day 2). The dotted line represents the "no-effect" line with a slope of 1.

DISCUSSION

Analytical method

Calibration samples were prepared in water. Attempted standard addition to urine samples resulted in bad reproducibility of the response and therefore in bad correlation of the calibration curves. In this experiment the main goal was to detect differences in excretion rates of endogenous steroids, related to basal levels. Therefore, some bias in accuracy was accepted and the main focus was placed on the within- and between-batch precision.

In this study, methanolysis and β-glucuronidase hydrolysis were used as methods to deconjugate the steroids under investigation. During validation it was shown that both methods resulted in high conversion of targeted deconjugation of synthetic DHEA-S, AO-S and AO-G. In the applied Protocols I and II these high conversions were combined with a high extraction recovery. However, during sample analysis the recovery of the glucuronide fraction was sometimes higher than of the total fraction in case of T, EO, 5α,3α-ADIOL and 5β,3α-ADIOL. Possibly, side reactions occurred during methanolysis. Therefore, interpretation of the results is mostly based on the separate results of Protocol I and II.

Effect of ethanol on T/E ratio

Our results clearly show a significant increase of the T/E ratio as a result of ethanol administration based on both intra- and interindividual data. Falk *et al.* [2] and Karila *et al.* [5] did not observe any significant effects applying dose levels of 1.0 and 1.2 g/kg bodyweight, respectively. Subsequently, significance was obtained for a dose level of 2 g/kg. However, in both studies the first urine sampling was performed 12-13 hours after the first volume of ethanol was administered. As described by Seppenwoolde-Waasdorp *et al.* [3] and confirmed by the present study, the maximum effect of ethanol on steroid excretion can be expected before 11 hours after the first ethanol intake. As described in Figures 1-4 the T/E ratio increased immediately after the first administration, and lasted until 8-12 hours afterwards. The maximum T/E ratio was detected mostly within 5 hours after the detected maximum ethanol concentration in plasma.

The results of Mareck-Engelke *et al.* [6] illustrate that 2 g/kg is a very high dose, as 7 out of 11 volunteers could not reach this target dose level because of nausea. However, when correctly timed sampling is performed, a dose level of 1.0-1.2 g/kg is sufficient to observe significant steroid excretion differences.

As can be observed in Figure 5, the maximum T/E ratio shows a positive correlation to the mean basal T/E ratios. The higher slope of the presented least squares regression lines in case of females, illustrates that the maximum effect on the T/E ratio as determined in Protocol I and II is larger for females than for males.

The lower urinary T and E concentrations in females, leading to a higher analytical variability, could explain the higher correlation for the male compared to the female subjects. The positive correlation shows that the effect of ethanol consumption on the T/E ratio can be more or less predicted, based on the intra-subject mean T/E ratio. Theoretically, this information could be used to preliminary judge claims made by athletes, that a detected high T/E ratio was caused by ethanol consumption.

Mechanistic considerations

Several hypotheses have been posed for the endocrine response to ethanol metabolism that can explain the alteration of the T/E ratio. As also described by others [5,13], increase of T production by LH stimulation is unlikely, as plasma LH concentrations were stable.

Steroid metabolism and ethanol oxidation are both NAD^+-dependent. As a result, oxidation of ethanol increases the $NADH/NAD^+$ ratio and thereby influences the metabolic conversion of steroids. The effect of ethanol metabolism on 17β-hydroxysteroid dehydrogenase is described as a shift of the 17β-hydroxy/17-keto ratio of redox coupled steroid conjugates towards the reduced steroid [14,15]. This effect is also reported for monosulfates of Δ5-AEDIOL/DHEA [16,17], as determined by plasma concentrations. The 17β-hydroxy/17-keto ratios as determined for non-conjugated steroids were not increased. Our results showed the shift of the 17β-hydroxy/17-keto ratio in urine by the significantly increased Δ5-AEDIOL/DHEA and T/Δ4-AEDIONE ratio (total fraction of conjugates) and the decreased AO/5α,3α-ADIOL and EO/5β,3α-ADIOL ratio (in both studied fractions). This clearly shows that ethanol administration significantly influences steroid transformation.

It is difficult to interpret how these metabolic changes could affect the T/E ratio, as it in not clear what role oxidoreduction of steroid conjugates plays in steroid biosynthesis and metabolism. Androst-5-ene-3β,17α-diol (Δ5-17α-AEDIOL) that is produced from DHEA, has been identified as biosynthetic precursor of E [18]. It is therefore likely that other redox coupled steroid conjugates, as for example Δ5-17α-AEDIOL/DHEA, are involved in E production as in T production. The same accounts for E and T metabolism. An explanation of the effect of ethanol metabolism on the T/E ratio by means of redox effects on steroid conjugates should therefore be considered as a realistic possibility.

Falk *et al.* [2] suggested that the T/Δ4-AEDIONE ratio will increase as result of an increased $NADH/NAD^+$ ratio during ethanol metabolism. Therefore, the inter-conversion of T to E through Δ4-AEDIONE was suggested to be blocked. Although an increased T/Δ4-AEDIONE ratio was obtained in our results, three arguments counter the statement of Falk *et al.* First, Andersson *et al.* [14] showed that the conjugates of T and Δ4-AEDIONE are not redox coupled, as they are sulfated or glucuronidated at the C^3- and C^{17}-position, respectively. Second, Donike *et al.* [1] introduced the T/E ratio as a way to detect T administration on the basis of low a inter-conversion of T and E. Third, according the hypothesis, the elevation of the T/E ratio should coincide with a decrease of E excretion. Our results do not indicate this.

Ethanol metabolism will also affect NAD^+ dependent glucuronidation, by competition with UDPglucose dehydrogenase for NAD^+ [19]. This does not occur for sulfation. As

a result, a decreased excretion of extensively glucuronidated steroids as T, E, AO and EO should occur. This was the case for excretion of AO and EO. The stable excretion rates of DHEA and Δ5-AEDIOL could also match this hypothesis. However, it cannot explain the increased T/E ratio as T is glucuronidated more extensively as E. Theoretically, an opposite effect on the T/E ratio is expected.

Illustrated by Cobb *et al.* [20] ethanol and particularly its metabolite acetaldehyde stimulate the hypothalamic-pituitary-adrenal (HPA) axis. In cases of severe and chronic ethanol abuse this can lead to clinical and biochemical features of Cushing's syndrome [21]. Characteristically, HPA stimulation results in adrenal hormone production and following elevated plasma concentrations of cortisol, DHEA-sulfate, DHEA and Δ4-AEDIONE. Karila *et al.* [5] posed the elevated T/E ratio to be caused by the metabolism of these adrenal steroids to T. However, our results did not show significantly elevated plasma T concentrations. These findings are confirmed by earlier published results [22]. Furthermore, as DHEA-sulfate is readily excreted, this should lead to elevated DHEA urine concentrations in Protocol I. Also increased production of DHEA is expected to lead to increased formation of AO and EO as main metabolites (see Chapters 4 and 6). As our results contradicted these expectations, it is unlikely that the activity of the HPA axis can explain the T/E alterations.

Direct inhibition of T metabolism, as suggested by Karila *et al.* [5] is expected to decrease AO and EO production, which is supported by the decreased excretion rates of these metabolites in the present experiment. However, the increased excretion rate of other reduced T metabolites (androstanediols) is in contradiction with this suggestion.

Also stimulating effects of ethanol metabolism on T metabolism are described. As suggested by Cronholm *et al.* [23] in an experiment with rats, coenzyme pools used in different reductions at C^3 of corticosterone and Δ4-AEDIONE are metabolically related to NADH formed in the ethanol dehydrogenase reaction. The results suggest that ethanol metabolism could stimulate reduction of 3-keto steroids as T and E. This hypothesis can explain reduced plasma T concentrations and an increased excretion of androstanediols independently from the metabolic route to AO and EO. However, decreased excretion of AO and EO, the relative stable E excretion and the increased T/E ratio can not be explained by this mechanism.

CONCLUDING REMARKS

Ethanol consumption leads to increased T/E ratios for several hours in case of both males and females. In females the maximum effect is higher than for males. This occurs also at a more realistic dose level as has so far been assumed. The chance of T/E ratios rising above the cutoff point of 6 is realistic, and should be kept in mind especially in case of out-of-competition doping controls. Additional screening of ethanol in urine samples can be considered to prevent false-positive cases of T abuse. The mechanism that causes the increase of the T/E ratio is unclear. However, the competition between ethanol metabolism and steroid biosynthesis and metabolism for enzyme cofactors appear to play a predominant role.

ACKNOWLEDGEMENT

The analytical participation of Prof. Dr. L. Starka and Dr. R. Hampl, who performed the analyses of epitestosterone in plasma, and the medical assistance of Dr. T. Brouwer are greatly acknowledged.

LITERATURE

1. M. Donike, K.-R. Bärwald, K. Klostermann, W. Schänzer and J. Zimmermann. Nachweis von exogenem Testosteron. In *Sport: Leistung und Gesundheit*, H. Heck, W. Hollmann, H. Liesen, R. Rost, Eds. Deutscher Ärzte Verlag, Köln, 1983, pp 293-298.
2. O. Falk, E. Palonek and I. Björkhem. Effect of ethanol on the ratio between testosterone and epitestosterone in urine. *Clin. Chem.* **34:** 1462-1464 (1988).
3. T.J.A. Seppenwoolde-Waasdorp, D. de Boer, H.M.J. van Engelen, A.D. Vrijdag and R.A.A. Maes. Evaluation of endogenous steroid profiles in urine (2). Effects of ethanol intake reinvestigated. In *Recent advances in doping analysis (3)*, M. Donike, H. Geyer, A. Gotzmann and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, 1996, pp 157-165.
4. A. Leinonen, T. Karila and T. Seppälä. An increased testosterone to epitestosterone ratio due to high doses of ethanol - a case report on a female powerlifter. In *Recent advances in doping analysis (3)*, M. Donike, H. Geyer, A. Gotzmann and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, 1996, pp 167-176.
5. T. Karila, V. Kosunen, A. Leinonen, R. Tähtelä and T. Seppälä. High doses of ethanol increase urinary testosterone-to-epitestosterone ratio in females. *J. Chromatogr. B* **687:** 109-116 (1996).

6. U. Mareck-Engelke, H. Geyer, U. Schindler, U. Flenker, R. Iffland and M. Donike. Influence of ethanol on steroid profile parameters. In *Recent advances in doping analysis (3)*, M. Donike, H. Geyer, A. Gotzmann and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, 1996, pp 143-155.

7. M. Axelson, T. Cronholm, B.-L. Sahlberg and J. Sjövall. Changes in the metabolic profile of steroids in urine during ethanol metabolism in man. *J. Steroid Biochem.* **14:** 155-159 (1981).

8. L. Dehennin, P. Lafarge, Ph. Dailly, D. Bailloux and J.-P. Lafarge. Combined profile of androgen glucuro- and sulfoconjugates in post-competition urine of sportsmen: a simple screening procedure using gas chromatography-mass spectrometry. *J. Chromatogr. B* **687:** 85-91 (1996).

9. J.H. Thijssen, W.G. Wood, A.C. Kessler, H.W. Griesser, O. Bauer, C. Bieglmayer, J. Eugui, J.L. Mirabel, H. Gaessler and K. Klinga. Multicenter evaluation of new enzyme-linked immunoassays of follitropin and lutropin in serum or plasma. *Clin. Chem.* **37:** 1257-1263 (1991).

10. E.K. Harris. Some theory of reference values. I. Stratified (categorized) normal ranges and a method for following an individual's clinical laboratory values. *Clin.Chem.* **21:** 1457-1464 (1975).

11. H.E. Solberg and R. Grasbeck. Reference values. *Adv.Clin.Chem.* **27:** 1-79 (1989).

12. M. Donike, S. Rauth, U. Mareck-Engelke, H. Geyer and R. Nitschke. Evaluation of longitudinal studies, the determination of subject based reference ranges of the testosterone/epitestosterone ratio. In *Recent advances in doping analysis*, M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke and S. Rauth, Eds. Sport und Buch Strauss, Köln, 1994, pp 33-39.

13. J.H. Mendelson, N.K. Mello, and J. Ellingboe. Acute ethanol intake and pituitary gonadal hormones in normal human females. *J.Pharmacol.Exp.Ther.* **218:** 23-26 (1981).

14. S.H. Andersson, T. Cronholm and J. Sjovall. Effects of ethanol on the levels of unconjugated and conjugated androgens and estrogens in plasma of men. *J. Steroid Biochem.* **24:** 1193-1198 (1986).

15. S. Andersson. Effects of Ethanol on androgens and estrogens in vivo. Karolinska Institutet, Stockholm, 1985.

16. T. Cronholm and J. Sjövall. Effect of ethanol metabolism on redox state of steroid sulfates in man. *Eur. J. Biochem.* **13:** 124-131 (1970).

17. T. Cronholm and J. Sjövall. Effect of ethanol on the concentrations of solvolyzable plasma steroids. *Biochim. Biophys. Acta* **152:** 233-236 (1968).

18. Dehennin, L. Secretion by the human testis of epitestosterone, with its sulfoconjugate and precursor androgen 5-androstene-3 beta,17 alpha-diol. *J. Steroid Biochem. Mol. Biol.* **44:** 171-177 (1993).

19. P. Moldeus, B. Andersson and A. Norling. Interaction of ethanol oxidation with glucuronidation in isolated hepatocytes. *Biochem.Pharmacol.* **27:** 2583-2588 (1978).

20. C.F. Cobb, D.H. van Thiel, M.F. Ennis, J.S. Gavaler and R. Lester. Is acetaldehyde an adrenal stimulant? *Curr. Surg.* **36:** 431-434 (1979).

21. R. Groote Veldman and A.E. Meinders. On the mechanism of ethanol-induced pseudo-Cushing's syndrome. *Endocr. Rev.* **17:** 262-268 (1996).

22. R. Ylikahri, M. Huttunen, M. Harkonen and H. Adlercreutz. Hangover and testosterone. *Br. Med. J.* **2:** 445 (1974).

23. T. Cronholm. Steroid metabolism in rats given (1-2H2)ethanol. Biliary metabolites of corticosterone and administered 4-androstene-3,17-dione. *Eur. J. Biochem.* **27:** 10-22 (1972).

PART II:

food supplements
and
steroid profiling

3

SPECIFIC DETECTION OF FOOD SUPPLEMENT STEROIDS

ABSTRACT

Since the nineties steroid containing food supplement products have become widely available. After the introduction of these products, a frequently occurring abuse has been suspected in the sports community. Steroids present in food supplements are 19C-steroids as dehydroepiandrosterone (DHEA), androst-4-ene-3,17-dione (Δ4-AEDIONE) and 19-norsteroids as 19-norandrost-4-ene-3,17-dione. In this chapter an overview is given on the metabolism of food supplement steroids with DHEA and Δ4-AEDIONE in particular.

The detection of DHEA or Δ4-AEDIONE abuse in doping analysis is usually based on easily available metabolic pathways to *e.g.* androst-5-ene-3β,17β-diol, testosterone, epitestosterone, androsterone, etiocholanolone and androstanediols. However, also oxygenation reactions form relevant routes of biotransformation. DHEA is mostly converted by respectively 7α-, 7β- and 16α-hydroxylase activity. Also 7-keto-metabolites can be detected. Oxygenating enzymatic activities related to Δ4-AEDIONE are 6β-, 7α-, 16α- and 16β-hydroxylase.

When Δ4-AEDIONE is converted to estrone by aromatase, small quantities of 19-hydroxy-androst-4-ene-3,17-dione and 19-nor-androst-4-ene-3,17-dione could possibly be produced as aromatase intermediate products or byproducts. Detection of these steroids could provide relevant information about the exogenous or endogenous origin of 19-norsteroids.

Scientific data does not describe occurrence of 4-hydroxy-androst-4-ene-3,17-dione (4-OH-Δ4-AEDIONE) as endogenous steroid. However, it cannot be excluded that 4-OH-Δ4-AEDIONE is produced as metabolite after Δ4-AEDIONE administration.

INTRODUCTION

Since the abuse of testosterone (T) was first suspected during the Olympic Games of Moscow in 1980, many other endogenous steroids have been introduced as ergogenic aid in sports. During the following decade, the abused steroids were mostly limited to T and 5α-dihydrotestosterone derivatives. During the nineties, steroid containing food supplement products became widely available. The first supplement steroids introduced were dehydroepiandrosterone (DHEA) and androst-4-ene-3,17-dione (Δ4-AEDIONE). One or two years later these steroids were followed by androst-5-ene-3β,17β-diol (Δ5-AEDIOL), androst-4-ene-3β,17β-diol (Δ4-AEDIOL) and androst-5-ene-3,17-dione (Δ5-AEDIONE). The common feature of these steroids is that they are biosynthetic precursors of T and they are claimed to metabolize to T after oral administration, without pertaining anabolic effects themselves.

Simultaneous to the developments of the mentioned 19C-steroids, 19-norsteroids became popular as food supplement steroids. Based on metabolic conversion to 19-nortestosterone (nandrolone), products were developed that contained steroids as 19-nor-androst-4-ene-3,17-dione, 19-nor-androst-4-ene-3β,17β-diol and 19-nor-androst-5-ene-3β,17β-diol. Nowadays, the concept of the endogenous origin of nortestosterone's main metabolite norandrosterone has gained interest and scientific acceptance [1,2]. This illustrates that besides the mentioned 19C-steroids, also the 19-norsteroids are metabolically related to the endocrine system.

Food supplement steroids are mostly sold in capsules or other formulations requiring oral administration. As no chemical modification has been performed, as for example has been the case for 17α-methyl-substituted anabolic steroids, extensive first-pass metabolism occurs after oral administration. Besides a low absorption, this leads to a high metabolic and renal clearance and a low half-life time.

In this short overview, a summary will be given of the metabolic pathways of DHEA and Δ4-AEDIONE. These will serve as model compounds for other supplement steroids mentioned above, as limited knowledge is available on the oxygenation of 19-nor steroids.

"NON-SPECIFIC" METABOLISM OF FOOD SUPPLEMENT STEROIDS

In doping analysis a limited number of analytical tools are available to tackle the detection of endogenous compounds or corresponding biosynthetic precursors. One of these is isotope ratio mass spectrometry that discriminates between endogenous

and exogenous origin of steroids [3]. This technique was initially developed for analytical confirmation of T administration [4] and is still under development as an efficient screening technique for T [5] and as a confirmation technique for other endogenous steroids [6,7].

Other analytical tools are based on easily available metabolic pathways of the food supplement steroids. This mostly implies the metabolism as summarized in Figure 1. The presented model is limited to 19C-steroids, but the enzymatic activities can be extrapolated to 19-norsteroids. Metabolites that are mostly studied in relation to administration of 19C-steroids are T, E, androsterone (AO), etiocholanolone (EO), 5α- and 5β-isomers of dihydrotestosterone (5α-DHT and 5β-DHT) and different stereoisomers of androstanediol (5α,3α-ADIOL; 5α,3β-ADIOL; 5β,3α-ADIOL and 5β,3β-ADIOL).

Based on the presented metabolism, steroid ratios have been introduced for the detection of T [8,9] and 5α-DHT [10,11]. Several studies have focussed on the effect of administration of food supplement 19C-steroids on the ratio of testosterone/epitestosterone (T/E ratio) [12-16]. Although the T/E ratio mostly increases, the response is limited. Also the AO/EO ratio or the corresponding 5α/5β-ratio for androstanediols has been studied [17]. Saturation of 5α-reductase by androst-4-ene steroids could lead to an increased activity of 5β-reductase. Administration of high dose levels can therefore be expected to decrease the AO/EO (5α/5β) ratio. So far, the AO/EO ratio has mostly been studied in a case study design [15-17] and requires additional investigation.

The main virtue of the described analytical tools is the general detection of unknown 19C-steroids or 19-norsteroids, by applying a rather arbitrary and qualitative pattern recognition methodology [15,16]. It is difficult to obtain sufficient specific information from a steroid profile based on the steroids shown in Figure 1, to identify the steroid that has been administered. The large number of steroids that are available today as supplement products challenges for more specific analytical methodologies. Additionally, an increasing number of claims are made of unintentional steroid administration, due to contamination of permitted (non-steroid) food supplement products. In general, these claims are supported by some scientific observations [18,19]. This illustrates a need for more specificity in doping analysis.

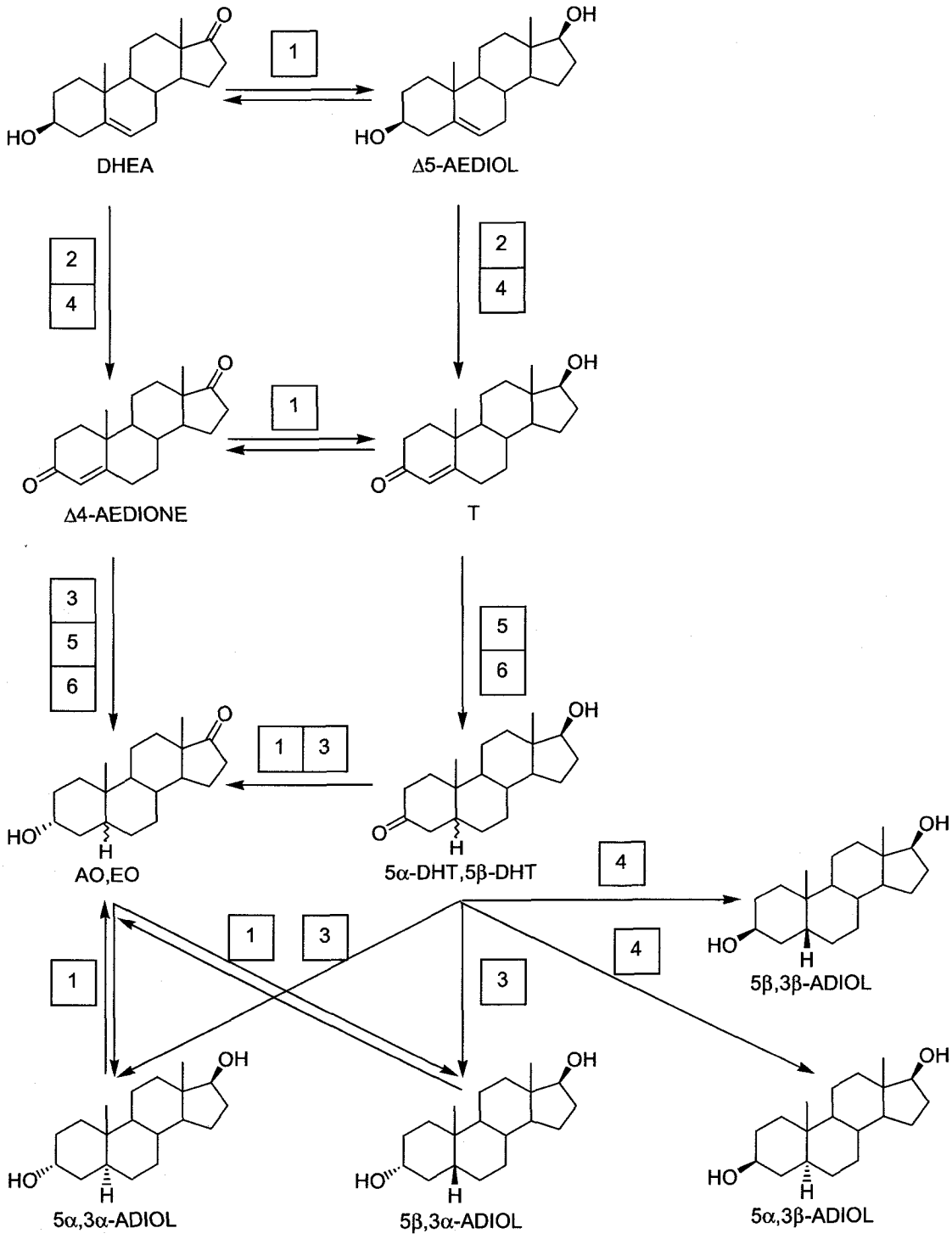


Figure 1: "Non-specific" metabolism of DHEA and Δ4-AEDIONE. Enzymatic activities involved are: (1) 17β-dehydrogenase; (2) 3,4-isomerase; (3) 3α-dehydrogenase; (4) 3β-dehydrogenase; (5) 5α-reductase; (6) 5β-reductase.

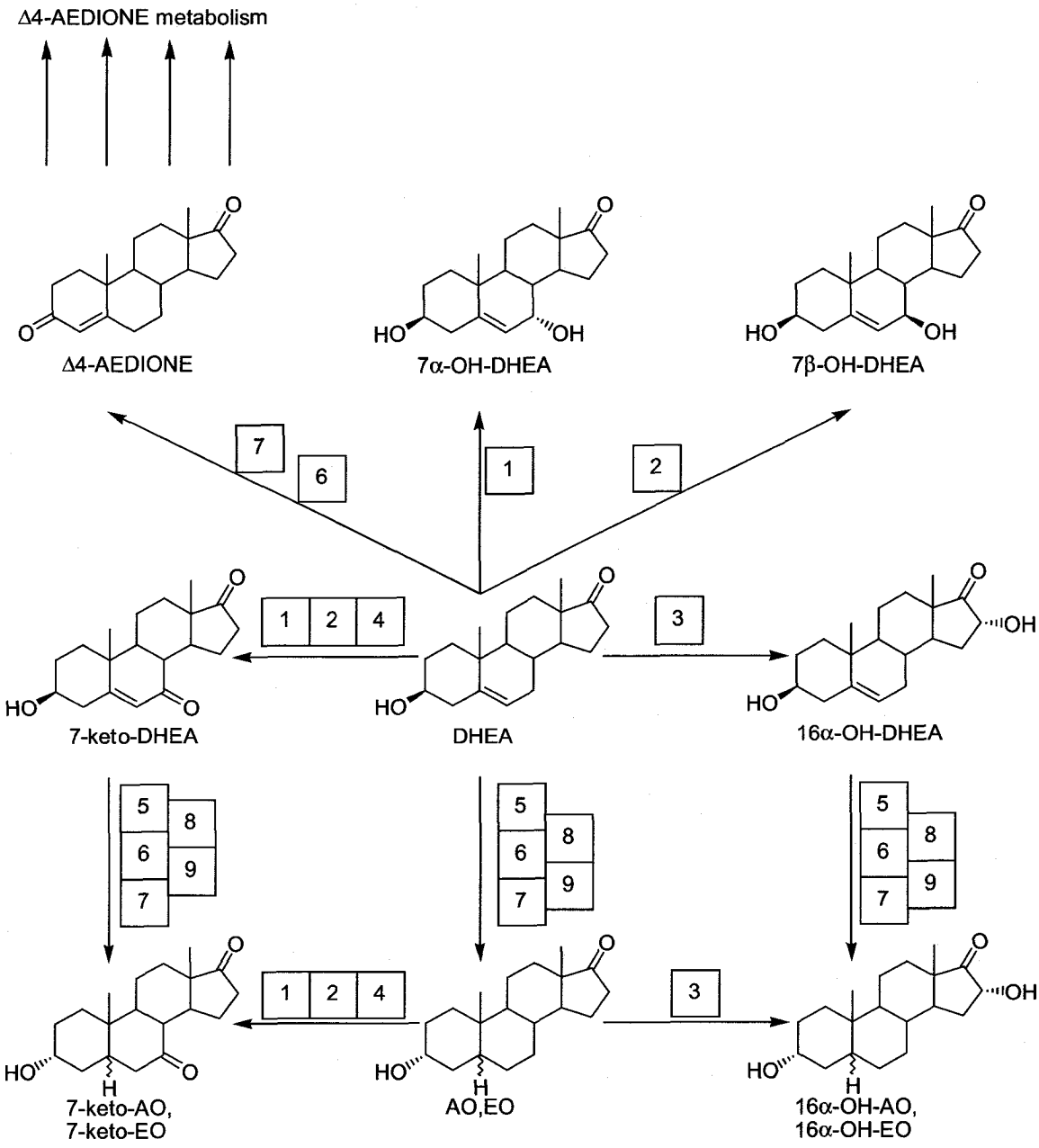


Figure 2: Suggested model for "specific" metabolism of DHEA. Involved enzymatic activities are: (1) 7α-hydroxylase; (2) 7β-hydroxylase; (3) 16α-hydroxylase; (4) 7-hydroxy dehydrogenase; (5) 3α-dehydrogenase; (6) 3β-dehydrogenase; (7) 4-5 isomerase; (8) 5α-reductase; (9) 5β-reductase

"SPECIFIC" METABOLISM OF DHEA AND Δ4-AEDIONE

Endoplasmic reticulum-bound cytochrome P-450 plays a central role in the oxidative metabolism of lipophilic compounds as steroids [20]. In mammals, the microsomal cytochromes are predominantly present in hepatic tissues, where they catalyze NADPH-dependent monooxygenation, *e.g.* hydroxylation. There are multiple pathways for this kind of steroid biotransformation, with a high degree of specificity, depending on the chemical properties of the steroid. Usually, not much knowledge is present about the possible endocrine function of such metabolites.

DHEA is readily converted to several oxygenated metabolites, as summarized in Figure 2. The main hydroxylation pathways are 7α-, 7β- and 16α-hydroxylation, resulting in 7α-hydroxy-DHEA (7α-OH-DHEA), 7β-hydroxy-DHEA (7β-OH-DHEA) and 16α-hydroxy-DHEA (16α-OH-DHEA) [21-25]. 7α-Hydroxylation is the first and rate-limiting step in the metabolic pathway of steroids, leading to bile acids [26]. Only suggestive biological relevance has been described for 7α- or 7β-hydroxylation of DHEA, as immunomodulatory action of 7α- and 7β-OH-DHEA in semen [27] and increased production of 7α-OH-DHEA in Alzheimer's disease [28].

Also 7-keto-DHEA has been detected in human urine samples [29]. It can be expected that 7-keto-DHEA is produced through 7α-, or 7β-hydroxy dehydrogenation of the mentioned 7α- or 7β-OH-metabolites of DHEA. Although no biological effect is known, this steroid is sold nowadays as food supplement steroid, either as 7-keto-DHEA or 3-acetyl-7-keto-DHEA (see Appendix). The commercially based claim for the biological effects are the same as for DHEA, but excluding androgenic side effects. No literature is available about the metabolism to 7-keto-androsterone (7-keto-AO) and 7-keto-etiocholanolone (7-keto-EO). Based on the described metabolic steps, these steroids can be expected as main metabolites of 7-keto-DHEA. However, the presence of these metabolites in human plasma or urine could also originate from direct 7-oxygenation of AO and EO.

16α-Hydroxylation of DHEA to 16α-OH-DHEA is one of the most described hydroxylation reactions of DHEA, as this metabolite is present in relatively high concentrations in urine [23,30,31]. It is a known intermediate product of estriol (1,3,5(10)-estratriene-3,16α,17β-triol) during pregnancy. Based on the presented metabolic steps, it is suggested that 16α-hydroxy-androsterone and -etiocholanolone (16α-OH-AO and 16α-OH-EO) could be detected as main metabolites of 16α-OH-DHEA. However, as mentioned before, these metabolites could also be produced by direct 16α-hydroxylase conversion of AO and EO [32].

Another reported minor oxygenation pathway is 18-hydroxylation, as determined in *in vitro* incubation experiments with human liver microsomes [33]. No further *in vivo* data are available.

Several oxygenation routes are described for Δ4-AEDIONE. A summary is given in Figure 3. Based on *in vitro* experiments with liver microsomes the best-described reactions are 6β-, 7α-, 16α- and 16β-hydroxylation [20,34]. The fact that the same pathways are found for the hydroxylation of T [34,35] suggests that these reactions are specific for androst-4-ene-3-one steroids. For both steroids, the major part is accounted by 6β-hydroxylation (≥ 70% for Δ4-AEDIONE [20]). This is also the case for some synthetic derivatives of T [36]. No data is available about 6α-hydroxylation and metabolic conversion to 6-keto-Δ4-AEDIONE. However, analogous to DHEA metabolism, the presence of 6-keto-metabolites could occur after dehydrogenation of 6β-OH-Δ4-AEDIONE.

In order of decreasing conversion rate, hydroxylation at C^{16} of Δ4-AEDIONE is the second route [20,34,35]. In contrast to DHEA metabolism, 16α- as well as 16β-hydroxylation takes place at Δ4-AEDIONE. Also, multiple hydroxylation to 6β,16α-diol and 6β,16β-diol metabolites has been reported for rats [37]. As is also suggested above for the metabolism of DHEA, likely products to be expected are the 16α- and 16β-hydroxy metabolites of AO and EO.

Described by Ryan *et al.* [38], local production of estrogens occurs from androgens, catalyzed by an aromatase complex in human placenta tissue. However, this reaction has also been established *in vitro* in microsomes obtained from other tissues [38]. The conversion of Δ4-AEDIONE to estrone (3-hydroxy-1,3,5(10)-oestratriene-17-one) is expected to occur with 19-hydroxy-Δ4-AEDIONE (19-OH-Δ4-AEDIONE) as one of the intermediate products or byproducts [39,40]. Kelly *et al.* [41] showed that 19-OH-Δ4-AEDIONE is readily excreted in urine as glucuronide and sulfate conjugate. The conversion of Δ4-AEDIONE to 19-OH-Δ4-AEDIONE is, however, relatively small and it is expected that little 19-OH-Δ4-AEDIONE leaves the site of aromatization. It cannot be ruled out that conjugation occurs at C^{19} [40], and it is unknown whether these conjugates can be deconjugated by the applied enzymatic β-glucuronidase and solvolysis methods of hydrolysis [40,41].

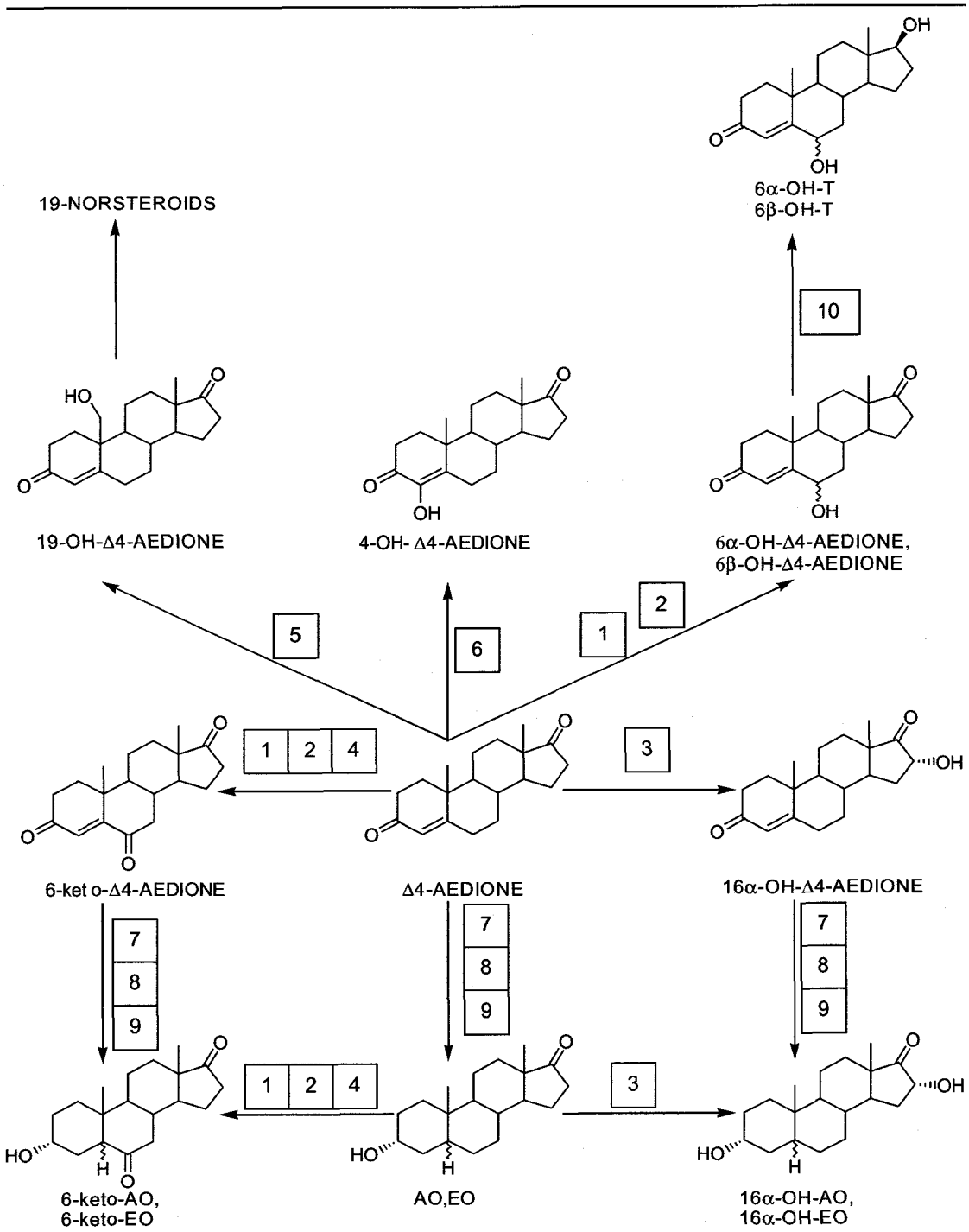


Figure 3: Suggested model of "specific" metabolism of Δ4-AEDIONE. Supposedly involved enzymatic activities are: (1) 6α-hydroxylase; (2) 6β-hydroxylase; (3) 16α-hydroxylase; (4) 6-hydroxy dehydrogenase. (5) 19-hydroxylase; (6) 4-hydroxylase; (7) 3α-dehydrogenase; (8) 5α-reductase; (9) 5β-reductase; (10) 17β-dehydrogenase

During aromatization also small quantities of 19-norsteroids (19-nortestosterone and 19-nor-Δ4-AEDIONE) are produced. This has been shown by *in vitro* tests on aromatase rich tissues as the ovarian follicle [42] and by the placenta [43]. Dehennin *et al.* [1] posed the production of small quantities of 19-nor-steroids by less aromatase rich tissues as adipose tissue, skin, testis, adrenal, liver and muscle. Based on this theory, production of 19-norsteroids could be accompanied by production of similar quantities of 19-OH-Δ4-AEDIONE and 19-OH-T. Detection of these steroids could perhaps aid the analytical discrimination between urine samples taken after 19-norsteroid administration and samples containing only 19-norsteroids of endogenous origin. For this purpose, quantification of 19-hydroxy-steroids down to low ng/ml levels is necessary.

Metabolism of estrogens by hydroxylation at C^4 is an important metabolic step, leading to the production of catecholestrogens [44,45]. Hydroxylation at the Δ4-double bond in Δ4-AEDIONE, leading to 4-hydroxy-Δ4-AEDIONE (4-OH-Δ4-AEDIONE) has never been reported. During development studies on this steroid as aromatase inhibitor for treatment of breast cancer, no 4-OH-Δ4-AEDIONE was detected as endogenous substance [46]. A limit of quantification of 0.3 ng/ml was reported by Dowsett *et al.* for the applied radioimmunoassay of non-conjugated 4-OH-Δ4-AEDIONE [47]. This was done by an assay for Δ4-AEDIONE that showed 25% cross-reactivity for 4-OH-Δ4-AEDIONE. This suggests that 4-hydroxylation either does not occur, or in very small amounts, or that conjugation prevents radioimmunoassay detection.

Poon *et al.* [48] showed that orally administered 4-OH-Δ4-AEDIONE shows extensive first and second phase metabolism. This suggests that if production of 4-OH-Δ4-AEDIONE would occur after Δ4-AEDIONE administration, this would probably lead to efficient excretion as conjugates. As conjugation occurs at C^4, it is not clear whether enzymatic deconjugation results in a high recovery. Concluding, insufficient results are available to exclude the endogenous origin of 4-OH-Δ4-AEDIONE and the conversion to 4-OH-Δ4-AEDIONE after oral administration of Δ4-AEDIONE.

LITERATURE

1. L. Dehennin, Y. Bonnaire and P. Plou. Urinary excretion of 19-norandrosterone of endogenous origin in man: quantitative analysis by gas chromatography-mass spectrometry. *J Chromatogr. B* **721:** 301-307 (1999).

2. Y. Reznik, L. Dehennin, C. Coffin, J. Mahoudeau and P. Leymarie. Urinary nandrolone metabolites of endogenous origin in man: a confirmation by output regulation under human chorionic gonadotropin stimulation. *J. Clin. Endocrinol. Metab.* **86:** 146-50 (2001).

3. R. Aguilera, M. Becchi, H. Casabianca, C.K. Hatton, D.H. Catlin, B. Starcevic and H.G. Pope Jr. Improved method for detection of testosterone abuse by gas chromatography/combustion/isotope ratio mass spectrometry analysis of urinary steroids. *J. Mass Spectrom.* **31:** 169-176 (1996).

4. C.H.L. Shackleton, A. Phillips, T. Chang and Y. Li. Confirming testosterone administration by isotope ratio mass spectrometric analysis of urinary androstanediols. *Steroids* **62:** 379-387 (1997).

5. R. Aguilera, D.H. Catlin, M. Becchi, A. Philips, C. Wang, R.S. Swerdloff, H.G. Pope and C.K. Hatton. Screening urine for exogenous testosterone by isotope ratio mass spectrometric analysis of one pregnanediol and two androstanediols. *J. Chromatogr. B* **727:** 95-105 (1999).

6. C.H.L. Shackleton, E. Roitman, A. Phillips and T. Chang. Androstanediol and 5-androstenediol profiling for detecting exogenously administered dihydrotestosterone, epitestosterone, and dehydroepiandrosterone: Potential use in gas chromatography isotope ratio mass spectrometry. *Steroids* **62:** 665-673 (1997).

7. M. Ueki and M. Okano. Analysis of exogenous dehydroepiandrosterone excretion in urine by gas chromatography/combustion/isotope ratio mass spectrometry. *Rapid Commun. Mass Spectrom.* **13:** 2237-2243 (1999).

8. M. Donike, K.-R. Bärwald, K. Klostermann, W. Schänzer and J. Zimmermann. Nachweis von exogenem Testosteron. In *Sport: Leistung und Gesundheit*, H. Heck, W. Hollmann, H. Liesen, R. Rost, Eds. Deutscher Ärzte Verlag, Köln, 1983, pp 293-298.

9. M. Donike. Steroid profiling in Cologne. In *Proceedings of the 10th Cologne workshop on dope analysis*, M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke and S. Rauth, Eds. Sport und Buch Strauss, Köln, 1993, pp 47-68.

10. A.T. Kicman, S.B. Coutts, C.J. Walker and D.A. Cowan. Proposed confirmatory procedure for detecting 5α-dihydrotestosterone doping in male athletes. *Clin. Chem.* **41:** 1617-1627 (1995).

11. M. Donike, M. Ueki, Y. Kuroda, H. Geyer, E. Nolteernsting, S. Rauth, W. Schanzer, U. Schindler, E. Volker and M. Fujisaki. Detection of dihydrotestosterone (DHT) doping: alterations in the steroid profile and reference ranges for DHT and its 5 alpha-metabolites. *J. Sports Med. Phys. Fitness.* **35:** 235-250 (1995).

12. L. Dehennin, M. Ferry, P. Lafarge, G. Pérès and J.-P. Lafarge. Oral administration of dehydroepiandrosterone to healthy men: Alteration of the urinary androgen profile and consequences for the detection of abuse in sport by gas chromatography-mass spectrometry. *Steroids* **63:** 80-87 (1998).

13. L. Bowers. Oral dehydroepiandrosterone supplementation can increase the testosterone/epitestosterone ratio. *Clin. Chem.* **45:** 295-297 (1999).

14. T.Z. Bosy, K.A. Moore and A. Poklis. The effect of oral dehydroepiandrosterone (DHEA) on the urinary testosterone/epitestosterone (T/E) ratio in human male volunteers. *J. Anal. Toxicol.* **22:** 455-459 (1998).

15. V.P. Uralets and P.A. Gillette. Over-the-counter anabolic steroids 4-androsten-3,17-dione; 4-androsten-3beta,17beta-diol; and 19-nor-4-androsten-3,17-dione: excretion studies in men. *J. Anal. Toxicol.* **23:** 357-366 (1999).

16. V.P. Uralets and P.A. Gillette. Over-the-counter delta5 anabolic steroids 5-androsten-3,17-dione; 5-androsten-3beta, 17beta-diol; dehydroepiandrosterone; and 19-nor-5-androsten-3,17-dione: excretion studies in men. *J. Anal. Toxicol.* **24:** 188-193 (2000).

17. R. Kazlauskas. Effects of dehydroepiandrosterone on urinary steroids. In *Recent advances in doping analysis (5)*, W. Schänzer, H. Geyer, A. Gotzmann and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, 1998, pp 83-90.

18. D.H. Catlin, B.Z. Leder, B. Ahrens, B. Starcevic, C.K. Hatton, G.A. Green, J.S. Finkelstein. Trace contamination of over-the-counter androstenedione and positive urine test results for a nandrolone metabolite. *Jama* **284:** 2618-2621 (2000).

19. H. Geyer, U. Mareck-Engelke, U. Reinhart, M. Thevis and W.Schanzer. The analysis of nutritional supplements for anabolic-androgenic steroids. In *Recent advances in doping analysis (8),* W. Schänzer, H. Geyer, A. Gotzmann and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, 2000, pp 23-32.

20. D.J. Waxman. Interactions of hepatic cytochromes P-450 with steroid hormones. Regioselectivity and stereospecificity of steroid metabolism and hormonal regulation of rat P-450 enzyme expression. *Biochem. Pharmacol.* **37:** 71-84 (1988).

21. O. Lapcik, R. Hampl, M. Hill, M. Bicikova and L. Starka. Immunoassay of 7-hydroxysteroids: 1. Radioimmunoassay of 7beta-hydroxy dehydroepiandrosterone. *J. Steroid Biochem. Mol. Biol.* **67:** 439-445 (1998).

22. O. Lapcik, R. Hampl, M. Hill and L. Starka. Immunoassay of 7-hydroxysteroids: 2. Radioimmunoassay of 7alpha-hydroxy-dehydroepiandrosterone. *J. Steroid Biochem. Mol. Biol.* **71:** 231-237 (1999).

23. R. Hampl and L. Starka. Minireview: 16alpha-hydroxylated metabolites of dehydroepiandrosterone and their biological significance. *Endocr. Regul.* **34:** 161-163 (2000).

24. J.L. Fitzpatrick, S.L. Ripp, N.B. Smith, W.M. Pierce Jr and R.A. Prough. Metabolism of DHEA by cytochromes P450 in rat and human liver microsomal fractions. *Arch. Biochem. Biophys.* **389:** 278-287 (2001).

25. J.F. Levesque and C. Ayotte. The oral administration of DHEA: The efficiency of steroid profiling. In *Recent advances in doping analysis (7),* W. Schänzer, H. Geyer, A. Gotzmann and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, 2000, pp 213-221.

26. R. Hampl, R. Morfin and L. Starka. Minireview 7-Hydroxylated Derivatives of Dehydroepiandrosterone: What Are They Good For? *Endocr. Regul.* **31:** 211-218 (1997).

27. R. Hampl, M. Hill, I. Sterzl and L. Starka. Immunomodulatory 7-hydroxylated metabolites of dehydroepiandrosterone are present in human semen. *J. Steroid Biochem. Mol. Biol.* **75:** 273-276 (2000).

28. S. Attal-Khemis, V. Dalmeyda, J.L. Michot, M. Roudier and R. Morfin. Increased total 7 alpha-hydroxy-dehydroepiandrosterone in serum of patients with Alzheimer's disease. *J. Gerontol. A Biol. Sci. Med. Sci.* **53:** B125-32 (1998).

29. D.K. Fukushima, A.D. Kemp, R. Schneider, M.B. Stokem and T.F. Gallagher. Steroid metabolism. XXV. Isolation and characterization of new urinary steroids. *J. Biol. Chem.* **210:** 129-137 (1954).

30. F. Callies, W. Arlt, L. Siekmann, D. Hubler, F. Bidlingmaier and B. Allolio. Influence of oral dehydroepiandrosterone (DHEA) on urinary steroid metabolites in males and females. *Steroids* **65:** 98-102 (2000).

31. C.H. Shackleton, T.W. Kelly, P.M. Adhikary, C.J. Brooks, R.A. Harkness, P.J. Sykes and F.L. Mitchell. The identification and measurement of a new steroid 16 beta-hydroxydehydroepiandrosterone in infant urine. *Steroids* **12:** 705-716 (1968).

32. M. Ingelman-Sundberg, A. Rane and J.A. Gustafasson. Properties of hydroxylase systems in the human fetal liver active on free and sulfoconjugated steroids. *Biochemistry* **14:** 429-437 (1975).

33. K. Einarsson, J.A. Gustafsson, T. Ihre and M. Ingelman-Sundberg. Specific metabolic pathways of steroid sulfates in human liver microsomes. *J. Clin. Endocrinol. Metab.* **43:** 56-63 (1976).

34. D.J. Waxman, C. Attisano, F.P. Guengerich and D.P. Lapenson. Human liver microsomal steroid metabolism: identification of the major microsomal steroid hormone 6 beta-hydroxylase cytochrome P-450 enzyme. *Arch. Biochem. Biophys.* **263:** 424-436 (1988).

35. S.A. Testino Jr., J. Ozarowski, A.W. Thurston, R.F. Arrendale and G. Patonay. Determination of testosterone and 6beta-hydroxytestosterone by gas chromatography-selected ion monitoring-mass spectrometry for the characterization of cytochrome p450 3A activity. *J. Chromatogr. B* **734:** 73-81 (1999).

36. W. Schanzer, S. Horning and M. Donike. Metabolism of anabolic steroids in humans: synthesis of 6 beta-hydroxy metabolites of 4-chloro-1,2-dehydro-17 alpha-methyltestosterone, fluoxymesterone, and metandienone. *Steroids* **60:** 353-366 (1995).

37. J.J. Sheets and R.W. Estabrook. Multiple sites of steroid hydroxylation by the liver microsomal cytochrome P-450 system: primary and secondary metabolism of androstenedione. *Biochemistry* **24:** 6591-6597 (1985).

38. K.J. Ryan. Biochemistry of aromatase: significance to female reproductive physiology. *Cancer Res.* 1982. **42(Suppl):** 3342s-3344s (1982).

39. M. Akhtar, M.R. Calder, D.L. Corina and J.N. Wright. Mechanistic studies on C-19 demethylation in oestrogen biosynthesis. *Biochem. J.* **201:** 569-580 (1982).

40. W.G. Kelly, O. De Leon and T.H. Rizkallah. The role of 19-hydroxy-delta4-androstene-3,17-dione in the conversion of circulating delta4-androstene-3, 17-dione to estrone. *J. Clin. Endocrinol. Metab.* **43:** 190-194 (1976).

41. W.G. Kelly, O. de Leon and T.H. Rizkallah. Metabolism of 19-hydroxyandrostenedione in human subjects. Urinary excretion of conjugates. *J. Clin. Endocrinol. Metab.* **46:** 445-451 (1978).

42. L. Dehennin, M. Jondet and R. Scholler. Androgen and 19-norsteroid profiles in human preovulatory follicles from stimulated cycles: an isotope dilution-mass spectrometric study. *J. Steroid Biochem.* **26:** 399-405 (1987).

43. Y. Reznik, M. Herrou, L. Dehennin, M. Lemaireand P. Leymarie. Rising plasma levels of 19-nortestosterone throughout pregnancy: determination by radioimmunoassay and validation by gas chromatography-mass spectrometry. *J. Clin. Endocrinol. Metab.* **64:** 1086-1088 (1987).

44. D.C. Spink, B.C. Spink, X. Zhuo, M.M. Hussain, J.F. Gierthy and X. Ding. NADPH- and hydroperoxide-supported 17beta-estradiol hydroxylation catalyzed by a variant form (432L, 453S) of human cytochrome P450 1B1. *J. Steroid Biochem. Mol. Biol.* **74:** 11-18 (2000).

45. C.L. Hayes, D.C. Spink, B.C. Spink, J.Q. Cao, N.J. Walker and T.R. Sutter. 17 beta-estradiol hydroxylation catalyzed by human cytochrome P450 1B1. *Proc. Natl. Acad. Sci.* **93:** 9776-9781 (1996).

46. P.E. Goss, T.J. Powles, M. Dowsett, G. Hutchison, A.M. Brodie, J.C. Gazet and R.C. Coombes. Treatment of advanced postmenopausal breast cancer with an aromatase inhibitor, 4-hydroxyandrostenedione: phase II report. *Cancer Res.* **46:** 4823-4826 (1986).

47. M. Dowsett, P.E. Goss, T.J. Powles, G. Hutchinson, A.M. Brodie, S.L. Jeffcoate and R.C. Coombes. Use of the aromatase inhibitor 4-hydroxyandrostenedione in postmenopausal breast cancer: optimization of therapeutic dose and route. *Cancer Res.* **47:** 1957-1961 (1987).

48. G.K. Poon, M. Jarman, M.G. Rowlands, M. Dowsett and J. Firth. Determination of 4--hydroxyandrost-4-ene-3,17-dione metabolism in breast cancer patients using high-performance liquid chromatography-mass spectrometry. *J. Chromatogr.* **565:** 75-88 (1991).

4

PROFILING SPECIFIC METABOLITES OF DEUTERIUM LABELED DEHYDROEPIANDROSTERONE (DHEA)

ABSTRACT

During recent years a number of products containing the steroid dehydroepiandrosterone (DHEA) have appeared on the food supplement market. The International Olympic Committee placed this steroid on the list of forbidden substances. However, establishment of oral DHEA administration in doping control is aggravated by extensive metabolic clearance, and an assumed non-specific metabolism. In this study, a preliminary investigation is performed to construct a DHEA-selective profile of non-oxygenated metabolites (androsterone, etiocholanolone, testosterone, epitestosterone, androst-5-ene-3β,17β-diol, 5α-androst-3α,17β-diol and 5β-androstan-3α-17β-diol) and oxygenated DHEA metabolites (7α-hydroxy-DHEA, 7β-hydroxy-DHEA, 16α-hydroxy-DHEA, 7-keto-DHEA, 16α-hydroxy-androsterone, 16α-hydroxy-etiocholanolone and 7-keto-androsterone).

Labeled DHEA (3β-hydroxy-[16,16-$^{2}H_{2}$]androst-5-en-17-one) was administered to 2 male volunteers (25.5 and 52.5 mg, respectively) and urine samples were collected. Sample analysis was performed on the total fraction of glucuronides, sulfates and non-conjugated steroids with gas chromatography-mass spectrometry. The most sensitive parameters during the first 10 hours after administration were androsterone, etiocholanolone, testosterone, 7β-hydroxy-DHEA and 7-keto-DHEA. Etiocholanolone and 5α-androst-3α,17β-diol showed the highest sensitivity during 10 to 30 hours after administration. The total conversion of DHEA to the studied metabolites was 39.7-42.8%. The conversion of DHEA to oxygenated and non-oxygenated metabolites was 0.8-1.32% and 38.4-42.0%, respectively. Although the conversion to oxygenated metabolites is limited, profiling these specific steroids may lead to additional information about the identity of the administered steroid.

INTRODUCTION

An increasing number of steroid containing products has appeared on the food supplement market. The first supplements sold contained dehydroepiandrosterone (DHEA) and androst-4-ene-3,17-dione (Δ4-AEDIONE), later followed by androst-5-ene-3β,17β-diol (Δ5-AEDIOL), androst-4-ene-3β,17β-diol (Δ4-AEDIOL), the nor-steroids 19-nor-androst-4-ene-3,17-dione and 19-nor-androst-4-ene-3β,17β-diol [1,2]. The proclaimed pharmacological effect by metabolism to steroids with potential anabolic activity made these products mostly popular amongst bodybuilders. During the years 1997 to 1999 the mentioned steroids have been placed on the IOC list of forbidden substances.

Analytical methods have been developed to establish the abuse of steroid supplements as DHEA by profiling of main metabolites as androsterone (AO), etiocholanolone (EO), testosterone (T) and androstanediols [1-6]. However, establishment of DHEA abuse in doping control is aggravated by extensive first-pass metabolism after oral administration, fast renal and metabolic clearance of DHEA and metabolites, and an assumed non-specific metabolism compared to other orally administered steroids as Δ4-AEDIONE [1,2].

The generally applied determination of glucuronides and/or sulfates of non-specific metabolites of DHEA as AO, EO, T and Δ4-AEDIONE [1-6], is generally insufficient to obtain quality information about the identity of the administered steroid. A study performed by Shackleton *et al.* [7] described a method based on gas chromatography-isotope ratio mass spectrometry to confirm administration of T, E, DHEA and dihydrotestosterone. However, the described method was also not specific for any of the studied steroids.

Several studies have been performed for the use of the testosterone/epitestosterone ratio (T/E ratio) as a marker of DHEA or Δ4-AEDIONE abuse [4,5,8]. In theory, the T/E ratio could be a non-specific but sensitive parameter since T is a known metabolite of DHEA, Δ4-AEDIONE, Δ5-AEDIONE, Δ4-AEDIOL and Δ5-AEDIOL and no E production has been proven after administration of testosterone. In reality, the T/E ratio appears to be an insensitive parameter of DHEA abuse [8].

The complication for doping analysis caused by the wide spread use of steroid supplements demands for profiling procedures of higher specificity. Determination of oxygenated metabolites of potentially used steroids could supply the necessary information for identification of abused steroids.

In this study, a preliminary investigation is performed to construct a conclusive profile of selected oxygenated and non-oxygenated DHEA metabolites, that are commercially available, in order to look for conclusive parameters for the detection of

DHEA abuse. Therefore, 3β-hydroxy-[16,16-$^2H_2$]androst-5-en-17-one (d_2-DHEA) was administered to 2 healthy male volunteers and urine samples were collected at set times before and after administration. Analysis of the collected urine samples was performed with gas chromatography-mass spectrometry (GC-MS).

EXPERIMENTAL

Chemicals

Reference steroids: Androst-5-en-3β-ol-17-one (dehydroepiandrosterone, DHEA), androst-4-en-17α-ol-3-one (epitestosterone, E), 5α–androstan-3α-ol-17-one (androsterone, AO), 5β–androstan-3α-ol-17-one (etiocholanolone, EO), androst-4-ene-3,17-dione (androstenedione, Δ4-AEDIONE), androst-5-ene-3β,17β-diol (androstenediol, Δ5-AEDIOL) and 17α-methyl-androst-4-en-11α,17β-diol-3-one (11α-hydroxy-methyltestosterone, 11α-OH-MeT) were obtained from Sigma, St. Louis, Missouri, USA. Androst-4-en-17β-ol-3-one (testosterone, T), androst-5-ene-3β,7α-diol-17-one (7α-hydroxy-dehydroepiandrosterone, 7α-OH-DHEA) and androst-5-ene-3β,7β-diol-17-one (7β-hydroxy-dehydroepiandrosterone, 7β–OH-DHEA) were obtained through courtesy of Organon, Oss, The Netherlands). Androst-5-en-3β-ol-7,17-dione (7-keto-dehydroepiandrosterone, 7-keto-DHEA), 5α-androstan-3α-ol-7,17-dione (7-keto-androsterone, 7-keto-AO), androst-5-ene-3β,16α-diol-17-one (16α-hydroxy-dehydroepiandrosterone, 16α–OH-DHEA), 5α–androstane-3α,16α-diol-17-one (16α-hydroxy-androsterone, 16α-OH-AO), 5β–androstane-3α,16α-diol-17-one (16α-hydroxy-etiocholanolone, 16α-OH-EO) were obtained from Steraloids, Newport, Rhode Island, USA. 3β-Hydroxy-[16,16-$^2H_2$]androst-5-en-17-one (isotopic purity 97.3%) was obtained from C/D/N Isotopes, Pointe-Claire, Quebec, Canada.

Chemicals: *N*-Methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA), ethanethiol (97%), phosphorous pentoxide, *tert*-butyldimethylsilyl chloride (TBDMSCl) and imidazole were obtained from Sigma-Aldrich Chemie Company, Steinheim, Germany. Ammonium iodide was obtained from Fluka Chemie, Buchs, Switzerland. Potassium hydroxide, sodium acetate trihydrate, glacial acetic acid (96%), diethyl ether, acetic acid and methanol were obtained from Merck, Darmstadt, Germany. Acetonitril was obtained from J.T. Baker B.V., Deventer, The Netherlands. All chemicals were of analytical grade, unless indicated otherwise.

Hydrolysis was performed with Helix pomatia (type HP-2, containing 110.000 IU/ml β-glucuronidase and 1000-5000 IU/ml arylsulfatase), obtained from Sigma-Aldrich, Steinheim, Germany.

Columns for solid-phase extraction were IST Isolute C_{18} columns (200 mg, non-endcapped) obtained from Sopachem, Lunteren, The Netherlands.

Sample collection

Two male volunteers (subject 1: 29 years, 88 kg; subject 2: 21 years, 75 kg), collected urine samples every 2 hours during 3 days. On the second day at 9 a.m. 3β-hydroxy-[16,16-$^2H_2$]androst-5-en-17-one (d_2-DHEA) was orally administered (volunteer 1, 25.5 mg; volunteer 2, 52.5 mg). For each collected urine sample the exact void time, volume and specific gravity (Urine Specific Gravity Refractometer, Atago, Japan) were recorded. Samples were stored at -20°C until time of analysis.

Sample preparation

The volume of urine that was sampled for the analysis was determined on the basis of specific gravity (d) of the urine, according to:

$$\text{Volume} = \frac{0.020}{d-1} * 5 \text{ ml} \qquad \textbf{(1)}$$

A maximum volume of 15 ml was applied. This was done to enable quantitative analysis of metabolites in low concentration in diluted urine samples. Solid-phase extraction was performed with C_{18} columns. Before applying urine samples, the column material was preconditioned by washing with 4 ml of methanol and equilibrating with 2 ml of water. Inorganic material was removed from the column by washing with 2 ml of water. The organic material was collected by elution with 4 ml of methanol. After evaporation to dryness, 2 ml of acetate buffer (0.1 M, pH 5.2) was added to the extract. Hydrolysis was performed by adding 100 µl of Helix pomatia and incubating for 1 hour at 55°C. Steroids were isolated from the buffer solution by extracting with 5 ml of diethyl ether twice. After extraction the phase separation was stimulated by centrifugation (4000 rpm, 5 min). The organic layer was removed and evaporated to dryness under a gentle stream of nitrogen at 40°C. The extract was dried overnight over P_2O_5/KOH under reduced pressure.

To retain the deuterium atoms in the steroid structures and to obtain suitable peakshape and mass spectrometric fragmentation, a non-enolizing derivatization procedure to *tert*-butyldimethylsilyl-derivatives was selected (Method A). A modified procedure of Dehennin *et al.* [4] was applied. Derivatization was performed by addition 100 µl of TBDMSCl/imidazole/acetonitrile (30:30:1000; m/w/v) to the extract and incubating at 60°C for 60 min. Afterwards the reaction was stopped by addition

of 10 drops of water and extraction was performed with 1.6 ml of heptane. After vortexing, the organic layer was removed and evaporated to dryness. To the dry extract 50 µl of heptane were added and transferred in autosampler vials after vortexing.

Due to co-elution a different silylation method based on enolization was selected for derivatization of T, E, 5α,3α-ADIOL and 5α,3β-ADIOL (Method B). Enolizing conditions could be applied in this case, because these are steroids with an androstan-17β-ol moiety and therefore the deuterium atoms are retained under enolizing conditions. Loss of deuterium atoms would have occurred, in case of androstan-17-one steroids. The same cleanup procedure was performed. Derivatization was performed by incubation of the extract in 100 µl of MSTFA/NH_4I/ethanethiol (1000:2:3; v/w/v).

Gas chromatography and mass spectrometry

Selected ion monitoring was performed with a Hewlett Packard gas chromatograph (Model 5890, Agilent Technologies, Waldbronn, Germany) coupled to a Hewlett Packard quadrupole mass spectrometer (Model 5972A). Ionization was performed in electron impact mode at 70 eV. Analysis was performed by selected ion monitoring of specific ions, representing the respective deuterated and non-deuterated metabolites (see Table 1). A check for possibly incomplete derivatization of Method B was performed by detection of ions representing mono-TMS derivatives of AO and EO at m/z 362.

Gas chromatography was performed with a HP-1 fused silica column (length 18 m, inner diameter 0.2 mm, film thickness 0.11 µm). Via electronic pressure control the column flow (helium) was constant 1 ml/min. Sample injection of 1 µl was performed in split mode (ratio 1/10). A Hewlett Packard autosampler (Model 7673, Agilent Technologies, Waldbronn, Germany) was used for auto-injection. The injector temperature was set to 250°C. The oven temperature program used was: initial temperature 180°C, 2°/min to 225°C, 30°/min up to 310°C, held for 5 min. The interface temperature was set to 280°C.

The quality of d_2-DHEA was checked by GC-MS in full scan mode prior to administration. No impurities could be detected in a concentrated sample.

Table 1: *Monitored m/z values of labeled and non-labeled steroids. Method A (TBDMS-derivatization) was used for the analysis of labeled androstan-17-one steroids. Method B (TMS-enolTMS derivatization) was used for all non-labeled steroids and labeled androstan-17β-ol steroids.*

Steroid derivative	m/z value non-labeled Method A	m/z value labeled Method A	m/z value non-labeled Method B	m/z value labeled Method B	number of deuterium atoms in derivative (n)
AO	347.3	349.3	434.3	-	2
EO	347.3	349.3	434.3	-	2
DHEA	345.3	347.3	432.3	-	2
Δ5-AEDIOL	461.3	463.3	434.3	-	2
T	-	-	432.3	434.3	2
E	-	-	432.3	434.3	2
5α,3α-ADIOL	-	-	241.2	243.2	2
5α,3β-ADIOL	-	-	241.2	243.2	2
7α-OH-DHEA	399.3	401.3	430.3	-	2
7β-OH-DHEA	400.3	402.3	430.3	-	2
7-keto-AO	361.3	363.3	430.3	-	2
7-keto-DHEA	359.3	361.3	517.4	-	2
16α-OH-DHEA	343.3	344.3	505.4	-	1
16α-OH-AO	345.3	346.3	507.4	-	1
16α-OH-EO	345.3	346.3	507.4	-	1

Quantification of non-labeled endogenous metabolites ([d_0-M^{endo}])

Calibration samples were prepared by adding 100 µl of methanolic standard solutions to 4 ml demineralized water samples. These samples were included in the cleanup procedure with the urine samples. Quantification of non-labeled endogenous steroids ([d_0-M^{endo}]) was performed using two calibration curves for each steroid.

One curve containing five data-points, covered respective ranges: AO (467-2333 ng), EO (469-2347 ng), T (20-100 ng), E (10-50 ng), DHEA (459-2293 ng), Δ5-AEDIOL (509-2547 ng), 16α-OH-DHEA (248-1240 ng), 7α-OH-DHEA (25-125 ng), 7β-OH-DHEA (75-375 ng), 16α-OH-AO (307-1533 ng), 16α-OH-EO (195-973 ng), 7-keto-AO (15-75 ng), 7-keto-DHEA (40-200 ng). The second calibration curve contained 6 data-points and covered the respective ranges: AO (2333-56000 ng), EO (2347-56320 ng), T (100-2400 ng), E (50-1200 ng), DHEA (2293-55040 ng), Δ5-AEDIOL (2547-61120 ng), 16α-OH-DHEA (1240-29760 ng), 7α-OH-DHEA (125-3000 ng), 7β-OH-DHEA (375-9008 ng), 16α-OH-AO (1533-36800 ng), 16α-OH-EO (973-23360 ng), 7-keto-AO (75-1808 ng), 7-keto-DHEA (200-4800 ng). 11α-OH-MeT (500

ng) was used as internal standard. Weighted regression analysis ($1/x^2$) was used for calculation of the response curves.

Quantification of labeled steroids

The labeled steroids [d_2-M^{exo}] were quantified in an indirect way, since synthetic labeled metabolites were not available and construction of calibration curves for the labeled steroids was impossible except for DHEA. The concentration of the labeled metabolites was established by quantification of the concentration of the non-labeled metabolites [d_0-M^{endo}] and recording the area ratio of the detected labeled metabolite (A(M+2)) vs. the non-labeled metabolite (A(M)). The concentration of labeled DHEA was calculated with equation (2).

$$\frac{A(M+2)}{A(M)} = 0.27 * \frac{[d_2 - M^{exo}]}{[d_0 - M^{endo}]} + 0.072 \qquad \textbf{(2)}$$

Equation (2) was deduced by applying the mean area ratio A(M+2)/A(M) determined in pre-administration samples (=0.072 for DHEA) and A(M+2)/A(M) in a standard of DHEA and d_2-DHEA (=0.57*([d_2-M^{exo}]/[d_0-M^{endo}]). The calculation was performed with the assumption that no proton-deuterium exchange occurred, so no non-labeled metabolites of exogenous origin were detected ([d_0-M^{exo}]=0). This was considered valid, as 2 deuterium atoms were present in the steroid structure of the metabolites, with the exception of the 16α-hydroxy-steroids. Metabolites 16α-OH-DHEA, 16α-OH-AO and 16α-OH-EO were therefore excluded from the quantification procedure. Equation (2) was corresponding to the results of Dehennin *et al.* [4]. Comparable equations were used for other metabolites, assuming that the isotope effect was constant for all metabolites. The mean area ratio A(M+2)/A(M) determined in pre-administration samples was specifically calculated for each metabolite.

Validation

Quality control (QC) samples have been prepared according the following procedure: A healthy male subject was administered two capsules of each 100 mg of DHEA. Three pooled urine samples were prepared: QC_L (collected during 16 hours before time of administration), QC_H (collected during 0-8 hours after time of administration) and QC_M (collected during 8-16 hours after time of administration). The QC samples were divided over sample tubes and stored with the other urine samples of the experiment at –20°C until time of analysis.

The intra-assay precision was below 10% for all parameters, except for 7α-OH-DHEA (QC_L) that was <15%. The respective inter-assay precisions were all <20% except for AO and EO (QC_H), 7-keto-AO (QC_L) and the 16α-OH-steroids that were <25%. A relatively high variability of AO and EO in QC_H was caused by column overloading. Due to efficiency reasons no re-analysis of AO and EO was performed after dilution.

The recovery was >95% for all steroid parameters except 16α-OH-DHEA (>90%). All steroid derivatives were stable at room temperature at least 70 hours, except 7-keto-DHEA that was stable at least 24 hours.

Previously, significant 3β-hydroxylase-Δ5-4-isomerase activity in Helix pomatia was reported, resulting in an artificial 20% T production from Δ5-AEDIOL [9]. Use of Helix pomatia for the analysis of a total fraction of non-conjugated steroids and their glucuronides and sulfates, has been validated in this experiment by checking side product formation, after incubation of reference steroids, for every used batch (incubation for 2 hours). No products from 3β-hydroxylase-Δ5-4-isomerase activity were detected. Artificial hydroxylation reactions were not significant (<1%).

Statistical analysis

The calculated $[d_2\text{-}M^{exo}]+[d_0\text{-}M^{endo}]$ values of post-administration samples compared to the mean and standard deviation values of pre-administration samples were tested for significance with Student's one tailed t-test, with $p<0.05$ considered as significant. Testing of differences in A(M+2)/A(M) values were also tested with Student's one tailed *t*-test, with $p<0.01$ considered as significant. Assumed normality of excretion rates was tested with the Kolmogorov test ($p<0.05$).

RESULTS

The relative effect of labeled DHEA administration to two male subjects was established by recording the area ratio A(M+n)/A(M) of the ions at m/z values described in Table 1. In Figures 1a and b the area ratio A(M+n)/A(M) is graphically presented as a function of time, before and after oral administration of labeled DHEA, respectively for non-oxygenated and oxygenated metabolites. Clear differences were observed in kinetic behavior. The response of most metabolites returned to baseline before 30 hours after time of administration. Exceptions were DHEA, Δ5-AEDIOL, EO, 5α,3α-ADIOL and 5β,3α-ADIOL that were still significantly elevated at 50 hours

after time of administration ($p<0.01$). Oxygenated metabolites all approached baseline level before 30 hours after time of administration.

Compounds that showed a large increase in A(M+n)/A(M) were DHEA, AO, EO, T, 5α,3α-ADIOL, 5β,3α-ADIOL, 7β-OH-DHEA, 7-keto-DHEA and 7-keto-AO. Metabolites showing a moderate to no response were Δ5-AEDIOL, E, 7α-OH-DHEA, 16α-OH-DHEA, 16α-OH-AO and 16α-OH-EO. Concentration of labeled 7-keto-AO was below limit of detection during the pre-administration period in subject 2 and during the complete collection period in subject 1. Labeled 7α-OH-DHEA was below limit of detection during the whole collection period in subject 2 and could only be established in some urine samples of subject 1.

The total excretion rate of labeled DHEA metabolites and the excretion rate of endogenous metabolites ($[d_2\text{-}M^{exo}]+[d_0\text{-}M^{endo}]$) is graphically presented as a function of time in Figures 2a and b. The metabolites 5α,3α-ADIOL, 5β,3α-ADIOL, 16α-OH-DHEA, 16α-OH-AO and 16α-OH-EO were not quantified. The effect of labeled DHEA administration is less pronounced than as presented in Figures 1a and b, as it is superposed on the bias caused by random biological variation and circadian rhythm in steroid excretion. Circadian rhythm is most clearly visible in case of DHEA and Δ5-AEDIOL. In Figure 3, the AO/EO and T/E ratios of the calculated $[d_2\text{-}M^{exo}]+[d_0\text{-}M^{endo}]$ values are presented as a function of time.

From the data of $[d_2\text{-}M^{exo}]$ the conversion of d_2-DHEA to the respective metabolites after 24 hours was calculated (see Tables 2 and 3). The conversion to oxygenated metabolites is relatively small compared to non-oxygenated metabolites. The conversion to the latter is, however, mostly contributed by the production of AO and EO.

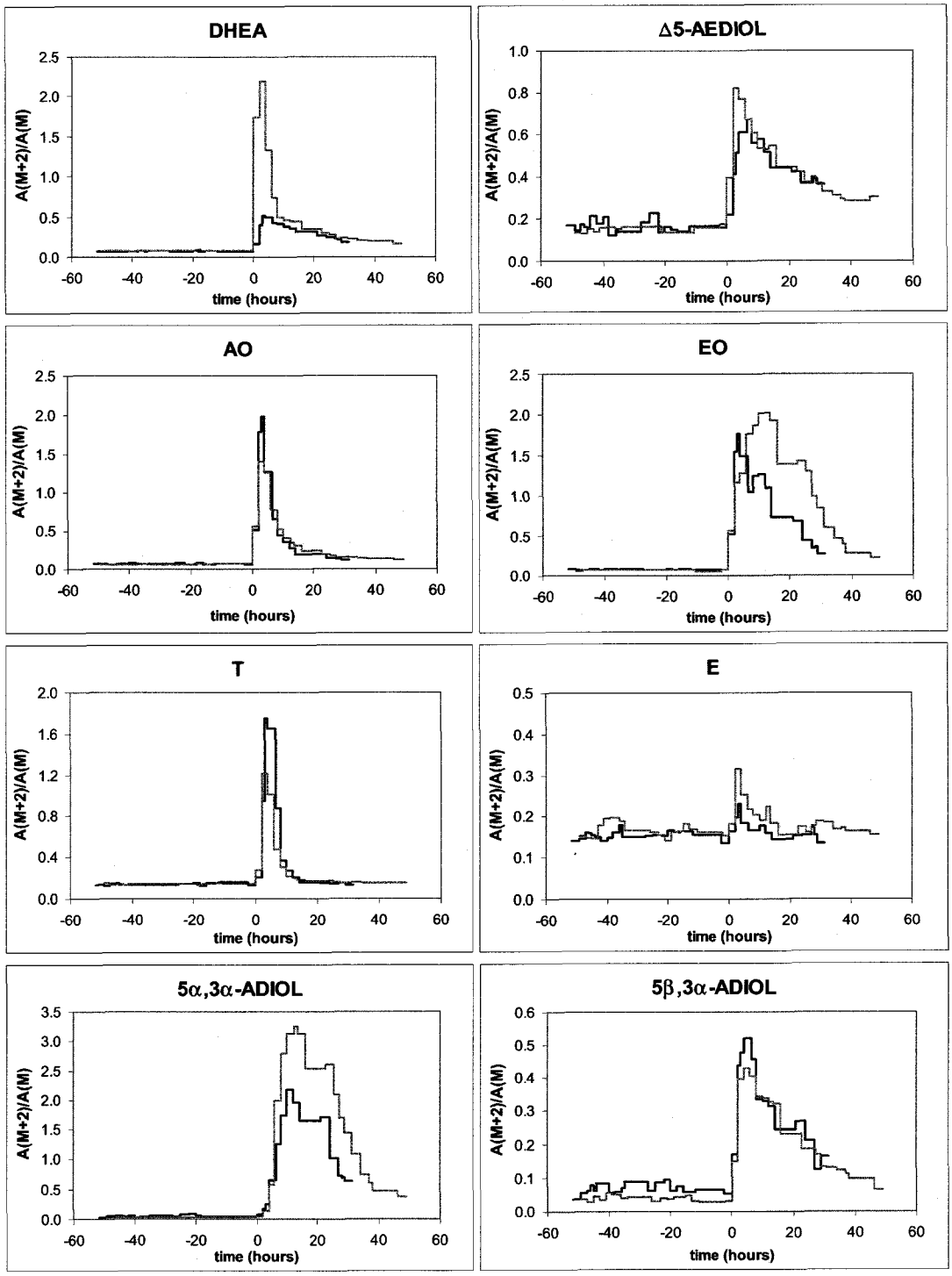


Figure 1a: The area ratio A(M+n)/A(M) of labeled vs. non-labeled DHEA or non-oxygenated metabolites of DHEA as a function of time, in subject 1 (——) and subject 2 (- - -). d_2-DHEA was administered at time-point 0 hours.

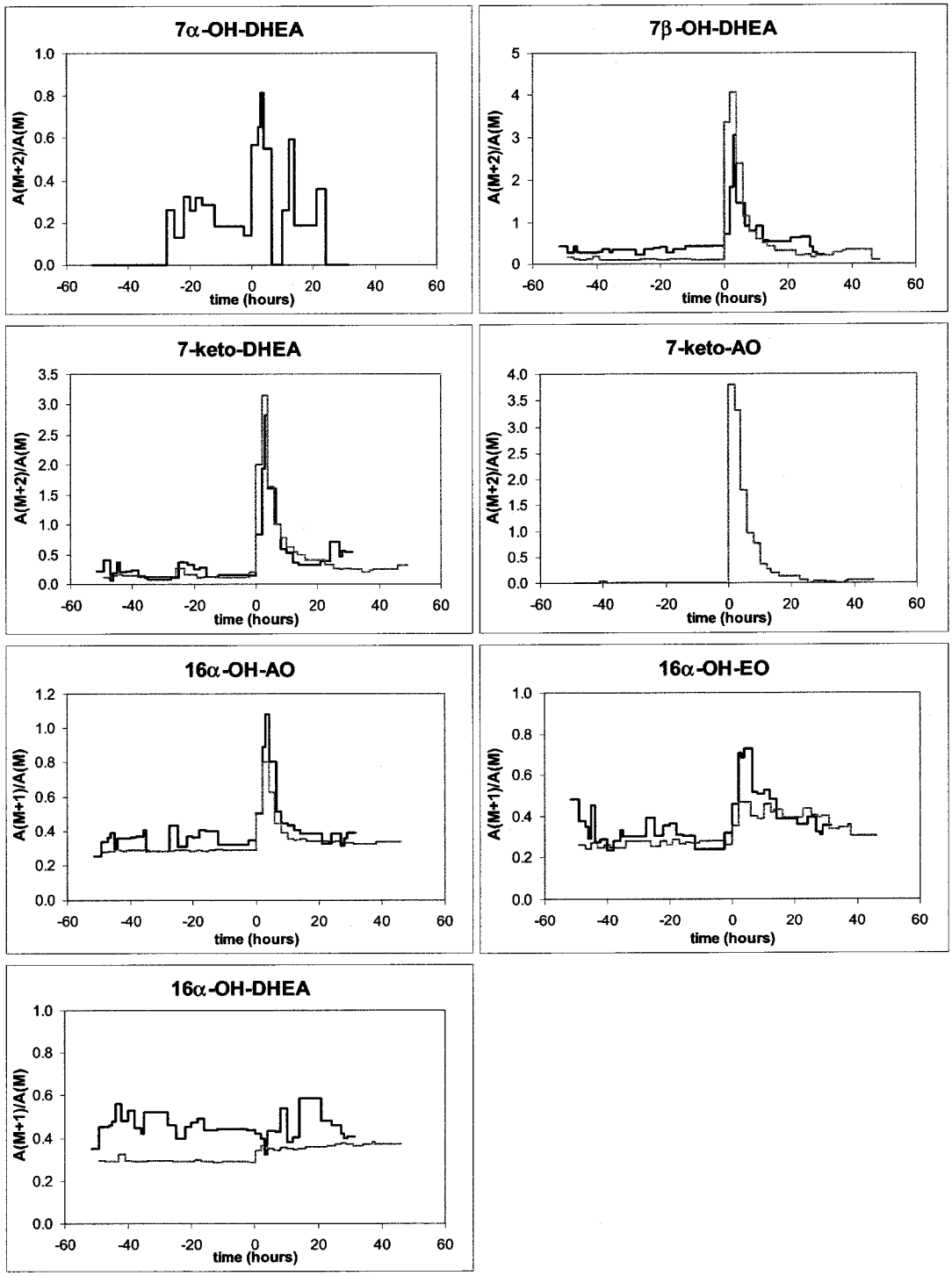


Figure 1b: The area ratio A(M+n)/A(M) of labeled vs. non-labeled DHEA or oxygenated metabolites of DHEA as a function of time, in subject 1 (——) and subject 2 (——).

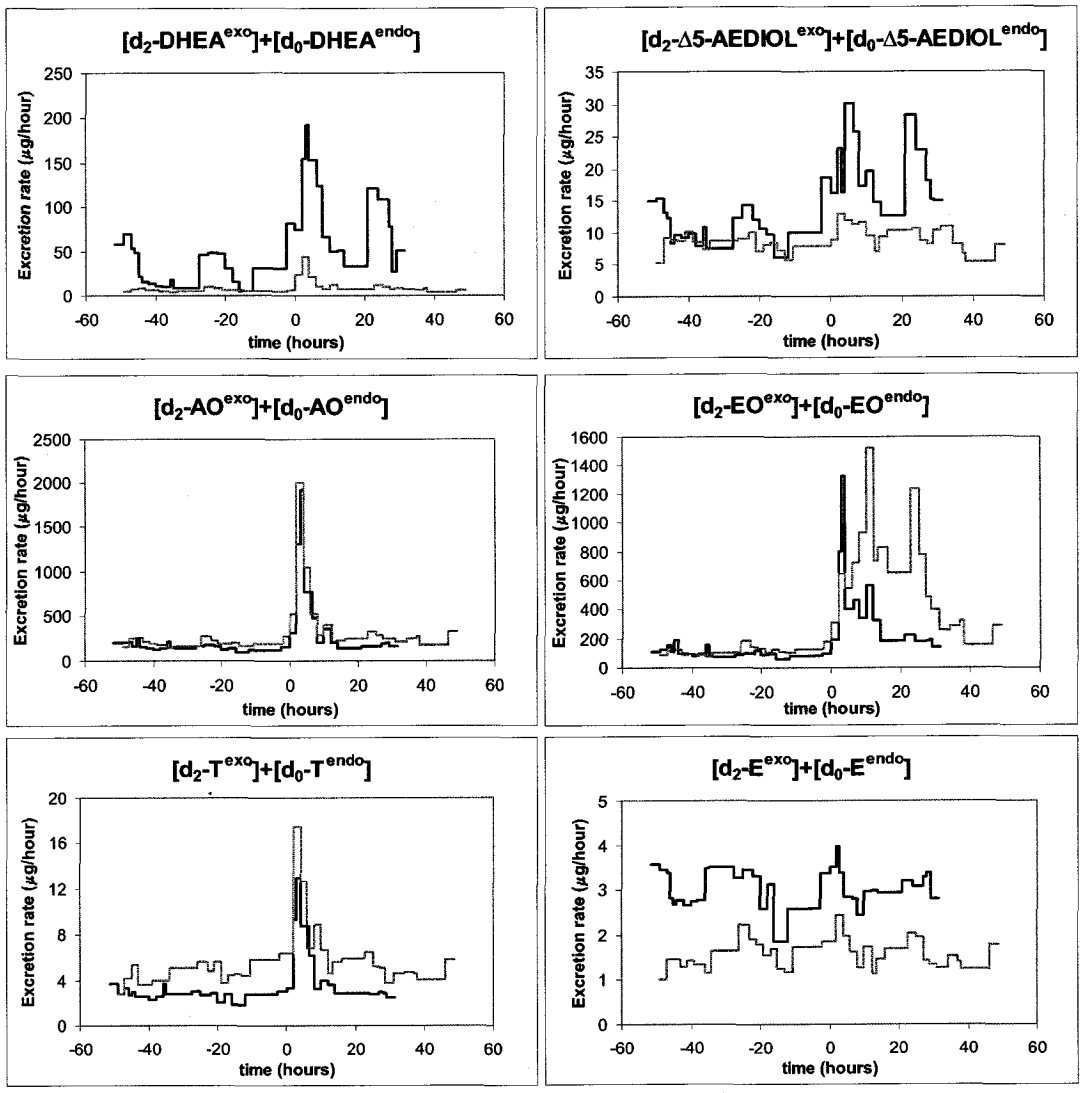
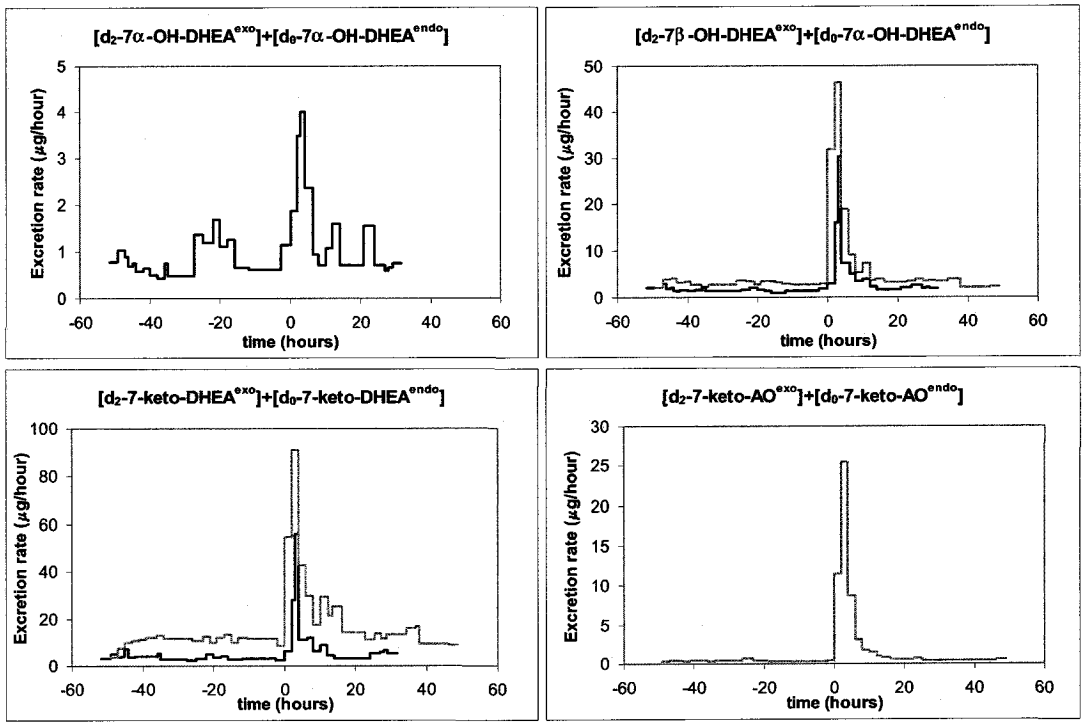


Figure 2a: Excretion rates of the combined labeled [d_2-M^{exo}] and non-labeled [d_0-M^{endo}] DHEA and non-oxygenated metabolites of DHEA as a function of time, in subject 1 (——) and subject 2 (——). d_2-DHEA was administered at time-point 0 hours.



Figuur 2b: Excretion rates of the combined labeled [d_2-M^{exo}] and non-labeled [d_0-M^{endo}] oxygenated metabolites of DHEA as a function of time, in subject 1 (——) and subject 2 (——).

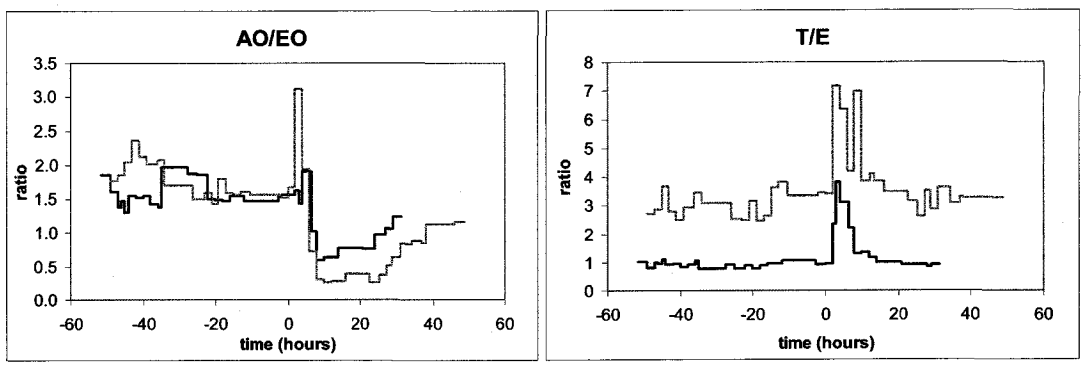


Figure 3: Ratios of metabolite concentrations calculated as [d_2-M^{exo}]+[d_0-M^{endo}], as a function of time, in subject 1 (——) and subject 2 (——). d_2-DHEA was administered at time-point 0 hours.

Table 2: *Conversion (%) of DHEA to the respective metabolites after 24 hours. N.D. = not determined.*

Metabolite (M)	Subject 1 (25.5 mg)	Subject 2 (52.5 mg)
DHEA	2.6	0.36
Δ5-AEDIOL	0.48	0.15
AO	19.9	12.0
EO	21.5	26.1
T	0.16	0.10
E	< 0.01	< 0.01
7α-OH-DHEA	0.03	N.D.
7β-OH-DHEA	0.34	0.45
7-keto-DHEA	0.46	0.72
7-keto-AO	N.D.	0.15

Table 3: *Conversion (%) of DHEA to different groups of metabolites after 24 hours.*

Group of metabolites	Subject 1 (25.5 mg)	Subject 2 (52.5 mg)
AO and EO	41.4	38.1
non-oxygenated	42.0	38.4
oxygenated	0.83	1.3
Total DHEA conversion	42.8	39.7
Total recovery DHEA + metabolites	45.4	40.0

DISCUSSION

Excretion kinetics

In this study the kinetics of DHEA and several of its metabolites were investigated. The excretion behavior of the sum of conjugated and non-conjugated DHEA and several of its metabolites as described in Figures 1a and b differ fundamentally. Roughly, the observed patterns can be divided in three classes:

1. *No change in excretion.* No or little production of the metabolite takes place after administration of d_2-DHEA, as accounts for E, 7α-OH-DHEA and 16α-OH-DHEA.
2. *Rapid and short increase of excretion.* Within 10 hours after administration of d_2-DHEA the respective compound is cleared in high speed, leading to a relatively fast return to baseline values, as accounts for AO, T, 7β-OH-DHEA, 7-keto-DHEA, 7-keto-AO, 16α-OH-AO and 16α-OH-EO.

3. *Rapid and prolonged increase of excretion.* Within 10 hours after administration of d_2-DHEA the excretion of the respective compound increases quickly and returns slowly to baseline values, as accounts for DHEA, EO, AEDIOL, 5α,3α-ADIOL and 5β,3α-ADIOL.

The differences between the kinetic patterns can be explained by the extensive first and second phase metabolic steps of DHEA. Our results show that orally administered DHEA is almost completely converted into metabolites. Through extensive first-pass metabolism, oral administration of DHEA will predominantly lead to production of DHEA sulfate (DHEA-S) [10]. In blood DHEA-S is mainly bound to albumin, in contrast to DHEA [11]. This results in much lower clearance rates for DHEA-S (13 L/day) compared to DHEA (2,000 L/day) and justifies our observation of prolonged excretion of small amounts of DHEA [12]. Conversion of DHEA to first phase metabolites followed by rapid sulfation and/or conversion of DHEA-S to other sulfated metabolites and their subsequent binding to albumin, could explain the prolonged excretion of certain metabolites [13-17]. Those metabolites that will be conjugated with glucuronic acid, are not bound to serum proteins and thus will be excreted rapidly. That could justify the rapid and short excretion of other metabolites.

Our data show a more prolonged excretion of EO than of AO, which indicates that a relation exists between EO and DHEA. It could suggest a relatively higher production of the sulfate of EO compared to that of AO. This presumption is however not supported by earlier results of Rosenfeld *et al.* [18] and Dehennin *et al.* [4] who performed separate analyses of glucuronides and sulfates after DHEA administration. Other possibilities are a rapid and stereoisomer specific conversion of relatively long circulating DHEA and an enterohepatic circulation of EO-glucuronide, resulting in lower clearance efficiency [19].

Sensitivity and specificity

When a parameter is selected as marker for substance abuse, the demands of sensitivity and selectivity should be fulfilled. In this respect, an evaluation of classical parameters for the detection of the abuse of endogenous steroids such as the ratios between AO and EO and between T and E, respectively, is of interest. Both ratios are known to have a limited specificity to establish administration of T, DHEA or Δ4-AEDIONE [1-5,8]. However, in terms of sensitivity they could be of use. In this investigation, the AO/EO ratio initially showed a small increase followed by a significant decrease below baseline level for at least 30-50 hours after DHEA administration (Figure 3). The T/E ratio was increased significantly during 10 hours and was therefore only of use for a shorter period than the AO/EO ratio. These characteristics have also been reported by Kazlauskas *et al.* [3].

The specificity and sensitivity of metabolites as parameter can be interpreted from Figures 1 and 2. The difference between the post-administration A(M+2)/A(M) ratio and the mean pre-administration ratio in Figure 1, is a measure of relative response towards DHEA administration.

Responding parameters, besides DHEA itself, in terms of sensitivity were the metabolites AO, EO, T, 5α3α-ADIOL, 5β,3α-ADIOL, Δ5-AEDIOL, 7β-OH-DHEA, 7-keto-DHEA and 7-keto-AO. Because of the high biological variation due to a high amplitude circadian rhythm, the response of DHEA and Δ5-AEDIOL resulted in only a limited sensitivity (Figure 2). 7-Keto-AO showed a large response in one subject, but could not be detected before and after administration in the other subject. The most sensitive parameters were therefore, AO, EO, T and the 7-oxygenated metabolites 7β-OH-DHEA and 7-keto-DHEA. No conclusions could be drawn about 5α,3α-ADIOL and 5β,3α-ADIOL as those were not quantified in this study.

Interpretation of the 16α-OH metabolites was not possible in these experiments as one deuterium was metabolically removed from the steroid structure due to the hydroxylation. A significant isotope effect due to deuterium-proton exchange can not be disregarded ($[d_2\text{-}M^{endo}]\neq 0$) for these metabolites. Equation (2) is therefore not applicable for 16α-OH-metabolites and have been excluded from further interpretation. Furthermore, significant physiological isotope effects have been reported in case of deuterium labeled substance administration [20]. Due to these physiological isotope effects the rate of metabolic deuterium removal is expected to be lower than the rate of equivalent proton removal, leading to a limitation of 16α-hydroxylation in this particular case.

In terms of specificity it could be argued that the non-oxygenated metabolites are not of interest, because they originate from various steroids. The oxygenated metabolites 7-keto-AO, 16α-OH-AO and 16β-OH-EO seem to be specific as they are reduced metabolites of 7-keto-DHEA and 16α-OH-DHEA. However, they could also be oxygenated metabolites of AO and EO. This is partly illustrated by 16α-hydroxylation of AO by human liver microsomes, as performed by Einarsson *et al.* [21]. The metabolites 7-keto-DHEA, 7α-OH-DHEA, 7β-OH-DHEA and 16α-OH-DHEA can theoretically be expected to be the most specific metabolites as they are directly converted from DHEA [21-25]. Moreover, as the double bond is retained at the Δ5-position in the steroid structure during metabolic conversion, a high sensitivity for DHEA and a low sensitivity towards Δ4-steroids as Δ4-AEDIONE and T can be expected. Based on this investigation at least the 7-oxygenated metabolites appear promising enough to study aspects of specificity in more detail.

LITERATURE

1. V.P. Uralets and P.A. Gillette. Over-the-counter delta5 anabolic steroids 5-androsten-3,17-dione; 5-androsten-3beta, 17beta-diol; dehydroepiandrosterone; and 19-nor-5-androsten-3,17-dione: excretion studies in men. *J. Anal. Toxicol.* **24:** 188-193 (2000).
2. V.P. Uralets and P.A. Gillette. Over-the-counter anabolic steroids 4-androsten-3,17-dione; 4-androsten-3beta,17beta-diol; and 19-nor-4-androsten-3,17-dione: excretion studies in men. *J. Anal. Toxicol.* **23:** 357-366 (1999).
3. R. Kazlauskas. Effects of dehydroepiandrosterone on urinary steroids. In *Recent advances in doping analysis (5)*, W. Schänzer, H. Geyer, A. Gotzmann and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, 1998, pp 83-90.
4. L. Dehennin, M. Ferry, P. Lafarge, G. Pérès and J.-P. Lafarge. Oral administration of dehydroepiandrosterone to healthy men: Alteration of the urinary androgen profile and consequences for the detection of abuse in sport by gas chromatography-mass spectrometry. *Steroids* **63:** 80-87 (1998).
5. T.Z. Bosy, K.A. Moore and A. Poklis. The effect of oral dehydroepiandrosterone (DHEA) on the urinary testosterone/epitestosterone (T/E) ratio in human male volunteers. *J. Anal. Toxicol.* **22:** 455-459 (1998).
6. F. Callies, W. Arlt, L. Siekmann, D. Hubler, F. Bidlingmaier and B. Allolio. Influence of oral dehydroepiandrosterone (DHEA) on urinary steroid metabolites in males and females. *Steroids* **65:** 98-102 (2000).
7. C.H.L. Shackleton, E. Roitman, A. Phillips and T. Chang. Androstanediol and 5-androstenediol profiling for detecting exogenously administered dihydrotestosterone, epitestosterone, and dehydroepiandrosterone: Potential use in gas chromatography isotope ratio mass spectrometry. *Steroids* **62:** 665-673 (1997).
8. L. Bowers. Oral dehydroepiandrosterone supplementation can increase the testosterone/epitestosterone ratio. *Clin. Chem.* **45:** 295-297 (1999).
9. R. Masse, C. Ayotte, and R. Dugal. Studies on anabolic steroids. I. Integrated methodological approach to the gas chromatographic-mass spectrometric analysis of anabolic steroid metabolites in urine. *J. Chromatogr.* **489:** 23-50 (1989).
10. C. Longcope. Metabolism of dehydroepiandrosterone. *Ann. NY Acad. Sci.* **774:** 143-148 (1995).
11. J.F. Dunn, B.C. Nisula and D. Rodbard. Transport of steroid hormones: binding of 21 endogenous steroids to both testosterone-binding globulin and corticosteroid-binding globulin in human plasma. *J. Clin. Endocrinol. Metab.* **53:** 58-68 (1981).
12. C. Longcope. Dehydroepiandrosterone metabolism. *J. Endocrinol.* **150 Suppl:** S125-127 (1996).

13. E.E. Baulieu, C. Corpechot and F. Dray. An andrenal-secreted 'androgen' dehydroepiandrosterone sulfate. Its metabolism and a tentative generalization on the metabolism of other steroid conjugates in man. *Recent Prog. Horm. Res.* **21:** 411-500 (1965).

14. D.Y. Wang and R.D. Bulbrook. Binding of the sulphate esters of dehydroepiandrosterone, testosterone, 17-acetoxypregnenolone and pregnenolone in the plasma of man, rabbit and rat. *J. Endocrinol.* **39:** 405-413 (1967).

15. D.Y. Wang, R.D. Bulbrook, F. Ellis and M. Coombs. Metabolic clearance rates of pregnenolone, 17-acetoxypregnenolone and their sulphate esters in man and in rabbit. *J. Endocrinol.* **39:** 395-403 (1967).

16. D.Y. Wang, R.D. Bulbrook, A. Sneddon and T. Hamilton. The metabolic clearance rates of dehydroepiandrosterone, testosterone and their sulphate esters in man, rat and rabbit. *J. Endocrinol.* **38:** 307-318 (1967).

17. H.E. Rosenthal, E. Pietrzak, W.R. Slaunwhite and A.A. Sandberg. Binding of estrone sulfate in human plasma. *J. Clin. Endocrinol. Metab.* **34:** 805-813 (1972).

18. R.S. Rosenfeld, L. Hellman and T.F. Gallagher. Metabolism and interconversion of dehydroisoandrosterone and dehydroisoandrosterone sulfate. *J. Clin. Endocrinol. Metab.* **35:** 187-193 (1972).

19. E. Hamalainen, T. Fotsis and H. Adlercreutz. A gas chromatographic method for the determination of neutral steroid profiles in urine, including studies on the effect of oxytetracycline administration on these profiles in men. *Clin. Chim. Acta* **199:** 205-220 (1991).

20. A. van Langenhove. Isotope effects: definitions and consequences for pharmacologic studies. *J. Clin. Pharmacol.* **26:** 383-389 (1986).

21. K. Einarsson, J.A. Gustafsson, T. Ihre and M. Ingelman-Sundberg. Specific metabolic pathways of steroid sulfates in human liver microsomes. *J. Clin. Endocrinol. Metab.* **43:** 56-63 (1976).

22. R. Hampl, R. Morfin and L. Starka. Minireview 7-Hydroxylated Derivatives of Dehydroepiandrosterone: What Are They Good For? *Endocr. Regul.* **31:** 211-218 (1997).

23. O. Lapcik, R. Hampl, M. Hill, M. Bicikova and L. Starka. Immunoassay of 7-hydroxysteroids: 1. Radioimmunoassay of 7beta-hydroxy dehydroepiandrosterone. *J. Steroid Biochem. Mol. Biol.* **67:** 439-445 (1998).

24. O. Lapcik, R. Hampl, M. Hill and L. Starka. Immunoassay of 7-hydroxysteroids: 2. Radioimmunoassay of 7alpha-hydroxy-dehydroepiandrosterone. *J. Steroid Biochem. Mol. Biol.* **71:** 231-237 (1999).

25. G.W. Oertel, G. Mappes and W. Wortmann. Metabolism of steroid conjugates. VI. In vivo perfusion of the human liver with synthetic 7-3H-dehydroepiandrostrone sulfatide. *Horm. Metab. Res.* **3:** 193-196 (1971).

5

PROFILING SPECIFIC METABOLITES OF DEUTERIUM LABELED ANDROST-4-ENE-3,17-DIONE

ABSTRACT

Androst-4-ene-3,17-dione (Δ4-AEDIONE) is a steroid that is easily obtainable as food supplement product. Because of assumed anabolic properties, the International Olympic Committee has placed Δ4-AEDIONE on the list of forbidden substances. So far, no techniques have become available to detect Δ4-AEDIONE abuse in a selective way. In this study, a preliminary investigation is performed to construct a Δ4-AEDIONE selective profile of non-oxygenated metabolites (dehydroepiandrosterone, androsterone, etiocholanolone, testosterone, epitestosterone, androst-5-ene-3β,17β-diol, 5α-androst-3α,17β-diol and 5β-androstan-3α-17β-diol) and oxygenated metabolites (6α/β-hydroxy-Δ4-AEDIONE, 4-hydroxy-Δ4-AEDIONE, 16α-hydroxy-Δ4-AEDIONE, 6-keto-Δ4-AEDIONE, 6α/β-hydroxy-testosterone, 16α-hydroxy-androsterone, 16α-hydroxy-etiocholanolone, 11β-hydroxy-androsterone and 11β-hydroxy-etiocholanolone).

Deuterium labeled Δ4-AEDIONE ([2,2,4,6,6,16,16-$^{7}H_2$]-androst-4-ene-3,17-dione) was administered to 2 healthy male volunteers (25.0 and 52.0 mg, respectively) and urine samples were collected. Sample analysis was performed of the total fraction of glucuronides, sulfates and non-conjugated steroids with gas chromatography-mass spectrometry. The most sensitive parameters during the first 10 hours after administration were androsterone, etiocholanolone, testosterone, 6α/β-hydroxy-Δ4-AEDIONE, 4-hydroxy-Δ4-AEDIONE and 6α/β-hydroxy-testosterone. Differences in excretion kinetics were observed between the studied metabolites. The total conversion of Δ4-AEDIONE to the studied metabolites was 39.3-42.1% after 48 hours. The conversion to oxygenated and non-oxygenated metabolites was 0.24-0.90% and 39.1-41.2%, respectively. Although the conversion to oxygenated metabolites was limited, the sensitivity and expected specificity for Δ4-AEDIONE detection of the corresponding metabolites make these potential parameters for possible use in doping analysis applications.

INTRODUCTION

The introduction of steroid containing food supplements on an easily accessible market had a great impact on doping analysis and the doping control system. Androst-4-ene-3,17-dione (Δ4-AEDIONE) was one of the first supplement steroids that was commercially available for athletes. The International Olympic Committee (IOC) placed Δ4-AEDIONE on the List of Forbidden Substances in 1997. The steroids that appeared on the food supplement market extended to androst-5-ene-3β,17β-diol, androst-4-ene-3β,17β-diol and nor-steroids 19-nor-androst-4-ene-3,17-dione and 19-nor-androst-4-ene-3β,17β-diol [1,2].

Non-specific metabolism, extensive first-pass metabolism and fast renal and metabolic clearance aggravate the analysis of orally administered supplement steroids. Analytical procedures for detecting Δ4-AEDIONE have mostly been limited to straightforward steroid profiling techniques, as originally developed for other endogenous steroids as testosterone (T), epitestosterone (E) [3,4] and 5α-dihydrotestosterone [5,6] in urine samples. A typical steroid profile that is analyzed in doping analysis includes, besides the mentioned mother compounds, metabolites as androsterone (AO), etiocholanolone (EO) and androstanediol epimers. This approach leads to mostly unspecific and therefore insufficiently conclusive information about the identity of the administered steroid.

In this study, a preliminary investigation is performed to construct a more steroid specific profile of selected oxygenated and/or non-oxygenated metabolites of Δ4-AEDIONE. Therefore, [2,2,4,6,6,16,16-$^{7}H_2$]-androst-4-ene-3,17-dione (d_7-Δ4-AEDIONE) was administered to 2 healthy male volunteers and urine samples were collected at set times before and after administration. Analysis of the collected urine samples was performed with gas chromatography-mass spectrometry (GC-MS).

EXPERIMENTAL

Chemicals

Reference steroids: Androst-5-en-3β-ol-17-one (dehydroepiandrosterone, DHEA), androst-4-en-17α-ol-3-one (epitestosterone, E), 5α–androstan-3α-ol-17-one (androsterone, AO), 5β–androstan-3α-ol-17-one (etiocholanolone, EO), androst-4-ene-3,17-dione (androstenedione, Δ4-AEDIONE), androst-5-ene-3β,17β-diol (androstenediol, Δ5-AEDIOL), 5α-androstan-3α,11β-diol-17-one (11β-hydroxy-androsterone, 11β-OH-AO), 5β-androstan-3α,11β-diol-17-one (11β-hydroxy-

etiocholanolone, 11β-OH-EO) and 17α-methyl-androst-4-en-11α,17β-diol-3-one (11α-hydroxy-methyltestosterone, 11α-OH-MeT) were obtained from Sigma, St. Louis, Missouri, USA. Androst-4-en-17β-ol-3-one (testosterone, T) was obtained through courtesy of Organon, Oss, The Netherlands. Androst-4-en-6α-ol-7,17-dione (6α-hydroxy-androstenedione, 6α-OH-Δ4-AEDIONE), androst-4-en-6β-ol-7,17-dione (6β-hydroxy-androstenedione, 6β-OH-Δ4-AEDIONE), androst-4-ene-6,7,17-trione (6-keto-androstenedione, 6-keto-Δ4-AEDIONE), androst-4-en-6β,17β-ol-7-one (6β-hydroxy-testosterone, 6β-OH-T), androst-4-en-4-ol-3,17-dione (4-hydroxy-androstenedione, 4-OH-Δ4-AEDIONE), androst-4-en-16α-ol-3,17-dione (16α-hydroxy-androstenedione, 16α-OH-Δ4-AEDIONE), 5α–androstane-3α,16α-diol-17-one (16α-hydroxy-androsterone, 16α-OH-AO), 5β–androstane-3α,16α-diol-17-one (16α-hydroxy-etiocholanolone, 16α-OH-EO) were obtained from Steraloids, Newport, Rhode Island, USA. [2,2,4,6,6,16,16-$^{7}H_2$]-Androst-4-ene-3,17-dione (d_7-Δ4-AEDIONE, isotopic purity 98.4%) was obtained from C/D/N Isotopes, Pointe-Claire, Quebec, Canada.

Chemicals: *N*-Methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA), ethanethiol (97%) and phosphorous pentoxide were obtained from Sigma-Aldrich Chemie Company, Steinheim, Germany. Ammonium iodide was obtained from Fluka Chemie, Buchs, Switzerland. Potassium hydroxide, sodium acetate trihydrate, glacial acetic acid (96%), diethyl ether, acetic acid and methanol were obtained from Merck, Darmstadt, Germany. All chemicals were of analytical grade, unless indicated otherwise.

Hydrolysis was performed with Helix pomatia (type HP-2, containing 110.000 IU/ml β-glucuronidase and 1000-5000 IU/ml arylsulfatase), obtained from Sigma, St. Louis, Missouri, USA.

Columns for solid-phase extraction were IST Isolute C_{18} columns (200 mg, non-endcapped) obtained from Sopachem, Lunteren, The Netherlands.

Sample collection

Two male volunteers (subject 1: 29 years, 88 kg; subject 2: 21 years, 75 kg), collected urine samples every 2 hours during 3 days. On the second day at 9 a.m. d_7-Δ4-AEDIONE was orally administered (subject 1, 25.0 mg; subject 2, 52.0 mg). For each collected urine sample the exact void time, volume and specific gravity (Urine Specific Gravity Refractometer, Atago, Japan) was recorded. Samples were stored at -20°C until time of analysis.

Sample preparation

The volume of urine sampled for the analysis was determined on the basis of specific gravity (d) of the urine, according to:

$$\text{Volume} = \frac{0.020}{d-1} * 5 \text{ ml} \qquad (1)$$

A maximum volume of 15 ml was applied. Solid-phase extraction was performed with C_{18} columns. Before applying urine samples, the column material was preconditioned by washing with 4 ml of methanol and equilibrated with 2 ml of demineralized water. Inorganic material was removed from the column by washing with 2 ml of water. The remaining compounds were collected by elution with 4 ml of methanol. After evaporation to dryness, 2 ml of acetate buffer (0.1 M, pH 5.2) was added to the extract. Hydrolysis was performed by addition 100µl of Helix pomatia followed by incubation for 1 hour at 55°C. Steroids were isolated from the buffer solution by extraction with 5 ml of diethyl ether twice. After extraction the phase separation was optimized by centrifugation (4000 rpm, 5 min). The organic layer was removed and evaporated to dryness under a stream of nitrogen at 40°C. The extract was dried overnight over P_2O_5/KOH under reduced pressure. Derivatization was performed by incubation of the extract in 100 µl of MSTFA/NH_4I/ethanethiol (1000:2:3; v/w/v) at 80°C for 30 min.

Gas chromatography and mass spectrometry

Selected ion monitoring (SIM) was performed with a Hewlett Packard gas chromatograph (Model 5890, Agilent Technologies, Waldbronn, Germany) coupled to a Hewlett Packard quadrupole mass spectrometer (Model 5972A). Ionization was performed in electron ionization mode at 70 eV. Analysis was performed by SIM analysis of specific ions representing the respective deuterated and non-deuterated metabolites (see Table 1). The number of deuterium atoms in Table 1 was based on maximum response after screening of all possible m/z values from $[M]^{\bullet+}$ to $[M+7]^{\bullet+}$. A check for possibly incomplete derivatization was performed by detection of ions representing mono-TMS derivatives of AO and EO at m/z 362.

Gas chromatography was performed with a HP-1 fused silica column (length 18 m, inner diameter 0.2 mm, film thickness 0.11 µm). Via electronic pressure control the column flow (helium) was constant at 1 ml/min. Sample injection of 1 µl was performed in split mode (ratio 1/10). A Hewlett Packard autosampler (Model 7673, Agilent Technologies, Waldbronn, Germany) was used for auto-injection. The injector temperature was set to 250°C. The oven temperature program used was: initial

temperature 180°C, 2°/min to 225°C, 30°/min up to 310°C, held for 5 min. The interface temperature was set to 280°C.

The quality of d_7-Δ4-AEDIONE was checked by gas chromatography-mass spectrometry (GC-MS) in full scan mode prior to administration. No impurities could be detected in a concentrated sample.

Quantification of non-labeled endogenous metabolites ([d_0-M^{endo}])

Calibration samples were prepared by adding 100 µl of methanolic standard solutions to samples of 4 ml of demineralized water. These samples were included in the cleanup procedure with the urine samples. Quantification of non-labeled endogenous steroids ([d_0-M^{endo}]) was performed using two calibration curves for each steroid.

One curve containing five data-points, covered respective ranges: Δ4-AEDIONE (10-50 ng), AO (645-3227 ng), EO (629-3147 ng), T (80-400 ng), E (20-100 ng), DHEA (493-2467 ng), Δ5-AEDIOL (432-2160 ng), 16α-OH-Δ4-AEDIONE (25-125 ng), 6α-OH-Δ4-AEDIONE (10-50 ng), 6β-OH-Δ4-AEDIONE (10-50 ng), 6β-OH-T (12-60 ng), 16α-OH-AO (347-1733 ng), 16α-OH-EO (339-1016 ng), 6-keto-Δ4-AEDIONE (20-100 ng). The second calibration curve contained 6 data-points and covered the respective ranges: Δ4-AEDIONE (50-960 ng), AO (3227-61951 ng), EO (3147-60415 ng), T (400-7680 ng), E (100-1,920 ng), DHEA (2467-47360 ng), Δ5-AEDIOL (2160-41472 ng), 16α-OH-Δ4-AEDIONE (125-2400 ng), 6α-OH-Δ4-AEDIONE (50-960 ng), 6β-OH-Δ4-AEDION (50-960 ng), 6β-OH-T (60-1152 ng), 16α-OH-AO (1733-33280 ng), 16α-OH-EO (1016-32512 ng), 6-keto-Δ4-AEDIONE (100-1920 ng). 11α-OH-MeT (500 ng) was used as internal standard. Weighted regression analysis ($1/x^2$) was used for calculation of the calibration curve.

As described in Chapter 8, androst-4-ene-3-one steroids that are hydroxylated on C^6 lose the stereochemical integrity at this position after derivatization under enolizing conditions [7,8]. This resulted in the conversion of 6α-OH-Δ4-AEDIONE and 6β-OH-Δ4-AEDIONE to identical 3,5,16-triene-3,6,17-triol TMS products (6α/β-OH-AEDIONE). This was also the case for 6α-OH-T and 6β-OH-T (6α/β-OH-T). The applied range of quantification for 6α/β-OH-Δ4-AEDIONE was therefore 20-100 ng and 100-1920 ng, respectively for the used calibration curves.

Table 1: *Monitored m/z values of labeled and non-labeled derivatives of Δ4-AEDIONE and metabolites.*

Steroid derivative	m/z value non-labeled	m/z value labeled	number of deuterium atoms in derivative (n)
Δ4-AEDIONE	430.3	435.3	5
AO	434.3	440.3	6
EO	434.3	440.3	6
DHEA	432.3	437.3	5
Δ5-AEDIOL	434.3	440.3	6
T	432.3	438.3	6
E	432.3	438.3	6
5α,3α-ADIOL	241.2	246.2	5
5α,3β-ADIOL	241.2	246.2	5
6α/β-OH-Δ4-AEDIONE	518.4	522.4	4
4-OH-Δ4-AEDIONE	518.4	522.4	4
16α-OH-Δ4-AEDIONE	503.4	507.4	4
6-keto-Δ4-AEDIONE	516.4	520.4	4
16α-OH-AO	507.4	512.4	5
16α-OH-EO	507.4	512.4	5
6α/β-OH-T	520.4	525.4	5
11β-OH-AO	522.4	528.4	6
11β-OH-EO	522.4	528.4	6

Quantification of labeled steroids

The concentration of labeled steroids [d_n-M^{exo}] that were excreted could only be quantified in an indirect way, since synthetic labeled metabolites were not available. The values of [d_n-M^{exo}] were established by calculation of the concentration of unlabeled metabolites [d_0-M^{endo}], and the area ratio of the detected labeled metabolites (A(M+n)) vs. the respective non-labeled metabolites (A(M)). [d_0-M^{endo}] was established by calibration as described above. Calculation of [d_n-M^{exo}] was performed with equation (2) (see also Dehennin *et al.* [9]).

$$R = \frac{A(M+n)}{A(M)} = 0.795 * \frac{[d_n - M^{exo}]}{[d_0 - M^{endo}]} + R^0 \qquad \textbf{(2)}$$

Equation (2) was deduced by applying the mean area ratio A(M+2)/A(M), as determined in pre-administration samples (=R^0) and A(M+n)/A(M) in standard of Δ4-AEDIONE and d_7-Δ4-AEDIONE (=0.795*([d_n-M^{exo}]/[d_0-M^{endo}]). The calculation was performed with the assumption that no proton-deuterium exchange occurred, so no non-labeled metabolites of exogenous origin were detected ([d_0-M^{exo}]=0). This was

considered valid, as 4-6 deuterium atoms were present in the steroid structure of the metabolites.

R^0 was equal to zero for most metabolites, as $[d_n\text{-}M^{endo}]$ was negligible. Due to insufficient specificity of the applied GC-MS method, $R^0>0$ in case of Δ4-AEDIONE, DHEA, E, 16α-OH-Δ4-AEDIONE and 6α/β-OH-Δ4-AEDIONE.

Validation

Quality control (QC) samples were prepared according the following procedure: A healthy male subject was administered two capsules of each 100 mg of Δ4-AEDIONE. Three pooled urine samples were prepared: QC_L (collected during 16 hours before time of administration), QC_H (collected during 0-8 hours after time of administration) and QC_M (collected during 8-16 hours after time of administration). The QC samples were divided over sample tubes and stored with the other urine samples of the experiment at –20°C until time of analysis.

The intra-assay precision was below 10% for all parameters, except 16α-OH-AO (QC_H) and 6α/β-OH-T (QC_L and QC_M) were <15%. Δ4-AEDIONE (all QC samples) was <20% and 6-keto-Δ4-AEDIONE (QC_M) was <40%. The respective inter-assay precisions were <20 %; except for AO and EO (QC_H), 4-OH-Δ4-AEDIONE and 6α/β-OH-Δ4-AEDIONE (QC_L) and 16α-OH-steroids that were <25%; Δ4-AEDIONE and 6-keto-Δ4-AEDIONE (QC_L and QC_M) were <35%. Based on these results the decision was made to exclude Δ4-AEDIONE and 6-keto-Δ4-AEDIONE from the quantification procedure. The relatively high variability of AO and EO in QC_H was caused by column overloading.

The recovery was >95% for all steroid parameters. All steroid derivatives were stable at room temperature for at least 70 hours. As also described in Chapter 4, the significance of side-reactions occurring in the Helix pomatia incubation mixture was tested. No products from 3β-hydroxylase-Δ5-4-isomerase activity were detected. Artificial hydroxylation reactions were not significant (<1%).

Statistical analysis

The calculated $[d_n\text{-}M^{exo}]+[d_0\text{-}M^{endo}]$ values of post-administration samples compared to the mean and standard deviation values of pre-administration samples were tested for significance with Student's one tailed t-test, with $p<0.05$ considered as significant. Assumed normality of excretion rates was tested with the Kolmogorov test ($p<0.05$).

RESULTS

In Figure 1 the recorded A(M+n)/A(M) ratio of Δ4-AEDIONE is shown as a function of time for both subjects. In blank urine samples only low concentrations of Δ4-AEDIONE were detected. Also, after administration minor quantities of d_7-Δ4-AEDIONE were recovered.

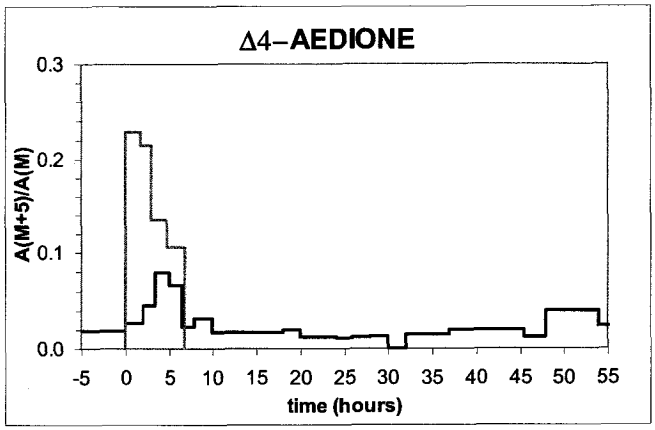


Figure 1: The area ratio A(M+n)/A(M) of labeled vs. non-labeled Δ4-AEDIONE as a function of time, in subject 1 (——) and subject 2 (——). d_7-Δ4-AEDIONE was administered at time-point 0 hours.

In Figures 2a and b the recorded A(M+n)/A(M) ratio is shown as a function of time for the non-oxygenated and oxygenated metabolites. In these results [d_0-6-keto-Δ4-AEDIONE] was below the limit of detection in both subjects, and was therefore excluded. Due to chromatographic co-elution of 5α,3α-ADIOL and 5β,3α-ADIOL could only be determined in samples of one subject.

Excretion of the labeled steroids returned to baseline within 20 hours, except for 5α,3α-ADIOL, 5β,3α-ADIOL, Δ5-AEDIOL and EO that were increased for more than 30 hours after time of administration. The maximum response of AO was around 10% higher than that of EO, but between 5-35 hours after time of administration the response of EO was higher. The parameters that showed the largest response in A(M+n)/A(M) were AO, EO, T, 6α/β-OH-Δ4-AEDIONE, 6α/β-OH-T and 4-OH-Δ4-AEDIONE.

In Figures 3a and b the total excretion rate of labeled and endogenous metabolites ($[d_n\text{-}M^{exo}]+[d_0\text{-}M^{endo}]$) is presented as a function of time for both subjects. The parameters Δ4-AEDIONE, 6-keto-Δ4-AEDIONE, 5α,3α-ADIOL, 5β,3α-ADIOL, 11β-OH-AO and 11β-OH-EO were not included in the quantification procedure. As the excretion of labeled Δ4-ADIONE metabolites is superposed on the biological variation of non-labeled steroid excretion, the presented response in Figures 3a and b is less pronounced as presented in Figures 2a and b. The excretion rate of all metabolites was back to baseline level at around 10 hours after administration. The metabolites that show the most significant effect were AO, EO, T, 6α/β-OH-Δ4-AEDIONE, 4-OH-Δ4-AEDIONE and 6α/β-OH-T.

The calculated AO/EO and T/E ratio based on $[d_n\text{-}M^{exo}]+[d_0\text{-}M^{endo}]$ values are shown in Figure 4. In subject 1 the AO/EO showed an increase during the first 10 hours with a maximum of 40%, followed by a decrease (40% maximum) until 25-30 hours after time of administration. In subject 2 the AO/EO ratio was only decreased (70% maximum decrease) during 25-30 hours after time of administration. For subject 1 and 2, the T/E ratio was increased from 0.9 to 2.4 (170%) and 2.5 to 14.2 (470%), respectively.

In Tables 2 and 3 an outline is given of the calculated conversion of Δ4-AEDIONE to the respective metabolites after 24 and 48 hours, as calculated by the total excreted amount for each metabolite vs. the applied dose. For the major part, Δ4-AEDIONE was converted to AO and EO. Only 1-2 % was converted to other studied metabolites. Conversion to oxygenated metabolites was 0.2-0.9%. The total recovery of Δ4-AEDIONE that was converted after 48 hours was 39-42%. Of the total conversion, 90-95% occurred within the first 24 hours after administration.

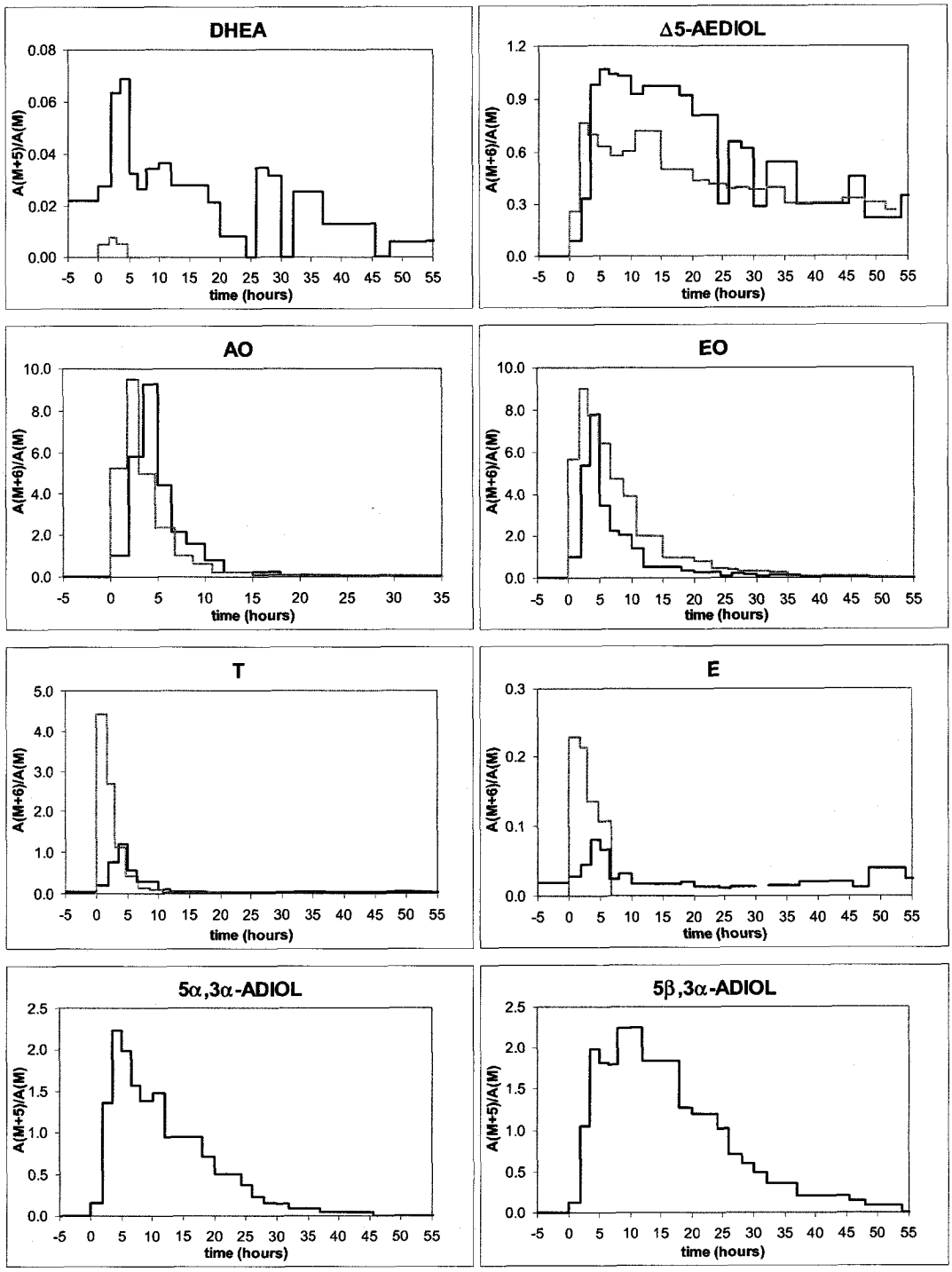


Figure 2a: The area ratio A(M+n)/A(M) of labeled vs. non-labeled, non-oxygenated metabolites of Δ4-AEDIONE as a function of time, in subject 1 (——) and subject 2 (——). d_7-Δ4-AEDIONE was administered at time-point 0 hours.

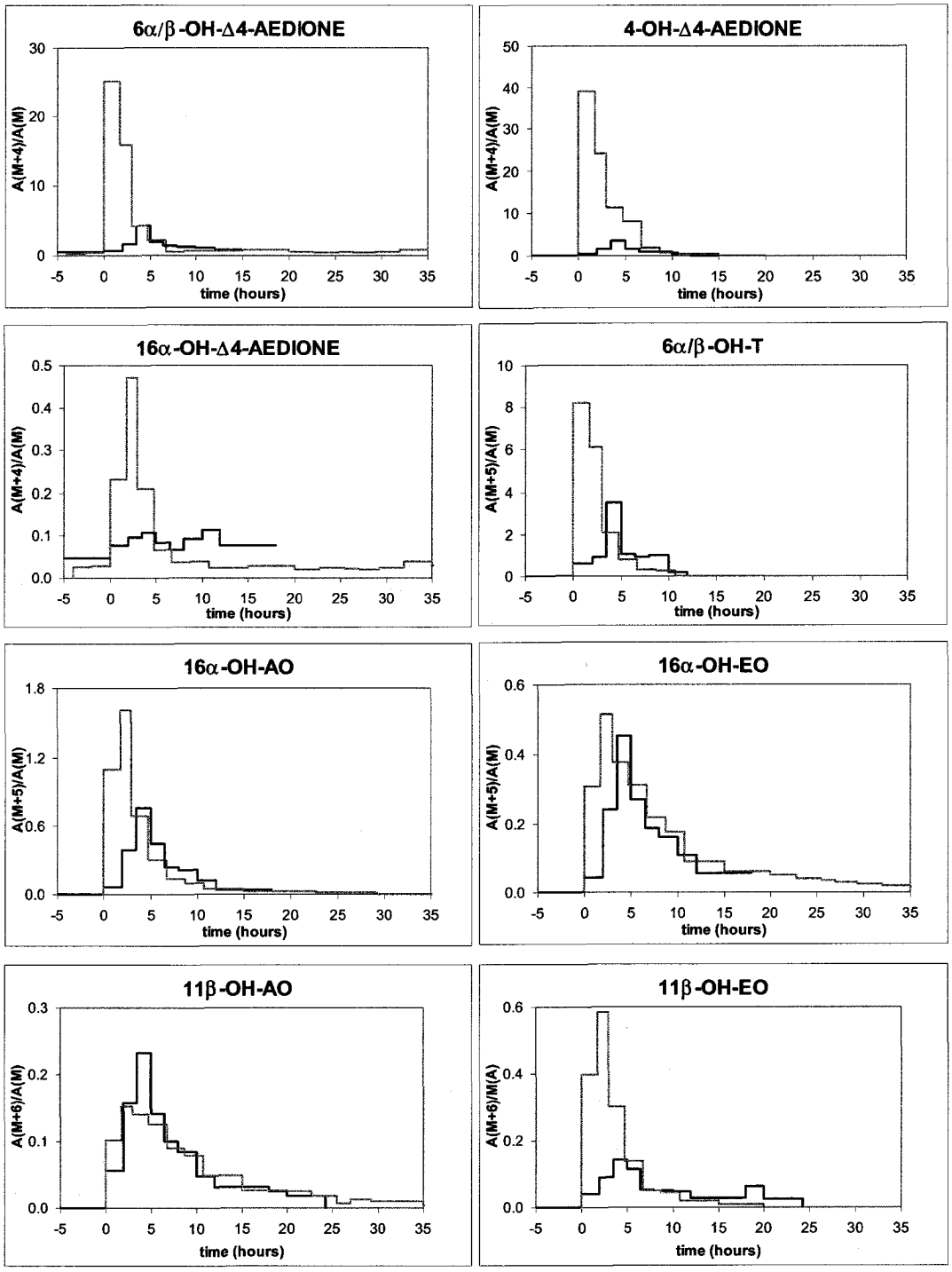


Figure 2b: The area ratio A(M+n)/A(M) of labeled vs. non-labeled, oxygenated metabolites of Δ4-AEDIONE as a function of time, in subject 1 (——) and subject 2 (- - - -).

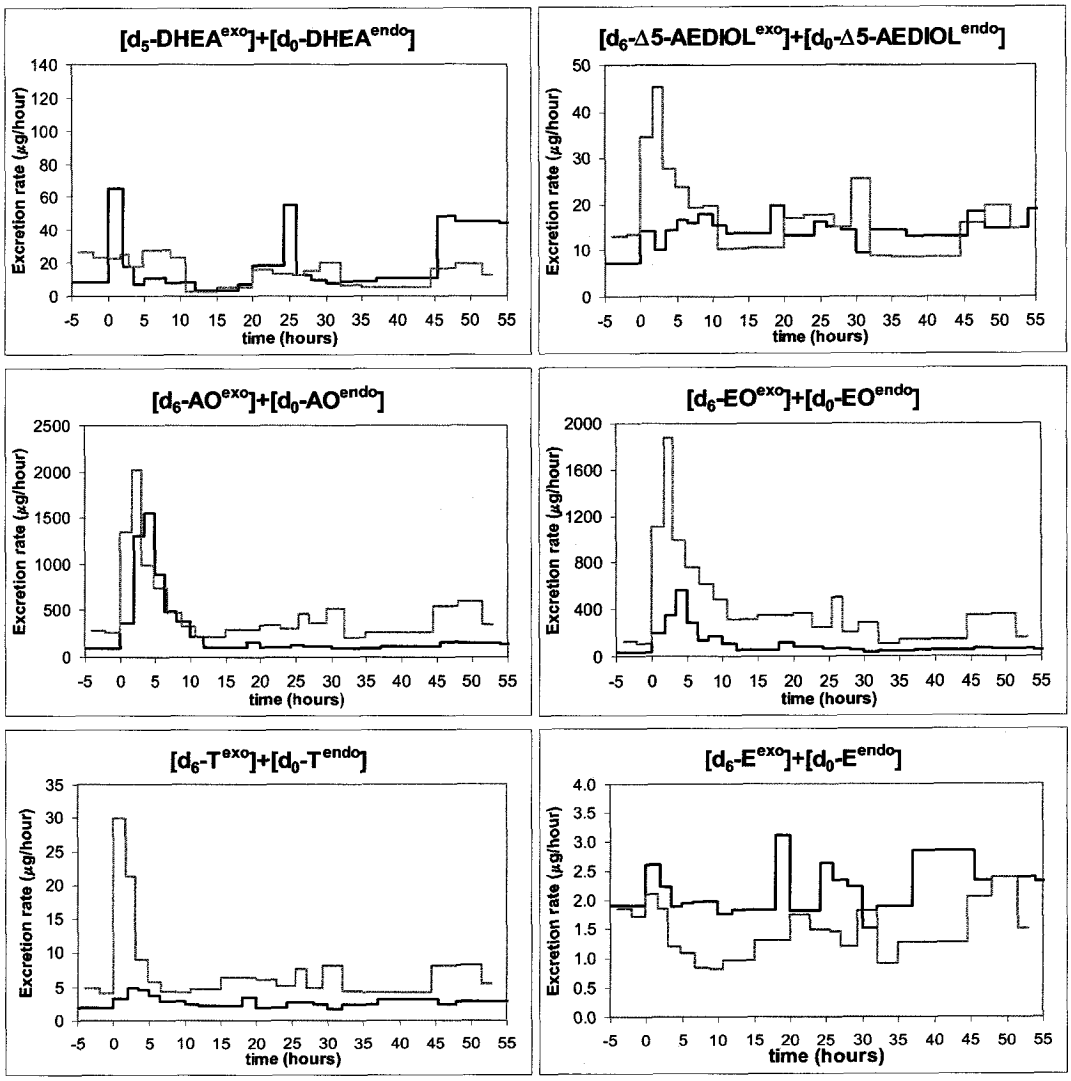
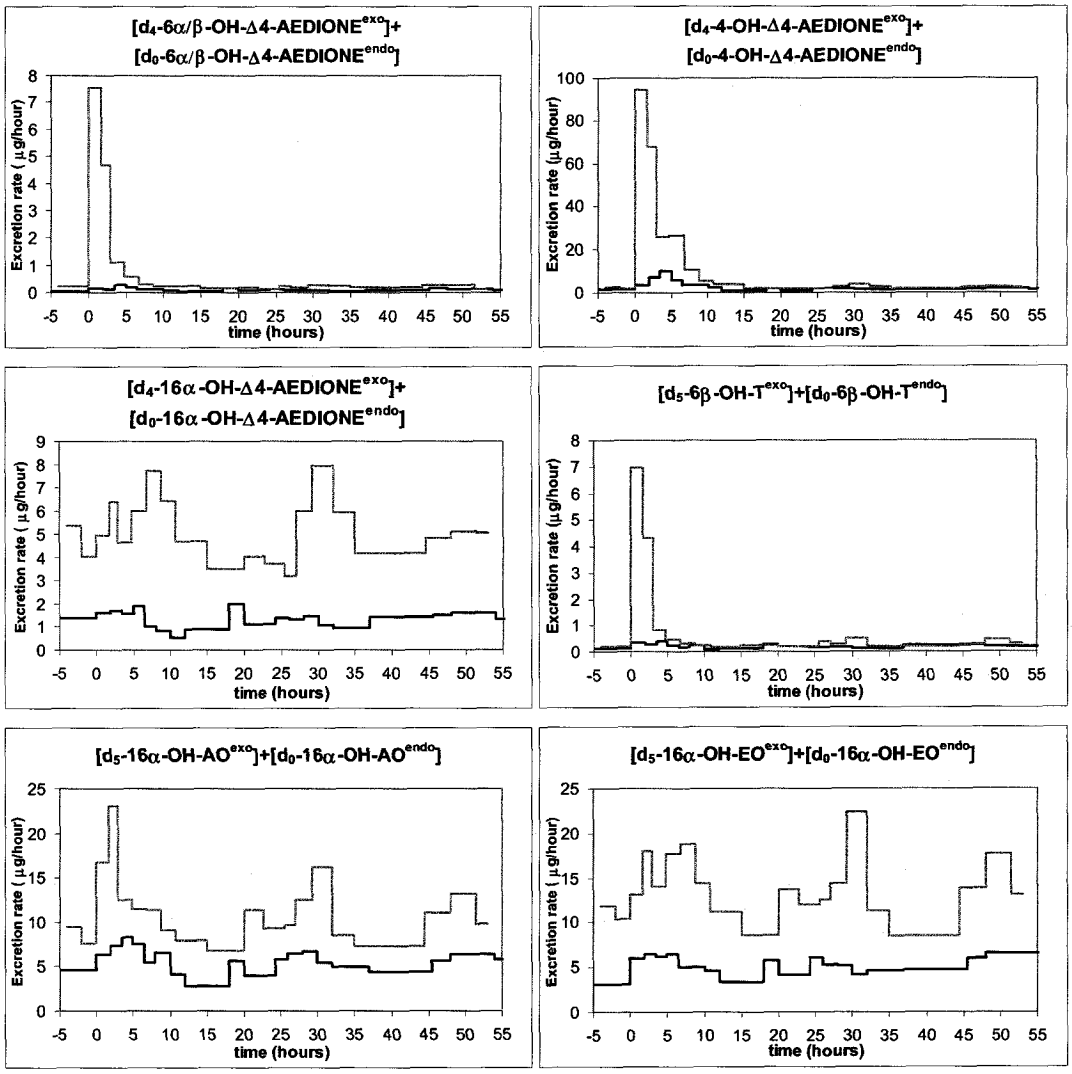


Figure 3a: Excretion rates of the combined labeled [d_n-M^{exo}] and non-labeled [d_0-M^{endo}], non-oxygenated metabolites of Δ4-AEDIONE as a function of time, in subject 1 (——) and subject 2 (——). d_7-Δ4-AEDIONE was administered at time-point 0 hours.



Figuur 3b: Excretion rates of the combined labeled [d_n-M^{exo}] and non-labeled [d_0-M^{endo}], non-oxygenated metabolites of Δ4-AEDIONE as a function of time, in subject 1 (——) and subject 2 (——).

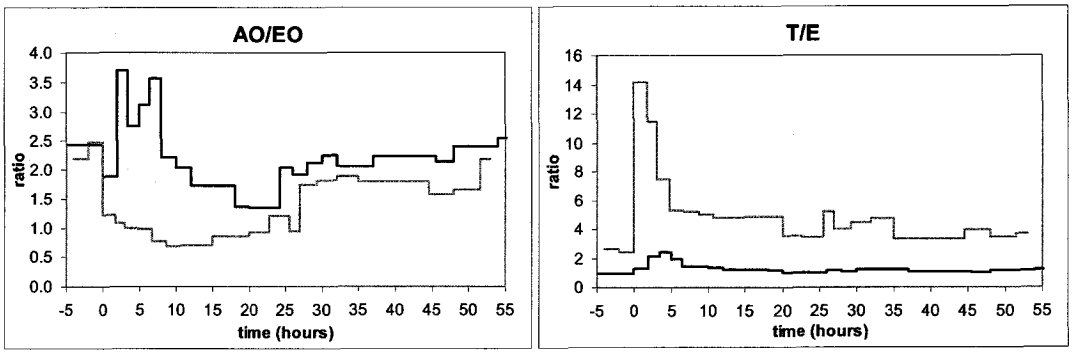


Figure 3: Ratio's of metabolite concentrations calculated as $[d_n\text{-}M^{exo}]+[d_0\text{-}M^{endo}]$, as a function of time, in subject 1 (——) and subject 2 (——). d_7-Δ4-AEDIONE was administered at time-point 0 hours.

Table 2: *Conversion (%) of Δ4-AEDIONE to the respective metabolites after 24 hours.*

Metabolite (M)	Subject 1 25.0 mg) after 24 hours	Subject 1 25.0 mg) after 48 hours	Subject 2 (52.0 mg) after 24 hours	Subject 2 (52.0 mg) after 48 hours
DHEA	0.02	0.02	<0.01	<0.01
Δ5-AEDIOL	0.70	1.2	0.35	0.57
AO	26.8	27.0	16.1	16.5
EO	10.3	10.8	21.6	24.0
T	0.05	0.05	0.16	0.16
E	<0.01	<0.01	<0.01	<0.01
6α/β-OH-Δ4-AEDIONE	<0.01	<0.01	0.04	0.04
6α/β-OH-T	<0.01	<0.01	0.03	0.03
4-OH-Δ4-AEDIONE	0.12	0.12	0.59	0.59
16α-OH-Δ4-AEDIONE	<0.01	<0.01	0.01	0.01
16α-OH-AO	0.07	0.07	0.10	0.11
16α-OH-EO	0.05	0.05	0.10	0.11

Table 3: *Conversion (%) of Δ4-AEDIONE to different groups of metabolites.*

Metabolites	Subject 1 (25.0 mg) after 24 hours	Subject 1 (25.0 mg) after 48 hours	Subject 2 (52.0 mg) after 24 hours	Subject 2 (52.0 mg) after 24 hours
AO and EO	37.1	37.8	37.7	40.5
non-oxygenated	37.9	39.1	38.2	41.2
oxygenated	0.24	0.24	0.87	0.89
Total	38.1	39.3	39.1	42.1

DISCUSSION

Excretion kinetics

Our results show different patterns for the excretion of produced metabolites (Figures 2a and b). DHEA, E, 16α-OH-Δ4-AEDIONE, 11β-OH-AO and 11β-OH-EO showed no, or a limited response to Δ4-AEDIONE administration (compare class 1 in Chapter 4). A rapid and short increase of excretion was observed for AO, T, 6α/β-OH-Δ4-AEDIONE, 4-OH-Δ4-AEDIONE, 6α/β-OH-T and 16α-OH-AO (class 2). Within 10 hours after administration, the respective metabolites were cleared in high speed, leading to relatively fast return to baseline values. The other studied metabolites Δ5-AEDIOL, EO, 5α,3α-ADIOL, 5β,3α-ADIOL and 16α-OH-EO showed a rapid and prolonged increase of excretion (class 3). Within 10 hours after administration the respective metabolites were cleared in relatively low speed, leading to a slow return to baseline level. This was illustrated most clearly in case of labeled Δ5-AEDIOL, which could be detected up to 100 hours post-administration (data not shown).

As has also been described in Chapter 4, kinetic differences can be explained by the extensive first and second phase metabolism of Δ4-AEDIONE. Our results show that Δ4-AEDIONE is almost completely metabolized, to mostly non-oxygenated metabolites. As in agreement with Uralets *et al.* [1], negligible amounts of labeled Δ4-AEDIONE itself were recovered.

A low renal clearance was shown previously for sulfate conjugates of DHEA, T, pregnenolone, estrone and cholesterol as compared to glucuronides, because in blood those steroids are mainly bound to albumin [10-13]. In contrast to DHEA metabolism, insufficient data are available about the second phase metabolism of orally administered Δ4-AEDIONE. As no separate analysis of the different conjugates was performed in this experiment, no further evidence was obtained.

As also reported by Uralets *et al.* [1] AO and EO are main metabolites of Δ4-AEDIONE. In the present experiment, data show a more prolonged excretion of EO compared to AO, leading to an AO/EO ratio that was significantly decreased until 30 hours after administration. This can be accounted to a relatively high production of the EO sulfate as compared to AO sulfate. Another explanation could be found in enterohepatic circulation of EO-glucuronide, resulting in lower clearance efficiency [14].

Sensitivity and selectivity

When sensitivity of a parameter is defined as the maximum response of either A(M+n)/A(M) or ($[d_n\text{-}M^{exo}]$ + $[d_0\text{-}M^{endo}]$), then AO, EO, T, 6α/β-OH-Δ4-AEDIONE, 4-

OH-Δ4-AEDIONE and 6α/β-OH-T can be regarded as most sensitive (see Figures 1 and 2). The T/E ratio can also be considered as a sensitive parameter in this experiment.

The detection of 6α-OH-Δ4-AEDIONE glucuronide after Δ4-AEDIONE administration was previously reported in a case study [15]. However, regarding the applied derivatization procedure the identification is unclear as 6α-OH-Δ4-AEDIONE and 6β-OH-Δ4-AEDIONE form an identical derivative (see Chapter 8). Also no excretion data were reported. In our results the combined 6α/β-OH-Δ4-AEDIONE showed a relatively high sensitivity. This was also the case for 6α/β-OH-T. As explained in Chapter 3, the sensitivity of these steroids can most likely be assigned to the 6β-OH-metabolites.

4-OH-Δ4-AEDIONE was also detected as a sensitive metabolite, as illustrated by its higher relative response in subject 2 than for AO and EO. The endogenous origin of this steroid has not been described previously. It has, however, frequently been applied as an effective aromatase inhibitor in cancer therapy. In a clinical trial report, no endogenous 4-OH-Δ4-AEDIONE was detected in plasma before administration of this steroid [16]. However, hydroxylation at C^4 is a relevant metabolic route for estrogens, leading to catecholestrogens [17,18]. As described in Chapter 3, insufficient evidence is available to exclude the endogenous origin of 4-OH-Δ4-AEDIONE or the conversion to this steroid after oral administration of Δ4-AEDIONE.

No production of Δ5-steroids was expected, as the conversion of Δ5- to Δ4-steroids is usually considered as an irreversible step in steroid biosynthesis. However, limited amounts of labeled Δ5-AEDIOL and negligible amounts of labeled DHEA were formed. This could suggest a relatively slow conversion of Δ4-AEDIONE to DHEA, possibly followed by immediate other metabolic steps as 17-hydrogenation to Δ5-AEDIOL or hydroxylation (as have been studied in Chapter 4).

In a previously reported case study [19], 11β-OH-AO and 11β-OH-EO were mentioned as suspected metabolites of Δ4-AEDIONE. The involved steroid 11β-hydroxylation is an important step in cortisol biosynthesis. In this case, the relative response of those steroids was very limited, which can be explained by the localization of 11β-hydroxylase activity in adrenal tissue [20].

Specificity for Δ4-AEDIONE was in the first place expected for oxygenated derivatives of this steroid, as 6α/β-OH-Δ4-AEDIONE, 4-OH-Δ4-AEDIONE, 16α-OH-Δ4-AEDIONE and 6-keto-Δ4-AEDIONE. All those parameters except 6-keto-Δ4-AEDIONE extensively responded to Δ4-AEDIONE administration. Prior to the experiment also 19-hydroxy-androst-4-ene-3,17-dione was evaluated in a pilot study (data not shown). This potential metabolite [21-23] could not be identified in blank

and post-administration samples, and was therefore excluded from this experiment. The reduced metabolites 16α-OH-AO and 16α-OH-EO were expected to be either reduced metabolites of 16α-OH-Δ4-AEDIONE or 16α-hydroxy metabolites of AO and EO, respectively. Whether 6α/β-OH-T are reduced metabolites of 6α/β-OH-Δ4-AEDIONE or are 6α/β-OH-metabolites of testosterone, is also not clear.

Care should be taken with the extrapolation of these data to a situation of non-labeled Δ4-AEDIONE administration. Significant physiological isotope effects have been reported in case of deuterium labeled substance administration [24]. Due to these physiological isotope effects the rate of metabolic deuterium removal is lower than of equivalent hydrogen removal, leading to a possible limitation of 4-, 6α-, 6β and 16α-hydroxylation in this particular case.

SUMMARY

Several oxygenated steroids were introduced that could provide sensitive and specific information about administration of Δ4-AEDIONE, although the conversion to those steroids was limited (<1%). The highest sensitivity for detection of Δ4-AEDIONE was obtained for AO, EO, T, 6α/β-OH-Δ4-AEDIONE, 4-OH-Δ4-AEDIONE and 6α/β-OH-T.

LITERATURE

1. V.P. Uralets and P.A. Gillette. Over-the-counter anabolic steroids 4-androsten-3,17-dione; 4-androsten-3beta,17beta-diol; and 19-nor-4-androsten-3,17-dione: excretion studies in men. *J. Anal. Toxicol.* **23:** 357-366 (1999).

2. V.P. Uralets and P.A. Gillette. Over-the-counter delta5 anabolic steroids 5-androsten-3,17-dione; 5-androsten-3beta, 17beta-diol; dehydroepiandro-sterone; and 19-nor-5-androsten-3,17-dione: excretion studies in men. *J. Anal. Toxicol.* **24:** 188-193 (2000).

3. M. Donike, K.-R. Bärwald, K. Klostermann, W. Schänzer and J. Zimmermann. Nachweis von exogenem Testosteron. In *Sport: Leistung und Gesundheit*, H. Heck, W. Hollmann, H. Liesen, R. Rost, Eds. Deutscher Ärzte Verlag, Köln, 1983, pp 293-298.

4. M. Donike. Steroid profiling in Cologne. In *Proceedings of the 10th Cologne workshop on dope analysis*, M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke and S. Rauth, Eds. Sport und Buch Strauss, Köln, 1993, pp 47-68.

5. A.T. Kicman, S.B. Coutts, C.J. Walker and D.A. Cowan. Proposed confirmatory procedure for detecting 5α-dihydrotestosterone doping in male athletes. *Clin. Chem.* **41:** 1617-1627 (1995).

6. M. Donike, M. Ueki, Y. Kuroda, H. Geyer, E. Nolteernsting, S. Rauth, W. Schanzer, U. Schindler, E. Volker and M. Fujisaki. Detection of dihydrotestosterone (DHT) doping: alterations in the steroid profile and reference ranges for DHT and its 5 alpha-metabolites. *J. Sports Med. Phys. Fitness.* **35:** 235-250 (1995).

7. D.H. van de Kerkhof, J.W. Zwikker, V.E.M. Kaats-Richters, K. Bouwman, R.D. van Ooijen, D. de Boer and R.A,.A. Maes. Trimethylsilylation of 3-keto-Δ4-ene steroids: thermodynamic versus kinetic control of enolization. In *Recent advances in doping analysis (8)*, W. Schänzer, H. Geyer, A. Gotzmann and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, 2000, pp 129-140.

8. W. Schanzer, S. Horning and M. Donike. Metabolism of anabolic steroids in humans: synthesis of 6 beta-hydroxy metabolites of 4-chloro-1,2-dehydro-17alpha-methyltestosterone, fluoxymesterone, and metandienone. *Steroids* **60:** 353-366 (1995).

9. L. Dehennin, M. Ferry, P. Lafarge, G. Pérès and J.-P. Lafarge. Oral administration of dehydroepiandrosterone to healthy men: Alteration of the urinary androgen profile and consequences for the detection of abuse in sport by gas chromatography-mass spectrometry. *Steroids* **63:** 80-87 (1998).

10. D.Y. Wang and R.D. Bulbrook. Binding of the sulphate esters of dehydroepiandrosterone, testosterone, 17-acetoxypregnenolone and pregnenolone in the plasma of man, rabbit and rat. *J. Endocrinol.* **39:** 405-413 (1967).

11. D.Y. Wang, R.D. Bulbrook, F. Ellis and M. Coombs. Metabolic clearance rates of pregnenolone, 17-acetoxypregnenolone and their sulphate esters in man and in rabbit. *J. Endocrinol.* **39:** 395-403 (1967).

12. D.Y. Wang, R.D. Bulbrook, A. Sneddon and T. Hamilton. The metabolic clearance rates of dehydroepiandrosterone, testosterone and their sulphate esters in man, rat and rabbit. *J. Endocrinol.* **38:** 307-318 (1967).

13. H.E. Rosenthal, E. Pietrzak, W.R. Slaunwhite and A.A. Sandberg. Binding of estrone sulfate in human plasma. *J. Clin. Endocrinol. Metab.* **34:** 805-813 (1972).

14. E. Hamalainen, T. Fotsis and H. Adlercreutz. A gas chromatographic method for the determination of neutral steroid profiles in urine, including studies on the effect of oxytetracycline administration on these profiles in men. *Clin. Chim. Acta* **199:** 205-220 (1991).

15. J.F. Levesque and Ch. Ayotte. Criteria for the detection of androstenedione oral administration. In *Recent advances in doping analysis (7),* W. Schänzer, H. Geyer, A. Gotzmann and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, 1999, pp 169-179.

16. P.E. Goss, T.J. Powles, M. Dowsett, G. Hutchison, A.M. Brodie, J.C. Gazet and R.C. Coombes. Treatment of advanced postmenopausal breast cancer with an aromatase inhibitor, 4-hydroxyandrostenedione: phase II report. *Cancer Res.* **46:** 4823-4826 (1986).

17. D.C. Spink, B.C. Spink, X. Zhuo, M.M. Hussain, J.F. Gierthy and X. Ding. NADPH- and hydroperoxide-supported 17beta-estradiol hydroxylation catalyzed by a variant form (432L, 453S) of human cytochrome P450 1B1. *J. Steroid Biochem. Mol. Biol.* **74:** 11-18 (2000).

18. C.L. Hayes, D.C. Spink, B.C. Spink, J.Q. Cao, N.J. Walker and T.R. Sutter. 17 beta-estradiol hydroxylation catalyzed by human cytochrome P450 1B1. *Proc. Natl. Acad. Sci.* **93:** 9776-9781 (1996).

19. P. van Eenoo, F.T. Delbeke, N. Desmet and P. De Backer. Excretion studies with 4-androstene-3,17-dione. In *Recent advances in doping analysis (6),* W. Schänzer, H. Geyer, A. Gotzmann and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, 1998, pp 171-180.

20. M. Peter, J.M. Dubuis and W.G. Sippell. Disorders of the aldosterone synthase and steroid 11beta-hydroxylase deficiencies. *Horm. Res.* **51:** 211-222 (1999).

21. W.G. Kelly, O. De Leon and T.H. Rizkallah. The role of 19-hydroxy-delta4-androstene-3,17-dione in the conversion of circulating delta4-androstene-3,17-dione to estrone. *J. Clin. Endocrinol. Metab.* **43:** 190-194 (1976).

22. W.G. Kelly, O. de Leon and T.H. Rizkallah. Metabolism of 19-hydroxyandrostenedione in human subjects. Urinary excretion of conjugates. *J. Clin. Endocrinol. Metab.* **46:** 445-451 (1978).

23. Y. Osawa, S. Ohnishi, C. Yarborough, S. Ohigashi, T. Kosaki, M. Hashino, T. Yanaihara and T. Nakayama. Serum level of 19-hydroxyandrostenedione during pregnancy and at delivery determined by gas chromatography/mass spectrometry. *Steroids* **55:** 165-169 (1990).

24. A. van Langenhove. Isotope effects: definitions and consequences for pharmacologic studies. *J. Clin. Pharmacol.* **26:** 383-389 (1986).

6

SPECIFIC DETECTION OF DEHYDROEPIANDROSTERONE (DHEA) ADMINISTRATION BY PROFILING OXYGENATED METABOLITES

ABSTRACT

In Chapters 4 and 5 several steroids were presented that could be used as sensitive parameters for establishing the abuse of dehydroepiandrosterone (DHEA) and androst-4-ene-3,17-dione (Δ4-AEDIONE). In this experiment these steroids were analyzed in urine samples taken before and after administration of 100 mg of DHEA to 8 male subjects. Sample analysis was performed on the total fraction of glucuronides, sulfates and non-conjugated steroids with gas chromatography-mass spectrometry.

In 24 hours, 39.5 ± 7.8 % was recovered from the administered dose as the total of DHEA and its metabolites. The conversion to non-oxygenated steroids was 19.7 ± 6.0 % and to oxygenated steroids 2.8 ± 0.9 %

The parameters that showed the best sensitivity for DHEA were 7β-hydroxy-DHEA, androst-5-ene-3β,17β-diol, 7-keto-androsterone, 6α/β-hydroxy-Δ4-AEDIONE, 16α-hydroxy-androsterone, 16α-hydroxy-etiocholanolone, androsterone, etiocholanolone and DHEA itself. Sensitivity was described as the relative increase in 24-hours excretion rate after administration, as compared to the mean basal excretion rate. 4-Hydroxy-Δ4-AEDIONE, 6α/β-hydroxy-T and T that were previously described as sensitive parameters for Δ4-AEDIONE administration, showed a relatively low increase in excretion after administration.

The 7β-hydroxy-DHEA/7α-hydroxy-DHEA and T/E ratio significantly increased. Mean intra-subject T/E ratios of the pre-administration period correlated with the corresponding maximum T/E ratios after DHEA administration.

INTRODUCTION

Metabolic precursors of testosterone have become commercially available as food supplements during the nineties. One of the pioneering steroids on this market was dehydroepiandrosterone (DHEA), which has been considered as a doping agent since the International Olympic Committee (IOC) added it to the List of Forbidden Substances in 1997.

In the past, steroid profiling was usually focussed on the analysis of reduced metabolites as androst-5-ene-3β,17β-diol (Δ5-AEDIOL), testosterone (T), epitestosterone (E), androsterone (AO), etiocholanolone (EO) and androstanediol stereoisomers [1]. However, the number of steroids found in food supplements that are expected to metabolize to several of these reduced compounds has increased to over five (DHEA, Δ5-AEDIOL, androst-5-ene-3,17-dione, androst-4-ene-3,17-dione, androst-4-ene-3β,17β-diol, pregnenolone) [1,2].

In Chapter 3 several oxygenated steroids were introduced that were related to the metabolism of DHEA and Δ4-AEDIONE. These could serve as parameters in steroid profiling to enable identification of either DHEA or Δ4-AEDIONE as administered doping agent. In Chapters 4 and 5 was illustrated that oxygenated metabolites provide sufficient sensitivity as compared to the mentioned non-oxygenated metabolites. Sensitivity was described as the relative increase in 24-hours excretion rate after administration, as compared to the basal excretion rate.

In Chapter 4, the best sensitivity for DHEA was ascribed to 7β-hydroxy-DHEA (7β-OH-DHEA) and 7-keto-DHEA, AO, EO and T. No preliminary conclusions could be drawn about the specificity of oxygenated steroids for DHEA, as these studied steroids were not analyzed after Δ4-AEDIONE administration.

In this experiment 100 mg of DHEA was administered to 8 healthy male subjects. The collected urine samples were analyzed with gas chromatography-mass spectrometry (GC-MS) for DHEA metabolites (see Chapter 4) in addition to oxygenated metabolites of Δ4-AEDIONE (see Chapter 5). Excretion data were compared for a preliminary evaluation of the specificity of these metabolites as parameters for DHEA. Additionally a re-evaluation was performed of the effect of oral DHEA administration on the T/E ratio.

EXPERIMENTAL

Chemicals

Reference steroids: Androst-5-en-3β-ol-17-one (dehydroepiandrosterone, DHEA), androst-4-en-17α-ol-3-one (epitestosterone, E), 5α–androstan-3α-ol-17-one (androsterone, AO), 5β–androstan-3α-ol-17-one (etiocholanolone, EO), androst-4-ene-3,17-dione (androstenedione, Δ4-AEDIONE), androst-5-ene-3β,17β-diol (androstenediol, Δ5-AEDIOL) and 17α-methyl-androst-4-en-11α,17β-diol-3-one (11α-hydroxymethyltestosterone, 11α-OH-MeT) were obtained from Sigma, St. Louis, Missouri, USA. Androst-4-en-17β-ol-3-one (testosterone, T), androst-5-ene-3β,7α-diol-17-one (7α-hydroxy-DHEA, 7α-OH-DHEA) and androst-5-ene-3β,7β-diol-17-one (7β-hydroxy-DHEA, 7β-OH-DHEA) were obtained through courtesy of Organon, Oss, The Netherlands). Androst-4-en-4-ol-3,17-dione (4-hydroxy-androstenedione, 4-OH-Δ4-AEDIONE), androst-4-en-6α-ol-7,17-dione (6α-hydroxy-androstenedione, 6α-OH-Δ4-AEDIONE), androst-4-en-6β-ol-7,17-dione (6β-hydroxy-androstenedione, 6β-OH-Δ4-AEDIONE), androst-4-en-16α-ol-3,17-dione (16α-hydroxy-androstenedione, 16α-OH-Δ4-AEDIONE), androst-4-ene-6,7,17-trione (6-keto-androstenedione, 6-keto-Δ4-AEDIONE), androst-5-ene-3β,16α-diol-17-one (16α-hydroxy-DHEA, 16α-OH-DHEA), androst-5-en-3β-ol-7,17-dione (7-keto-DHEA), androst-4-en-6β,17β-ol-7-one (6β-hydroxy-testosterone, 6β-OH-T), 5α–androstane-3α,16α-diol-17-one (16α-hydroxy-androsterone, 16α-OH-AO), 5β–androstane-3α,16α-diol-17-one (16α-hydroxy-etiocholanolone, 16α-OH-EO) and 5α-androstan-3α-ol-7,17-dione (7-keto-androsterone, 7-keto-AO) were obtained from Steraloids, Newport, Rhode Island, USA.

Chemicals: *N*-Methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) was obtained from Aldrich Chemical Company, Milwaukee, Winsconsin, USA. Ammonium iodide was obtained from Fluka Chemie, Buchs, Switzerland. Ethanethiol was obtained from Acros Organics, New Jersey, USA. Diethyl ether was obtained from Merck, Darmstadt, Germany. Hydrolysis was performed with Helix pomatia (type HP-2, containing 110.000 IU/ml β-glucuronidase and 1000-5000 IU/ml arylsulfatase), obtained from Sigma, St. Louis, Missouri, USA. Columns for solid-phase extraction were IST Isolute C_{18} columns (200 mg, non-endcapped) obtained from Sopachem, Lunteren, The Netherlands.

Sample collection

Urine samples of 8 male (ages 22-31 years, mean 25.4, standard error of the mean (SEM) 3.9; weights 57-105 kg, mean 75.4, SEM 15.9) were collected every 2 hours

and overnight during two successive days. On the second day at 9 a.m. an in-house prepared capsule containing 100 mg of DHEA was administered orally. For each collected urine sample the exact void time, volume and specific gravity (Urine Specific Gravity Refractometer, Atago, Japan) was recorded. Samples were stored at -20°C until time of analysis. Informed consent was obtained from the volunteers.

Sample preparation

The volume of urine that was sampled for the analysis was determined on the basis of specific gravity of the urine samples, according to:

$$V = \frac{0.020}{d-1} * 5 \text{ ml}$$

A maximum volume of 15 ml was applied. 11α-OH-MeT (500 ng) was added to the samples as internal standard. Solid-phase extraction was performed with C_{18} columns. Before applying urine samples, the column material was preconditioned by washing with 4 ml of methanol and equilibrated with 2 ml of water. Inorganic material was removed from the column by washing with 2 ml of water. The remaining compounds were collected by elution with 4 ml of methanol. After evaporation to dryness, 2 ml of acetate buffer (0.1 M, pH 5.2) were added to the extract. Hydrolysis was performed by addition of 100 µl of Helix pomatia followed by incubation for 1 hour at 55°C. Steroids were isolated from the buffer solution by extraction with 5 ml of diethyl ether, twice. After extraction the phase separation was optimized by centrifugation (4000 rpm, 5 min). The organic layer was removed and evaporated to dryness under a stream of nitrogen at 40°C. The extract was dried overnight over P_2O_5/KOH under reduced pressure. Derivatization was performed by incubation of the extract in 100 µl of MSTFA/NH_4I/ethanethiol (1000:2:3; v/w/v) at 80°C for 30 min.

Gas chromatography and mass spectrometry

Selected ion monitoring (SIM) was performed with a Hewlett Packard gas chromatograph (Model 5890, Agilent Technologies, Waldbronn, Germany) coupled to a Hewlett Packard quadrupole mass spectrometer (Model 5972A). Ionization was performed in electron ionization (EI) mode at 70 eV. Quantification of steroids was performed by monitoring ions at the following m/z values: 430 (Δ4-AEDIONE, 7α-OH-DHEA, 7β-OH-DHEA and 7-keto-AO), 432 (T, E and DHEA), 434 (AO, EO and Δ5-AEDIOL), 518 (6α/β-OHΔ4-AEDIONE, 4-OH-Δ4-AEDIONE), 520 (6α/β-OH-T, 16α-OH-DHEA), 507 (16α-OH-AO and 16α-OH-EO), 517 (7-keto-DHEA), 503 (16α-OH-

Δ4-AEDIONE) and 534 (11α-OH-MeT). A check for possibly incomplete derivatization was performed by detection of ions representing mono-TMS derivatives of AO and EO at m/z 362.

Gas chromatography was performed with a HP-1 fused silica column (length 18 m, inner diameter 0.2 mm, film thickness 0.11 μm). Through electronic pressure control the column flow (helium) was constantly 1 ml/min. Sample injection of 1 μl was performed in split mode (ratio 1/10). A Hewlett Packard autosampler (Model 7673, Agilent Technologies, Waldbronn, Germany) was used for autoinjection. The injector temperature was set to 250°C. The oven temperature program used was: initial temperature 180°C, 2°/min to 225°C, 30°/min up to 310°C, constant for 5 min. The interface temperature was set to 280°C.

The quality of DHEA was checked by GC-MS analysis in full scan mode prior to administration. No impurities could be detected in a concentrated derivatized sample of the substance.

Calibration, quantification and validation

The obtained urine samples were analyzed for DHEA and Δ4-AEDIONE metabolites in two separate analyses. The calibration procedure for 7α-OH-DHEA, 7β-OH-DHEA, 7-keto-DHEA, 16α-OH-DHEA and 7-keto-AO is described in Chapter 4. Calibration of all other metabolites is described in Chapter 5. The corresponding validation data are described in the same chapters (intra- and inter-assay variability, recovery, stability and side reactions during hydrolysis).

As mentioned in Chapter 5 and worked out in detail in Chapter 8, the applied derivatization method results in loss of stereochemical integrity at C^6 of Δ4-steroids. Therefore, 6α- and 6β-hydroxy-steroids are combined in one single derivative (indicated as 6α/β-OH).

Statistical analysis

Absolute difference in total 24-hours excretion rate were tested by Student's one-tailed paired t-test with $p<0.05$ considered as significant. Normalized excretion differences, confidence intervals and individual T/E ratio differences were tested by Student's one-tailed (non-paired) t-test with $p<0.05$ considered as significant. Assumed normality of excretion rates was tested with the Kolmogorov test ($p<0.05$).

RESULTS

The absolute 24-hours excretion rate before and after administration are listed in Table 1. As a marker of sensitivity, the difference in 24 hours excretion was normalized with the basal excretion (expressed as %).

Of the non-oxygenated steroids, DHEA and Δ5-AEDIOL showed the largest normalized excretion difference. Urinary concentrations of AO and EO were relatively high, but the effect of administration on the excretion rate was limited. With exception of 6α/β-OH-Δ4-AEDIONE, Δ4-steroids showed the least normalized difference with a minimum for T and E. Significantly increased excretion of oxygenated metabolites occurred , ranging from 55% for 6α/β-OH-T to 1163% for 7β-OH-DHEA.

For each studied parameter the mean DHEA conversion was calculated (see Table 1). In 24 hours of urine sample collection after time of administration, 39.5 ± 7.8 % (mean ± SEM) from the administered dose was recovered as the total of DHEA and its metabolites. The conversion to oxygenated steroids was small as compared to non-oxygenated steroids (see Table 2).

Calculated upper confidence limits ($p<0.05$), based on pre-administration excretion values, are listed in Table 3. The time period in which excretion exceeded the reference range was similar for most steroids (0-14 hours, see Table 3), except for DHEA, Δ5-AEDIOL and EO (24 hours) and T and E (6-8 hours).

In Figures 1 and 2 the mean excretion rates are shown for each individual time interval (in µg/hour). Because the timing of sample collection did not match the 2 hours interval as presented in the figures, two subjects were omitted from these results.

In Table 4, data are shown of the T/E ratio for each individual subject. A significant increase was observed for all subjects. Data for steroid ratios that were calculated for each time interval are shown in Figure 3. Where relatively small effects were observed for the T/E, AO/EO and 16α-OH-AO/16α-OH-EO ratio, the 7β-/7α-OH-DHEA ratio was significantly elevated for 14 hours.

Table 1: *Excretion data before and after administration of DHEA to 8 male Caucasian subjects. Results are placed in order of decreasing difference in excretion rate. Absolute and normalized excretion differences were all statistically significant ($p<0.05$).*

Parameter	µg/24 hours before administration mean ± SEM	µg/24 hours after administration mean ± SEM	normalized difference (%) in excretion rate mean ± SEM	conversion (% of DHEA) mean ± SEM
DHEA	1896.2 ± 1576.4	18880.5 ± 7185.1	2182 ± 2309	16.98 ± 6.29
7β-OH-DHEA	47.6 ± 39.0	470.5 ± 314.2	1163 ± 721	0.40 ± 0.27
Δ5-AEDIOL	427.7 ± 325.2	2192.6 ± 690.4	615 ± 488	1.71 ± 0.62
7-keto-AO	10.7 ± 8.9	40.4 ± 12.8	502 ± 449	0.03 ± 0.02
6α/β-OH-Δ4-AEDIONE	2.2 ± 1.5	11.2 ± 3.3	474 ± 272	0.009 ± 0.003
16α-OH-AO	127.1 ± 63.3	628.9 ± 250.2	451 ± 237	0.47 ± 0.20
16α-OH-EO	176.3 ± 108.9	670.8 ± 487.0	394 ± 336	0.47 ± 0.46
AO	3010.6 ± 1055.1	13240.7 ± 4033.1	388 ± 244	10.16 ± 3.77
EO	2659.9 ± 1187.8	10524.2 ± 2146.1	370 ± 236	7.81 ± 2.61
7α-OH-DHEA	32.6 ± 20.5	126.5 ± 61.8	334 ± 132	0.09 ± 0.04
16α-OH-DHEA	933.0 ± 665.5	2103.7 ± 800.0	318 ± 494	1.11 ± 0.54
7-keto-DHEA	89.0 ± 54.6	214.1 ± 91.8	189 ± 154	0.12 ± 0.07
4-OH-Δ4-AEDIONE	39.8 ± 8.1	94.1 ± 18.7	143 ± 64	0.052 ± 0.018
16α-OH-Δ4-AEDIONE	25.9 ± 8.5	46.9 ± 22.3	87 ± 59	0.020 ± 0.017
6α/β-OH-T	3.0 ± 2.1	4.9 ± 3.2	55 ± 34	0.0017 ± 0.0014
T	62.5 ± 30.4	91.1 ± 40.6	49 ± 30	0.029 ± 0.021
E	41.3 ± 9.3	48.4 ± 14.8	17 ± 20	0.007 ± 0.009

Table 2: *Conversion of DHEA to different groups of metabolites during first 24 hours ($n=8$).*

Group of metabolites	Average DHEA conversion (%)
AO and EO	18.0 ± 6.0
non-oxygenated metabolites	19.7 ± 6.0
oxygenated metabolites	2.8 ± 0.9
total of all metabolites	22.5 ± 6.1
total recovery DHEA + metabolites	39.5 ± 7.8

Table 3: *Confidence limits ($p<0.05$) for the mean excretion rate (µg/hour), calculated over the pre-administration day; and the post-administration time period in which the excretion rate of the respective steroid exceeded the upper confidence limit.*

Parameter	Calculated upper confidence limit ($p<0.05$, in µg/hour)	Period (hours) of significant post-administration elevation of excretion
DHEA	133.81	0-24
7β-OH-DHEA	3.34	0-14
Δ5-AEDIOL	31.00	0-24
7-keto-AO	0.76	0-14
6α/β-OH-Δ4-AE	0.14	0-24
16α-OH-AO	7.50	0-14
16α-OH-EO	11.13	0-14
16α-OH-DHEA	62.01	0-12
AO	162.13	0-14
EO	152.13	0-24
7α-OH-DHEA	2.07	0-14
7-keto-DHEA	5.61	0-10
4-OH-Δ4-AEDIONE	1.94	0-14
16α-OH-Δ4-AEDIONE	1.38	0-10
6α/β-OH-T	0.20	0-10
T	3.66	2-8
E	2.04	0-6

Table 4: *Individual results for the T/E ratio before and after DHEA administration.*

Subject	Mean T/E day 1	SEM T/E day 1	Maximum T/E day 2	Normalized difference (%)
1	0.091	0.0089	0.14	54
2	1.1	0.094	2.0	82
3	2.1	0.33	4.2	100
4	2.3	0.30	4.1	78
5	2.7	0.25	4.1	52
6	2.2	0.24	3.2	45
7	2.1	0.22	6.1	190
8	1.0	0.12	1.8	80

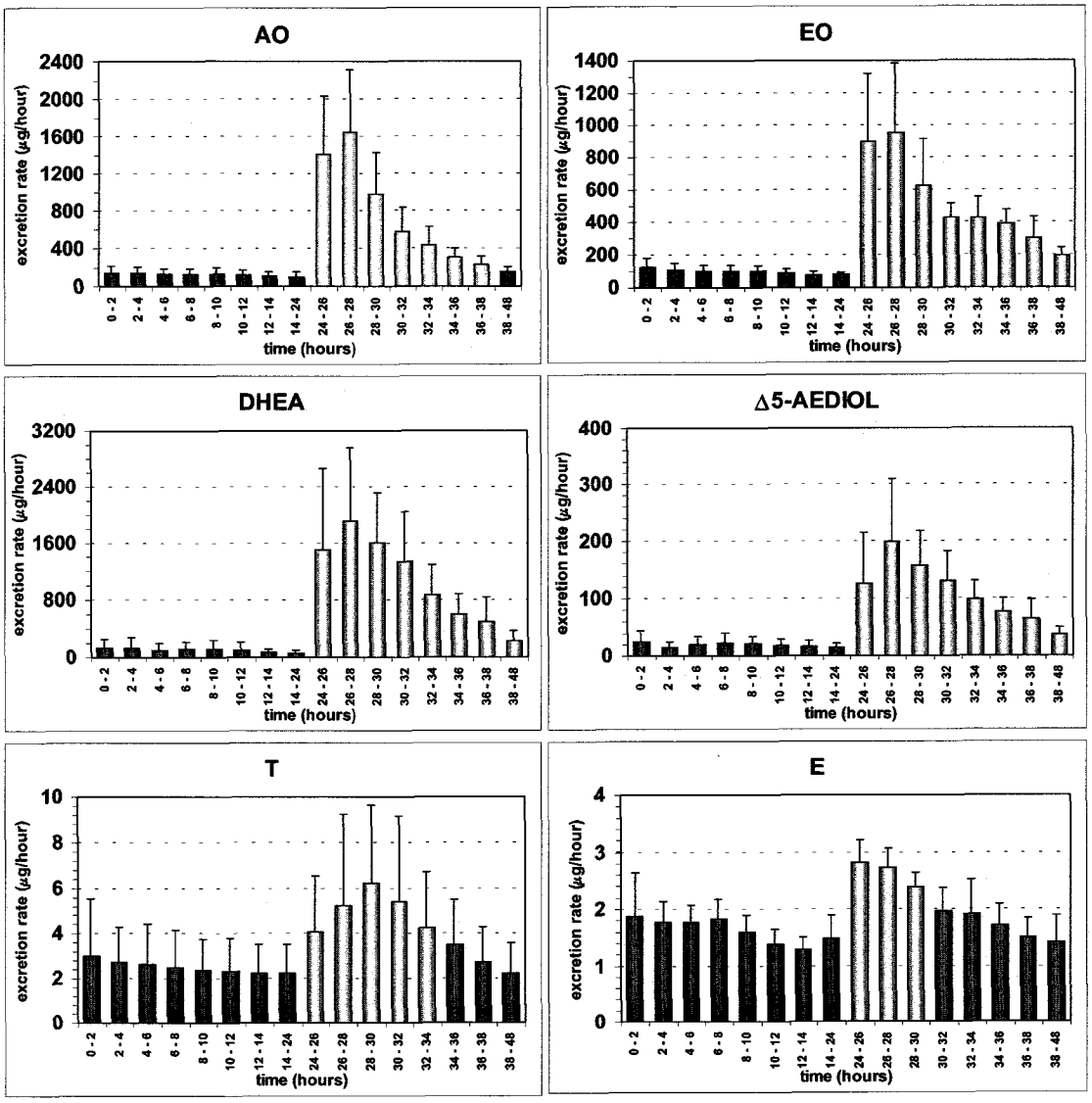


Figure 1: Excretion rate in µg/hour (mean ± SEM, n=6) of non-oxygenated steroids, before and after DHEA administration (at time point 24 hours). Increased mean excretion rates with statistical significance ($p<0.05$) are indicated as hatched columns (▨).

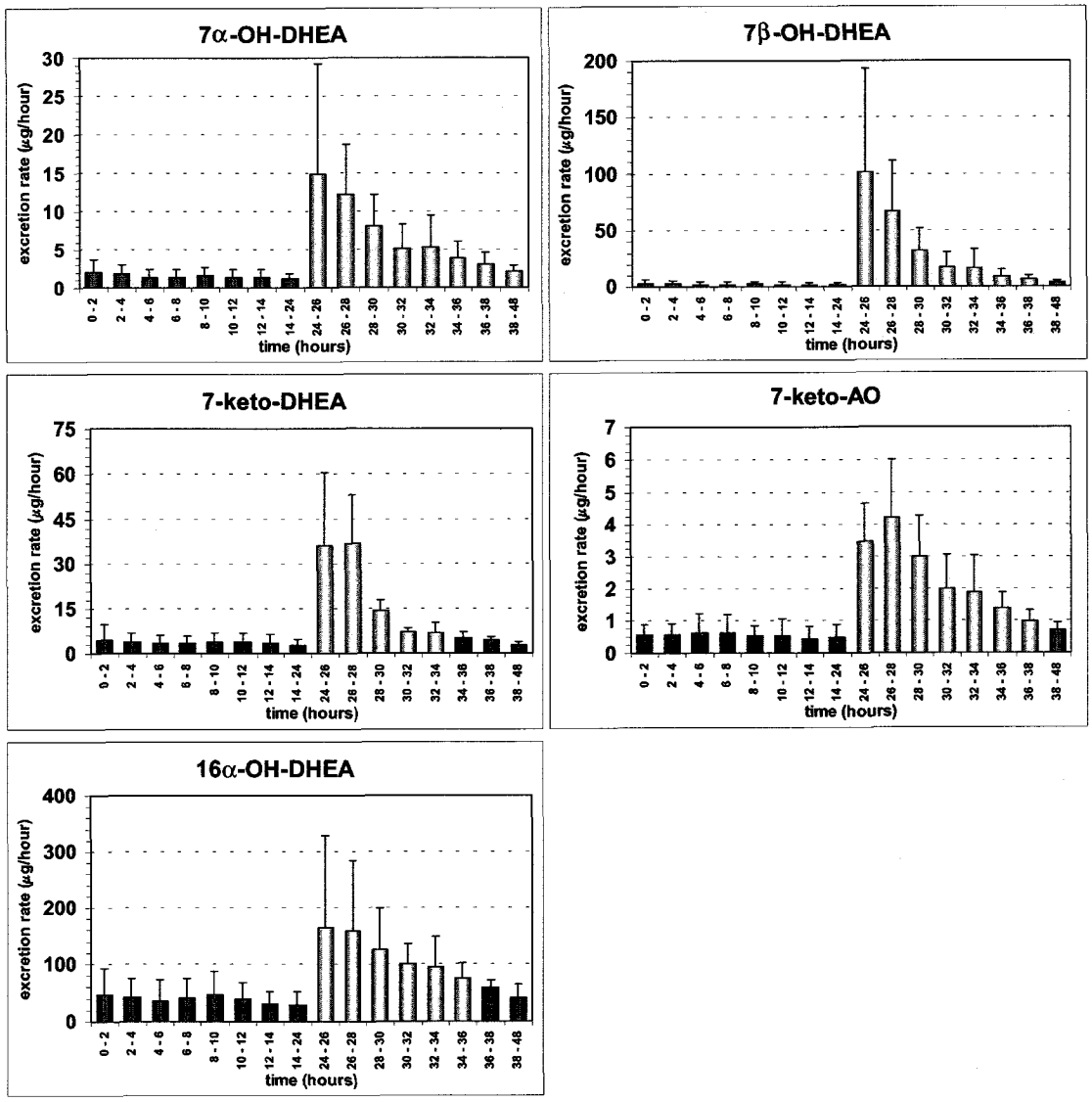


Figure 2a: Excretion rates in µg/hour (mean ± SEM, n=6) of oxygenated steroids, before and after DHEA administration (at time point 24 hours). Increased mean excretion rates with statistical significance ($p<0.05$) are indicated as hatched columns (▨).

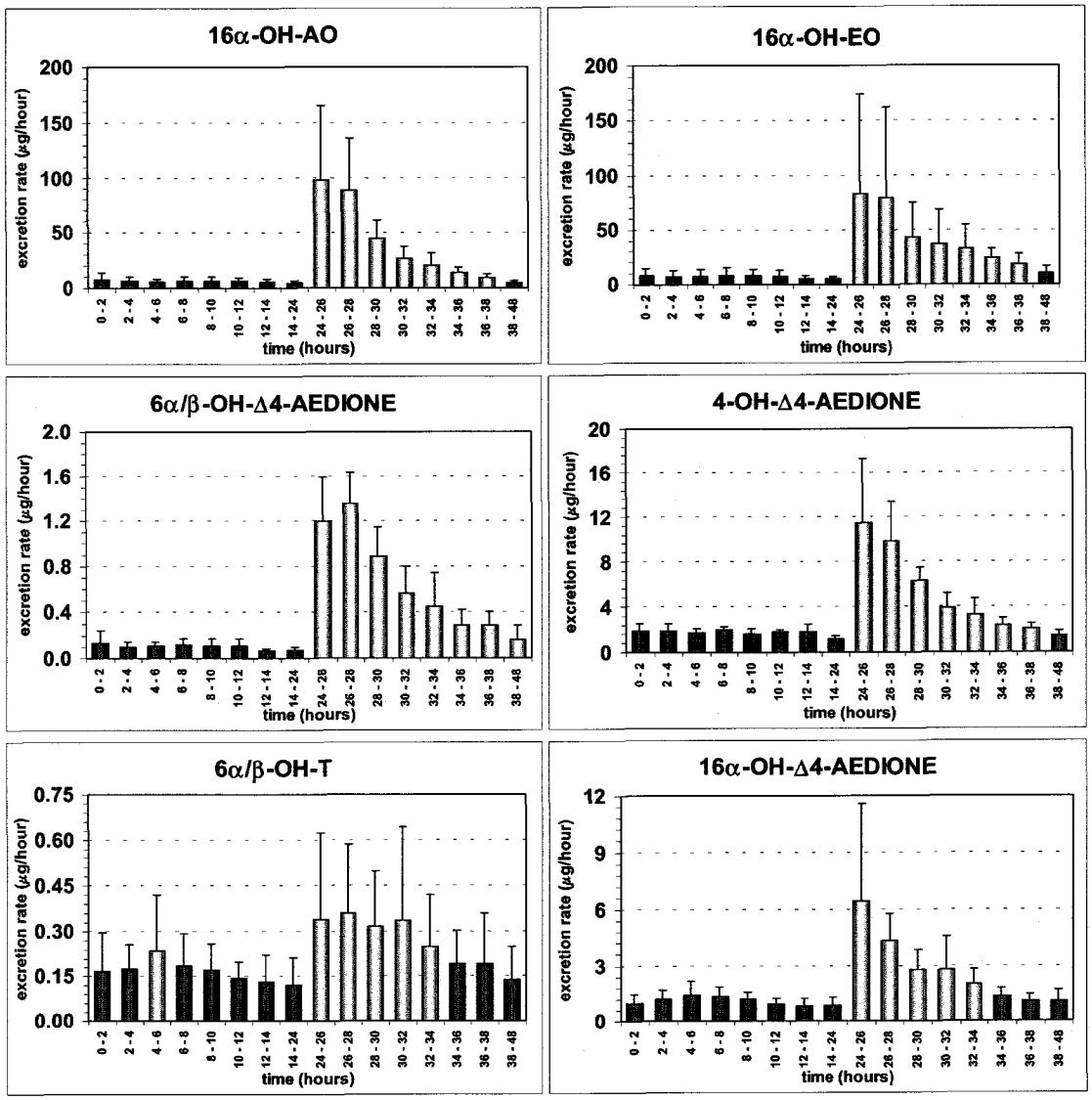


Figure 2b: Excretion rates in µg/hour (mean ± SEM, n=6) of oxygenated steroids, before and after DHEA administration (at time point 24 hours). Increased mean excretion rates with statistical significance ($p<0.05$) are indicated as hatched columns (▨).

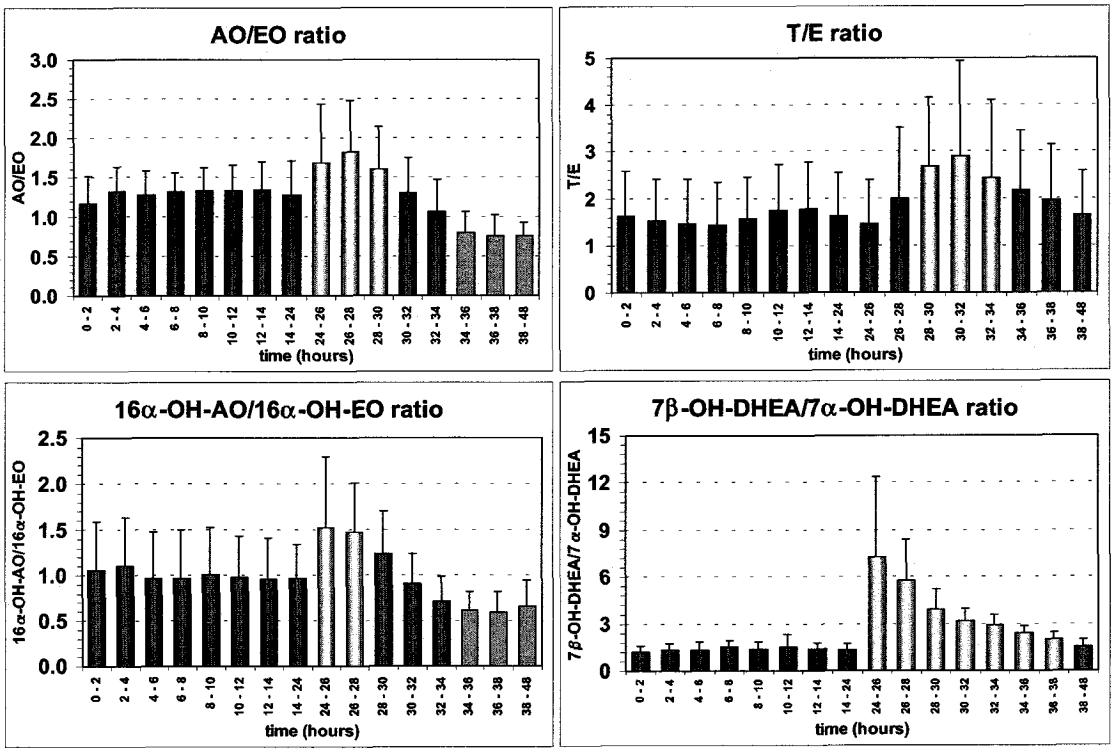


Figure 3: Ratio's (mean ± SEM, n=6) of excretion rates of several steroids, before and after DHEA administration (at time-point 24 hours). Increased mean excretion rates with statistical significance ($p<0.05$) are indicated as hatched columns (▨). Decreased mean excretion rates ($p<0.05$) are indicated by diagonal striping (▨).

DISCUSSION

DHEA conversion

The 40% total recovery of DHEA and its metabolites was comparable to the results in Chapter 4. However, in the present experiment a higher recovery for DHEA (17.0 ± 6.3% vs. 0.3% and 2.6% in Chapter 4) and a lower conversion to AO and EO was calculated. Also, a relatively high conversion to oxygenated metabolites was obtained compared to Chapter 4. As reported by Dehennin *et al.* [3] recovery of seemingly non-metablized DHEA can mostly be ascribed to excretion of DHEA-sulfate. Sulfate transferase could have less affinity for deuterium labeled DHEA as for DHEA itself, resulting in a relatively low second phase metabolism of DHEA and a

relatively high conversion to AO and EO. These can be regarded as isotope effects as has been discussed in Chapters 4 and 5 [4].

Sensitivity and specificity

AO and EO are metabolites of multi-origin that are excreted in large amounts (0.5-10 μg/ml) [5,6]. Published data mostly assign sensitivity to these metabolites for detection of DHEA [1,3,7-9]. However, as presented in Table 1, several other steroids showed a larger difference in mean excretion rate. Besides the Δ5-steroids DHEA, Δ5-AEDIOL and 7β-OH-DHEA, these were oxygenated derivatives of AO and EO, *i.e.* 7-keto-AO, 16α-OH-AO and 16α-OH-EO. The high sensitivity of 6α/β-OH-Δ4-AEDIONE is striking and can only be explained by the metabolism of Δ4-AEDIONE. This experiment confirms the conclusion of Chapter 4 that studying quantitatively minor routes of DHEA metabolism (oxygenation) can provide information of comparable sensitivity as the major routes.

Before DHEA administration, 7α-OH-DHEA concentration was smaller than 7β-OH-DHEA, resulting in a basal 7β-OH-DHEA/7α-OH-DHEA level between 1 and 2. The different sensitivity of the two stereoisomers for DHEA was illustrated by the significantly increased 7β-OH-DHEA/7α-OH-DHEA ratio to an average of 7, immediately after administration.

Such a difference in sensitivity was less obvious for AO/EO and 16α-OH-AO/16α-OH-EO, which only slightly exceeded the respective confidence limits. As also described in Chapter 4 the increase was followed by a decrease to a level that was significantly below baseline until 24 hours after administration.

With exception of 6α/β-OH-Δ4-AEDIONE, the sensitivity of Δ4-steroids was low (<150%). Of these steroids 4-OH-Δ4-AEDIONE, 6α/β-OH-T and T were identified as sensitive parameters for establishing Δ4-AEDIONE administration in Chapter 5. Therefore, these metabolites can be considered as specific parameters for Δ4-AEDIONE.

T/E ratio

In this experiment, T and E showed little sensitivity. The negligible conversion of orally administered DHEA to T in males was reported before, based on plasma data [10-12]. In females a small but significant T production was observed [13,14].

Several previously reported studies have focussed on the alteration of the T/E ratio after oral DHEA administration, with contradicting results. Bosy *et al.* [15] and Dehennin *et al.* [3] did not record significant changes after administration of 50 mg of

DHEA to 7 male volunteers. In other studies with limited numbers of subjects increased T/E ratios were observed for a dose regimen of 50-150 mg [1,8,9].

Our results showed a significantly increased T/E ratio in all 8 subjects with a maximum increase of 45-190% (see Table 4). Only one subject marginally passed the IOC criterion for the T/E ratio of 6. However, in doping analysis the T/E ratio and the corresponding cutoff level are based on the analysis of isolated glucuronides. In that case, the maximum T/E ratio should be higher than as observed in this experiment [16]. As shown in Figure 4, intra-subject mean T/E ratios during the pre-administration day correlate with the corresponding maximum T/E ratios after administration (coefficient of determination of 0.7448). This model implicates that a rough estimation can be made for the expected maximum T/E ratio, based on the intraindividual mean basal T/E ratio and the particular dose level.

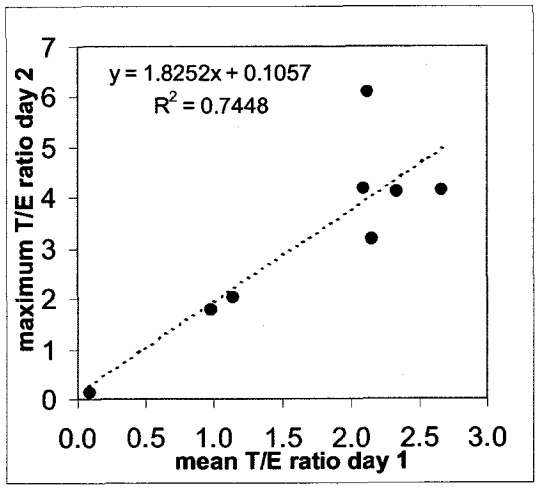


Figure 4: Correlation of the intra-subject mean T/E ratio during the pre-administration day (day 1) with the maximum T/E ratio during the post-administration day (day 2)

SUMMARY

In addition to AO and EO, the steroids DHEA, Δ5-AEDIOL and 7β-OH-DHEA, 7-keto-AO, 16α-OH-AO and 16α-OH-EO provided sensitive information about administration of DHEA. Furthermore, 4-OH-Δ4-AEDIONE, 6α/β-OH-T and T were indicated as specific parameters of Δ4-AEDIONE administration. In contrast to other previously reported studies, the T/E ratio significantly increased in all eight subjects. The extent of increase depended on the intra-subject mean T/E ratio before administration.

LITERATURE

1. V.P. Uralets and P.A. Gillette. Over-the-counter delta5 anabolic steroids 5-androsten-3,17-dione; 5-androsten-3beta, 17beta-diol; dehydroepiandrosterone; and 19-nor-5-androsten-3,17-dione: excretion studies in men. *J. Anal. Toxicol.* **24:** 188-193 (2000).
2. V.P. Uralets and P.A. Gillette. Over-the-counter anabolic steroids 4-androsten-3,17-dione; 4-androsten-3beta,17beta-diol; and 19-nor-4-androsten-3,17-dione: excretion studies in men. *J. Anal. Toxicol.* **23:** 357-366 (1999).
3. L. Dehennin, M. Ferry, P. Lafarge, G. Pérès and J.-P. Lafarge. Oral administration of dehydroepiandrosterone to healthy men: Alteration of the urinary androgen profile and consequences for the detection of abuse in sport by gas chromatography-mass spectrometry. *Steroids* **63:** 80-87 (1998).
4. A. van Langenhove. Isotope effects: definitions and consequences for pharmacologic studies. *J. Clin. Pharmacol.* **26:** 383-389 (1986).
5. M. Donike, S. Rauth and A. Wolansky. Reference ranges of urinary endogenous steroids determined by gas chromatography/mass spectrometry. In *Proceedings of the 10th Cologne workshop on dope analysis*, M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke and S. Rauth, Eds. Sport und Buch Strauss, Köln, 1993, pp 69-86.
6. C.H.L. Shackleton. Profiling steroid hormones and urinary steroids. *J. Chromatogr. B* **379:** 91-156 (1986).
7. F. Callies, W. Arlt, L. Siekmann, D. Hubler, F. Bidlingmaier and B. Allolio. Influence of oral dehydroepiandrosterone (DHEA) on urinary steroid metabolites in males and females. *Steroids* **65:** 98-102 (2000).
8. L. Bowers. Oral dehydroepiandrosterone supplementation can increase the testosterone/epitestosterone ratio. *Clin. Chem.* **45:** 295-297 (1999).
9. R. Kazlauskas. Effects of dehydroepiandrosterone on urinary steroids. In *Recent advances in doping analysis (5)*, W. Schänzer, H. Geyer, A. Gotzmann and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, 1998, pp 83-90..
10. G. Valenti, A. Banchini, L. Denti, M. Maggio, G. Ceresini and G.P. Ceda. Acute oral administration of dehydroepiandrosterone in male subjects: effect of age on bioavailability, sulfoconjugation and bioconversion in other steroids. *J. Endocrinol. Invest.* **22:** 24-28 (1999).
11. W. Arlt, J. Haas, F. Callies, M. Reincke, D. Hubler, M. Oettel, M. Ernst, H.M. Schulte and B. Allolio. Biotransformation of oral dehydroepiandrosterone in elderly men: significant increase in circulating estrogens. *J. Clin. Endocrinol. Metab.* **84:** 2170-2176 (1999).
12. G.A. Brown, M.D. Vukovich, R.L. Sharp, T.A. Reifenrath, K.A. Parsons and D.S. King. Effect of oral DHEA on serum testosterone and adaptations to resistance training in young men. *J. Appl. Physiol.* **87:** 2274-2283 (1999).

13. J.F. Mortola and S.S. Yen. The effects of oral dehydroepiandrosterone on endocrine-metabolic parameters in postmenopausal women. *J. Clin. Endocrinol. Metab.* **71:** 696-704 (1990).

14. A.J. Morales, J.J. Nolan, J.C. Nelson and S.S.C. Yen. Effects of replacement dose of dehydroepiandrosterone in men and women of advancing age. *J. Clin. Endocrin. Metab.* **78:** 1360-1367 (1994)

15. T.Z. Bosy, K.A. Moore and A. Poklis. The effect of oral dehydroepiandrosterone (DHEA) on the urinary testosterone/epitestosterone (T/E) ratio in human male volunteers. *J. Anal. Toxicol.* **22:** 455-459 (1998).

16. L. Dehennin. On the origin of physiologically high ratios of urinary testosterone to epitestosterone: Consequences for reliable detection of testosterone administration by male athletes. *J. Endocrinol.* **142:** 353-360 (1994).

7

SPECIFIC DETECTION OF ANDROST-4-ENE-3,17-DIONE ADMINISTRATION BY PROFILING OXYGENATED METABOLITES

Abstract

For the selective detection of androst-4-ene-3,17-dione (Δ4-AEDIONE) administration, the analysis of several oxygenated metabolites in urine was suggested previously to apply in steroid profiling techniques. In this experiment, excretion studies with Δ4-AEDIONE were performed with 5 male subjects, to provide information about sensitivity and specificity of these metabolites as parameters in steroid profiling. Analysis with gas chromatography with mass spectrometric detection was applied upon the combined fraction of glucuronides, sulfates and non-conjugated steroids.

Sensitivity, defined as the relative increase in 24-hours excretion rate after administration compared to the mean basal excretion rate, was ascribed to 4-hydroxy-Δ4-AEDIONE, 6α/β-hydroxy-Δ4-AEDIONE, androsterone, etiocholanolone and testosterone.

The metabolites that provided the best specificity for Δ4-AEDIONE were testosterone, 6α/β-hydroxy-testosterone and 4-hydroxy-Δ4-AEDIONE. The most specific steroid ratio was 7β-hydroxy-DHEA/7α-hydroxy-DHEA. DHEA, androst-5-ene-3β,17β-diol, 7β-hydroxy-DHEA and 7-keto-androsterone were confirmed as specific parameters for DHEA.

The T/E ratio was increased after administration in all studied subjects. A high correlation was observed between the mean T/E ratio during the pre-administration period, and the maximum T/E ratio that was detected after administration. This implicates that the expected maximum T/E ratio can be estimated, based on the intraindividual mean basal T/E ratio.

One Asian subject showed relatively low basal excretion rate values. Also, the effect of Δ4-AEDIONE administration on metabolite excretion was relatively large. Except a low basal T/E ratio, no relevant difference was observed in its response after administration.

INTRODUCTION

Recent years, an increasing number of steroids of endogenous origin are marketed as food supplements. Since 1997 several of these steroids, such as dehydroepiandrosterone (DHEA) and androst-4-ene-3,17-dione (Δ4-AEDIONE), have been placed on the list of forbidden substances of the International Olympic Committee. Usually applied profiling of non-specific androgens, as testosterone (T), epitestosterone (E), androsterone (AO) and etiocholanolone (EO) [1,2] provides insufficiently specific information, to enable identification of the administered steroid.

In Chapter 3 several oxygenated steroids were introduced that could serve as specific parameters for detection of DHEA or Δ4-AEDIONE abuse. Despite the fact that oxygenation reactions are quantitatively minor metabolic routes, it was shown in Chapters 4 and 5 that the analysis of these steroids provides a comparable range in sensitivity as the described non-specific parameters. Therefore, screening a profile of oxygenated metabolites could be useful to supply additional information needed for identification.

In this study, specificity and sensitivity of the presented profile of oxygenated metabolites (Chapter 5) for detection of Δ4-AEDIONE administration was investigated. Therefore, excretion studies with Δ4-AEDIONE were performed with 5 male subjects. The metabolism was studied by analyzing the collected urine samples for non-oxygenated and oxygenated metabolites of Δ4-AEDIONE (see Chapter 5) and DHEA (see Chapters 4 and 6).

The Asian race is often described as example of genetically determined variation in steroid metabolism. In doping analysis, this is most obvious for the relatively low T/E ratio as compared to other populations (see Chapter 1) [3]. In a case study by Uralets *et al.* [1], a different metabolism of Δ4-AEDIONE in Asian subjects was suggested, leading to significantly different effects on the T/E ratio. In the present study, differences in (non-)oxygenative Δ4-AEDIONE metabolism between one Asian and four Caucasian subjects was investigated.

EXPERIMENTAL

Chemicals

Reference steroids: Androst-5-en-3β-ol-17-one (dehydroepiandrosterone, DHEA), androst-4-en-17α-ol-3-one (epitestosterone, E), 5α–androstan-3α-ol-17-one (androsterone, AO), 5β–androstan-3α-ol-17-one (etiocholanolone, EO), androst-4-

ene-3,17-dione (androstenedione, Δ4-AEDIONE), androst-5-ene-3β,17β-diol (androstenediol, Δ5-AEDIOL) and 17α-methyl-androst-4-en-11α,17β-diol-3-one (11α-hydroxymethyltestosterone, 11α-OH-MeT) were obtained from Sigma, St. Louis, Missouri, USA. Androst-4-en-17β-ol-3-one (testosterone, T), androst-5-ene-3β,7α-diol-17-one (7α-hydroxy-DHEA, 7α-OH-DHEA) and androst-5-ene-3β,7β-diol-17-one (7β-hydroxy-DHEA, 7β-OH-DHEA) were obtained through courtesy of Organon, Oss, The Netherlands). Androst-4-en-4-ol-3,17-dione (4-hydroxy-androstenedione, 4-OH-Δ4-AEDIONE), androst-4-en-6α-ol-7,17-dione (6α-hydroxy-androstenedione, 6α-OH-Δ4-AEDIONE), androst-4-en-6β-ol-7,17-dione (6β-hydroxy-androstenedione, 6β-OH-Δ4-AEDIONE), androst-4-en-16α-ol-3,17-dione (16α-hydroxy-androstenedione, 16α-OH-Δ4-AEDIONE), androst-4-ene-6,7,17-trione (6-keto-androstenedione, 6-keto-Δ4-AEDIONE), androst-5-ene-3β,16α-diol-17-one (16α-hydroxy-DHEA, 16α-OH-DHEA), androst-5-en-3β-ol-7,17-dione (7-keto-DHEA), androst-4-en-6β,17β-ol-7-one (6β-hydroxy-testosterone, 6β-OH-T), 5α–androstane-3α,16α-diol-17-one (16α-hydroxy-androsterone, 16α-OH-AO), 5β–androstane-3α,16α-diol-17-one (16α-hydroxy-etiocholanolone, 16α-OH-EO) and 5α-androstan-3α-ol-7,17-dione (7-keto-androsterone, 7-keto-AO) were obtained from Steraloids, Newport, Rhode Island, USA.

Chemicals: *N*-Methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA), ethanethiol (97%), and phosphorous pentoxide were obtained from Sigma-Aldrich Chemie Company, Steinheim, Germany. Ammonium iodide was obtained from Fluka Chemie, Buchs, Switzerland. Potassium hydroxide, sodium acetate trihydrate, glacial acetic acid (96%), diethyl ether, acetic acid and methanol were obtained from Merck, Darmstadt, Germany. All chemicals were of analytical grade, unless indicated otherwise.

Hydrolysis was performed with Helix pomatia (type HP-2, containing 110.000 IU/ml β-glucuronidase and 1000-5000 IU/ml arylsulfatase), obtained from Sigma-Aldrich, Steinheim, Germany.

Columns for solid-phase extraction were IST Isolute C_{18} columns (200 mg, non-endcapped) obtained from Sopachem, Lunteren, The Netherlands.

Sample collection

Urine samples of 5 male subjects (age 22-39 years) were collected every 2 hours and overnight, during two days. On the second day at 9 a.m. an in-house prepared capsule containing 100 mg of Δ4-AEDIONE was administered orally. For each collected urine sample the exact void time, volume and specific gravity (Urine Specific Gravity Refractometer, Atago, Japan) was recorded. Samples were stored at -20°C until time of analysis.

Sample preparation

The volume of urine that was sampled for the analysis was determined on the basis of specific gravity of the urine, according to:

$$V = \frac{0.020}{d-1} * 5 \text{ ml}$$

A maximum volume of 15 ml was applied. 11α-OH-MeT (500 ng) was added to the samples as internal standard. Solid-phase extraction was performed with non-endcapped C_{18} columns. Before applying urine samples, the column material was preconditioned by washing with 4 ml of methanol and equilibrated with 2 ml of water. Inorganic material was removed from the column by washing with 2 ml of water. The remaining compounds were collected by elution with 4 ml of methanol. After evaporation to dryness, 2 ml of acetate buffer (0.1 M, pH 5.2) were added to the extract. Hydrolysis was performed by addition 100µl of Helix pomatia followed by incubation for 1 hour at 55°C. Steroids were isolated from the buffer solution by extraction with 5 ml of diethyl ether, twice. After extraction, the phase separation was optimized by centrifugation (4000 rpm, 5 min). The organic layer was removed and evaporated to dryness under a stream of nitrogen at 40°C. The extract was dried overnight over P_2O_5/KOH under reduced pressure. Derivatization was performed by incubation of the extract in 100 µl of MSTFA/NH_4I/ethanethiol (1000:2:3; v/w/v) at 80 °C for 30 min. The derivatization mixture was injected into the gas chromatography-mass spectrometry (GC-MS) system for analysis.

Gas chromatography and mass spectrometry

Selected ion monitoring (SIM) was performed with a Hewlett Packard gas chromatograph (Model 5890, Agilent Technologies, Waldbronn, Germany) coupled top a Hewlett Packard quadrupole mass spectrometer (Model 5972A). Ionization was performed in electron ionization (EI) mode at 70 eV. Quantification of steroids was performed by monitoring ions at the following m/z values: 430 (Δ4-AEDIONE, 7α-OH-DHEA, 7β-OH-DHEA and 7-keto-AO), 432 (T, E and DHEA), 434 (AO, EO and Δ5-AEDIOL), 518 (6α/β-OHΔ4-AEDIONE, 4-OH-Δ4-AEDIONE), 520 (6α/β-OH-T, 16α-OH-DHEA), 507 (16α-OH-AO and 16α-OH-EO), 517 (7-keto-DHEA), 503 (16α-OH-Δ4-AEDIONE) and 534 (11α-OH-MeT). A check for possibly incomplete derivatization was performed by detection of ions representing mono-TMS derivatives of AO and EO at m/z 362.

Gas chromatography was performed with a HP-1 fused silica column (length 18 m, inner diameter 0.2 mm, film thickness 0.11 µm). Through electronic pressure control

the column flow (helium) was constantly 1 ml/min. Sample injection of 1 µl was performed in split mode (ratio 1/10). A Hewlett Packard autosampler (Model 7673, Agilent Technologies, Waldbronn, Germany) was used for autoinjection. The injector temperature was set to 250°C. The oven temperature programme used was: initial temperature 180°C, 2°/min to 225°C, 30°/min up to 310°C, constant for 5 min. The interface temperature was set to 280°C.

The quality of Δ4-AEDIONE was checked by gas chromatography-mass spectrometry (GC-MS) in full scan mode prior to administration. No impurities could be detected in a concentrated sample.

Calibration, quantification and validation

The obtained urine samples were analyzed for DHEA and Δ4-AEDIONE metabolites in two separate analyses. The calibration procedure for 7α-OH-DHEA, 7β-OH-DHEA, 7-keto-DHEA, 16α-OH-DHEA and 7-keto-AO is described in Chapter 4. Calibration of all other metabolites is described in Chapter 5. The corresponding validation data are also described in Chapter 4 and 5 (intra- and inter-assay variability, recovery, stability and side reactions during hydrolysis).

Statistical analysis

Absolute differences in total 24-hours excretion rate were tested by Student's one-tailed paired t-test with $p<0.05$ considered as significant. Normalized excretion differences, confidence limits and differences in individual data for the T/E ratio were tested by Student's one-tailed t-test with $p<0.05$ considered as significant. One subject of Asian origin was excluded from the calculation of mean excretion rates, normalized excretion differences, conversion and confidence limits. Assumed normality of excretion rates was tested with the Kolmogorov test ($p<0.05$).

RESULTS

The 24-hour pre- and post-administration excretion data are listed in Table 1. As a marker of sensitivity, the difference in 24-hours excretion was normalized with the basal excretion (expressed as %). For each parameter the mean conversion of Δ4-AEDIONE was calculated.

The oxygenated steroids 4-OH-Δ4-AEDIONE and 6α/β-OH-Δ4-AEDIONE showed the largest normalized excretion difference. Negligible sensitivity was observed for 7β-OH-DHEA, 7-keto-DHEA and 16α-OH-DHEA. From the administered dose, 39.5 ± 3.2% was recovered as the total of all Δ4-AEDIONE metabolites in 24 hours after time of administration. The observed conversion was mostly explained by the metabolism to AO and EO. Relatively little Δ4-AEDIONE was converted to oxygenated metabolites (see Table 2).

Table 1: *Excretion data before and after administration of Δ4-AEDIONE of four male Caucasian subjects. Results are placed in order of decreasing difference in excretion rate.*

Parameter	µg/24 hours before administration mean ± SEM	µg/24 hours after administration mean ± SEM	normalized difference (%) in excretion rate mean ± SEM	conversion (% Δ4-AEDIONE) mean ± SEM	
4-OH-Δ4-AEDIONE	37.4 ± 7.9	491.5 ± 239.3	1304 ± 875	0.43 ± 0.23	*
6α/β-OH-Δ4-AEDIONE	4.7 ± 2.1	48.9 ± 16.9	1016 ± 389	0.042 ± 0.015	*
EO	3073.3 ± 1880.3	19867.6 ± 3322.0	697 ± 372	16.68 ± 2.62	*
AO	3077.7 ± 680.5	22983.7 ± 4377.1	677 ± 245	19.77 ± 4.49	*
T	38.8 ± 13.3	214.3 ± 132.1	426 ± 145	0.18 ± 0.12	*
6α/β-OH-T	4.2 ± 1.3	11.9 ± 3.5	186 ± 44	0.007 ± 0.002	*
16α-OH-AO	209.6 ± 179.8	471.3 ± 208.3	184 ± 121	0.62 ± 0.60	*
DHEA	779.3 ± 796.7	1747.0 ± 2020.4	114 ± 60	0.97 ± 1.24	**
7-keto-AO	3.6 ± 1.3	7.2 ± 2.5	104 ± 60	0.003 ± 0.002	*
16α-OH-EO	322.3 ± 186.7	564.9 ± 227.6	98 ± 60	0.63 ± 0.60	*
7α-OH-DHEA	48.6 ± 40.2	64.7 ± 39.5	88 ± 94	0.015 ± 0.032	
E	59.1 ± 8.9	85.6 ± 9.6	68 ± 23	0.034 ± 0.008	*
16α-OH-Δ4-AEDIONE	33.8 ± 16.1	48.6 ± 23.1	46 ± 19	0.014 ± 0.010	*
Δ5-AEDIOL	342.6 ± 142.3	488.0 ± 323.8	35 ± 39	0.14 ± 0.20	
16α-OH-DHEA	664.3 ± 691.7	658.2 ± 433.1	28 ± 39	-0.006 ± 0.34	
7-keto-DHEA	64.9 ± 24.4	62.4 ± 12.3	5 ± 33	-0.002 ± 0.013	
7β-OH-DHEA	37.6 ± 16.2	34.9 ± 8.7	-1 ± 20	-0.002 ± 0.007	

* = Statistical significance for the 24-hours excretion rate, as well as for the normalized difference (%).

** = Only statistical significance for the normalized difference (%).

Table 2: *Conversion of Δ4-AEDIONE to different groups of metabolites during the first 24 hours after administration (n=4).*

Group of metabolites	Δ4-AEDIONE conversion (mean ± SEM; in %)
AO and EO	36.5 ± 3.4
non-oxygenated	37.8 ± 3.6
oxygenated	1.76 ± 1.42
total	39.5 ± 3.2

Estimated upper confidence limits ($p<0.05$, $n=4$), based on 24-hours excretion values are listed in Table 3. All metabolite excretion rates returned to baseline level within 24 hours after administration.

Table 3: *Confidence limits ($p<0.05$) for the mean excretion rate (µg/hour), calculated over the pre-administration day; and the post-administration time period in which the excretion rate of the respective steroid exceeded the upper confidence limit.*

Parameter	Upper confidence limit ($p<0.05$; in µg/hour)	Period (hours) of significant post-administration elevation of excretion
4-OH-Δ4-AEDIONE	2.08	14-24
6α/β-OH-Δ4-AEDIONE	0.332	8-10
EO	252.62	12-14
AO	173.32	12-14
T	2.50	10-12
6α/β-OH-T	0.262	8-10
16α-OH-AO	14.38	8-10
DHEA	85.25	4-6
7-keto-AO	0.24	6-8
16α-OH-EO	28.72	4-6
7α-OH-DHEA	4.69	4-6
E	2.75	6-8
16α-OH-Δ4-AEDIONE	2.47	4-6
Δ5-AEDIOL	23.70	4-6
16α-OH-DHEA	73.50	-
7-keto-DHEA	4.32	-
7β-OH-DHEA	2.64	-

In Figures 1 and 2 the mean excretion rates are shown for each individual time interval (in µg/hour). The ratios AO/EO, 16α-OH-AO/16α-OH-EO, T/E and 7β-OH-DHEA/7α-OH-DHEA are shown in Figure 3. Both AO/EO and 16α-OH-AO/16α-OH-EO showed a non-significant increase followed by a decrease under baseline levels. The T/E ratio showed a significant increase and 7β-OH-DHEA/7α-OH-DHEA a significant decrease. T/E ratio data for each studied individual are presented in Table 4.

In Table 5, the 24-hours excretion data before and after administration are listed for the Asian subject. Relatively low basal excretion rates and high normalized excretion differences were observed for most studied steroids. The T/E ratio was low compared to the Caucasians, but no relevant kinetic differences were observed for any of the studied steroid ratios (see Figure 4).

A high correlation was observed between intra-subject mean T/E ratios during the pre-administration day and the corresponding maximum T/E ratios after administration is (coefficient of determination of 0.9882, see Figure 5). No such correlation was found for AO/EO, 16α-OH-AO/16α-OH-EO or 7β-OH-DHEA/7α-OH-DHEA (data not shown). As indicated in Figure 5 the effect on the T/E ratio in the Asian subject also matched this correlation.

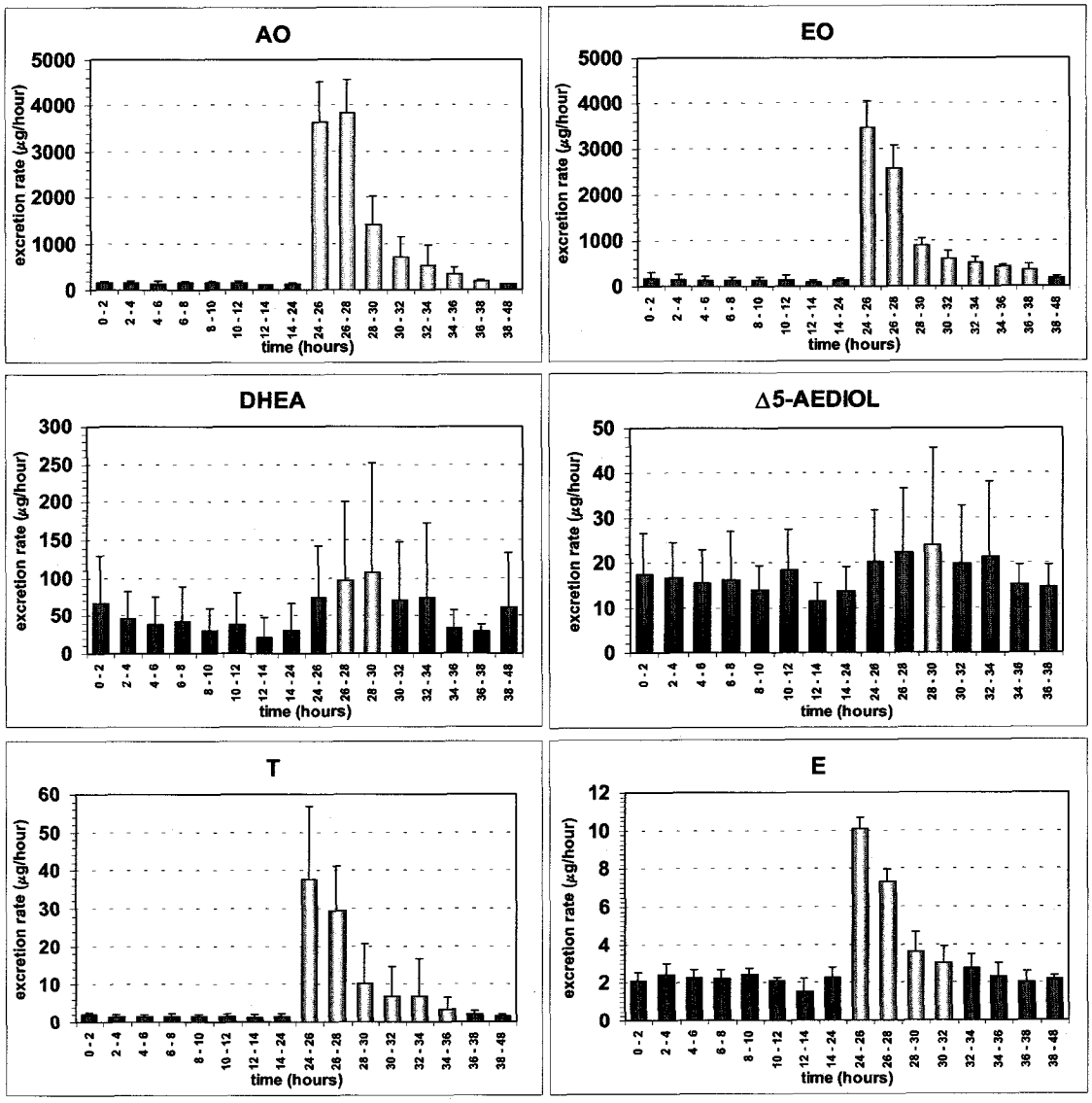


Figure 1: Excretion rates in µg/hour (mean ± SEM, n=4, Caucasians) of main non-oxygenated steroids, before and after Δ4-AEDIONE administration (at time point 24 hours). Increased mean excretion rates with statistical significance ($p<0.05$) are indicated as hatched columns (▨).

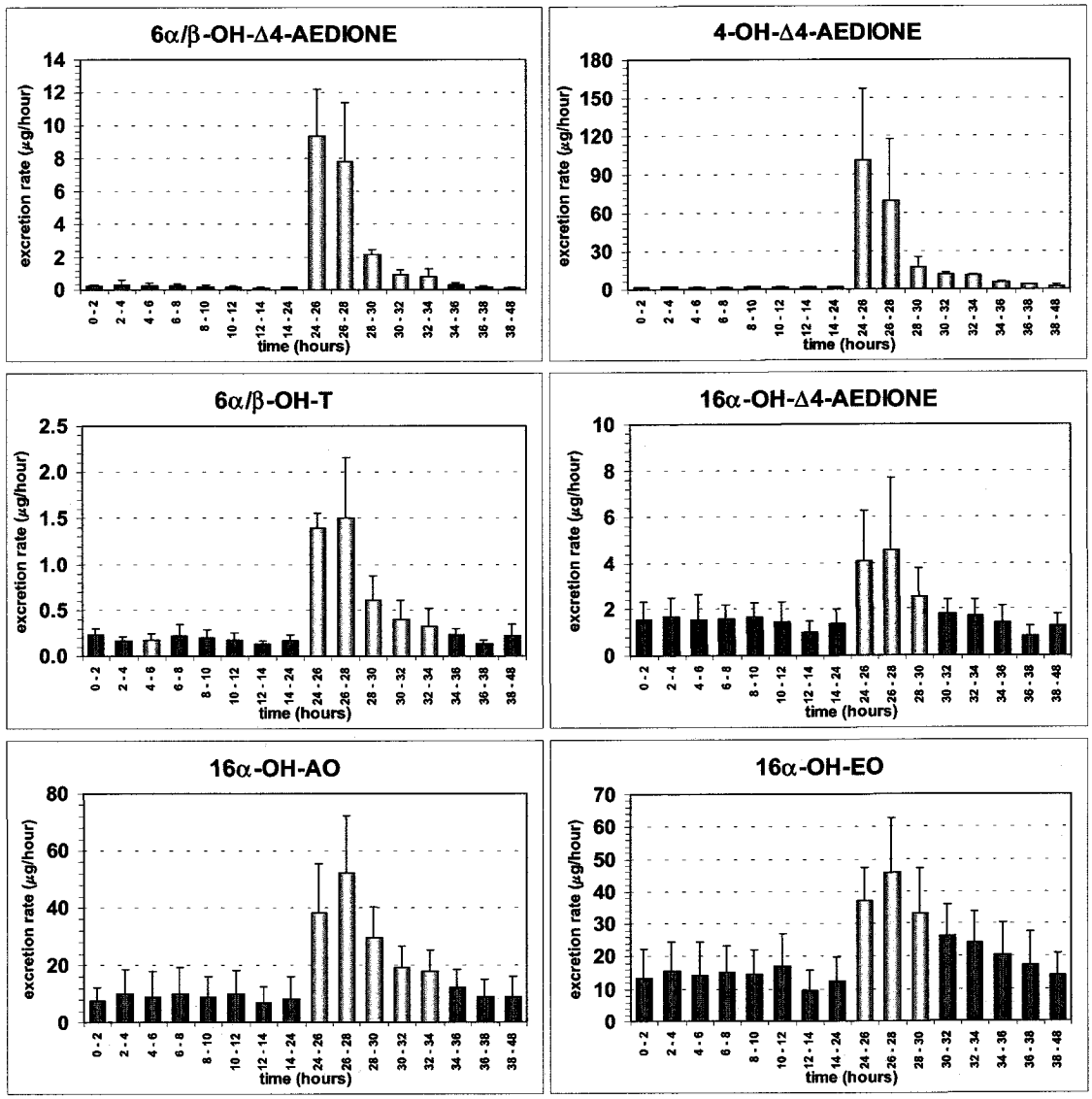


Figure 2a: Excretion rates in µg/hour (mean ± SEM, n=4, Caucasians) of oxygenated steroids, before and after Δ4-AEDIONE administration (at time point 24 hours). Increased mean excretion rates with statistical significance ($p<0.05$) are indicated as hatched columns (▨).

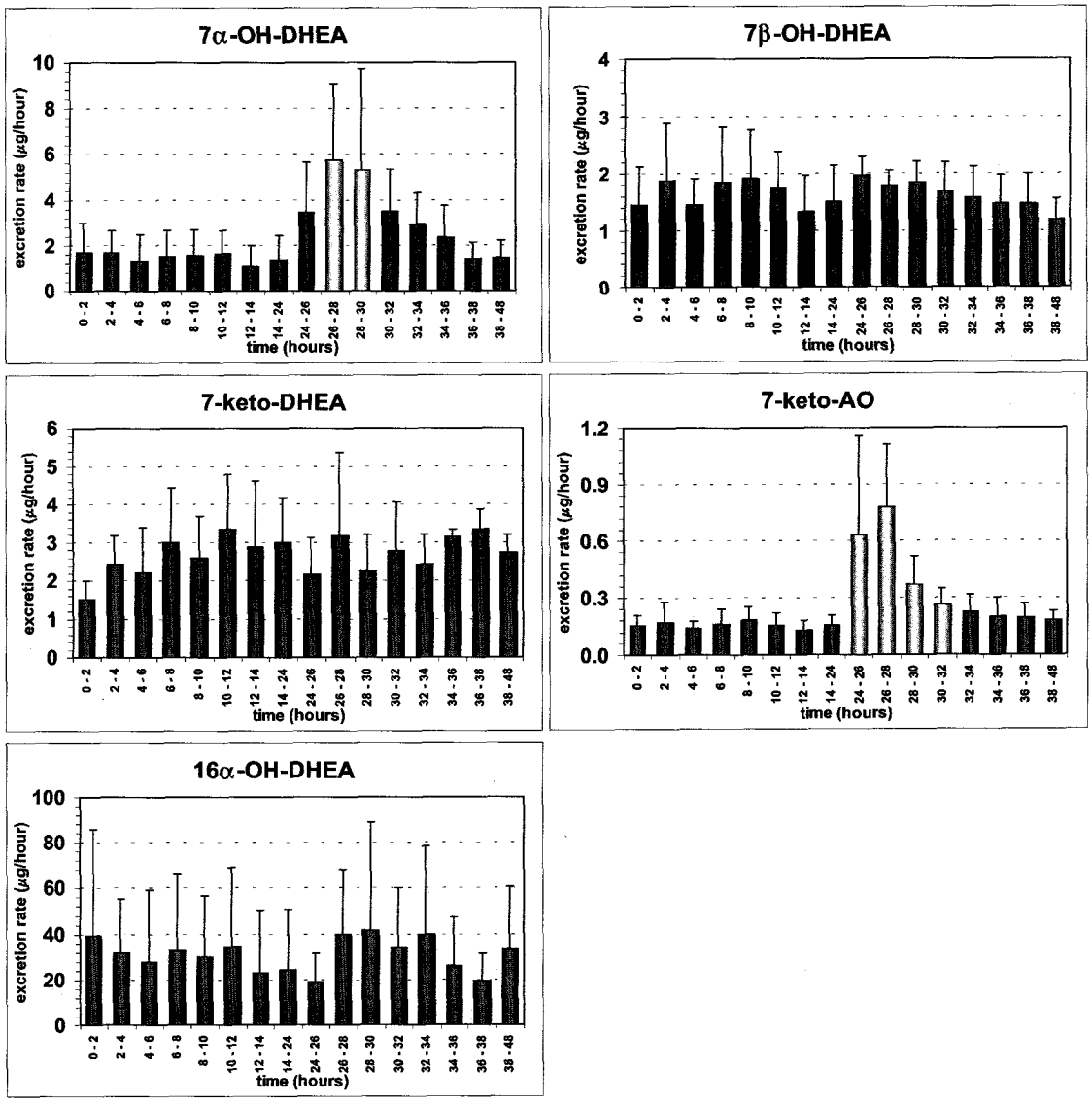


Figure 2b: Excretion rates in µg/hour (mean ± SEM, n=4, Caucasians) of oxygenated steroids, before and after Δ4-AEDIONE administration (at time point 24 hours). Increased mean excretion rates with statistical significance ($p<0.05$) are indicated as hatched columns (▨).

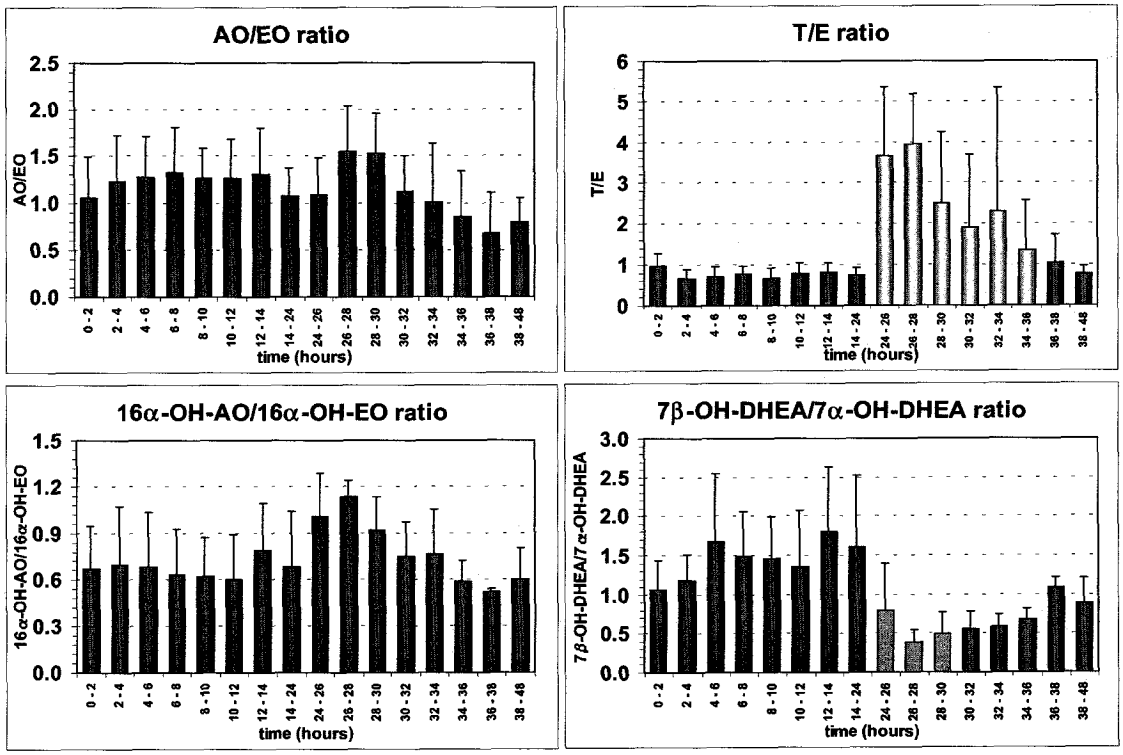


Figure 3: Ratios (mean ± SEM) of excretion rates of several steroids, before and after Δ4-AEDIONE administration (at time point 24 hours). Increased mean excretion rates with statistical significance ($p<0.05$) are indicated as hatched columns (▨). Decreased mean excretion rates ($p<0.05$) are indicated by diagonal stipings (▨).

Table 4: *Individual results for the T/E ratio before and after Δ4-AEDIONE administration.*

volunteer	mean T/E ± SEM day 1	CV (%)[1)] day 1	maximum T/E day 2	difference (%)
1	0.58 ± 0.06	10	2.82	386
2	1.04 ± 0.05	5	6.00	477
3	0.68 ± 0.28	41	3.49	413
4	0.73 ± 0.07	10	3.85	427
5[2)]	0.14 ± 0.01	7	0.63	350

[1)] Expressed as coefficient of variation (CV = SEM/mean*100%)

[2)] Subject of Asian origin

Table 5: *Excretion data of one male Asian before and after Δ4-AEDIONE administration .*

Parameter	µg/24 hours before administration mean ± SEM	µg/24 hours after administration mean ± SEM	normalized difference (%) in excretion rate mean ± SEM	conversion (% Δ4-AEDIONE) mean ± SEM
4-OH-Δ4-AEDIONE	10.7	971	8975	0.53
6α/β-OH-Δ4-AEDIONE	3.58	64.2	1693	0.045
EO	1356	15128	1016	16.23
AO	1451	12813	783	18.17
T	5.59	20.4	265	0.14
6α/β-OH-T	4.25	15.7	269	0.008
16α-OH-AO	132	806	511	0.53
DHEA	1078	2428	125	1.04
7-keto-AO	4.90	6.50	33	0.004
16α-OH-EO	208	692	233	0.56
7α-OH-DHEA	24.2	174	619	0.041
E	39.6	73.1	85	0.034
16α-OH-Δ4-AEDIONE	25.4	43.3	70	0.015
Δ5-AEDIOL	439	702	60	0.17
16α-OH-DHEA	470	650	38	0.029
7-keto-DHEA	35.3	48.0	36	-0.015
7β-OH-DHEA	29.3	38.6	32	0.000

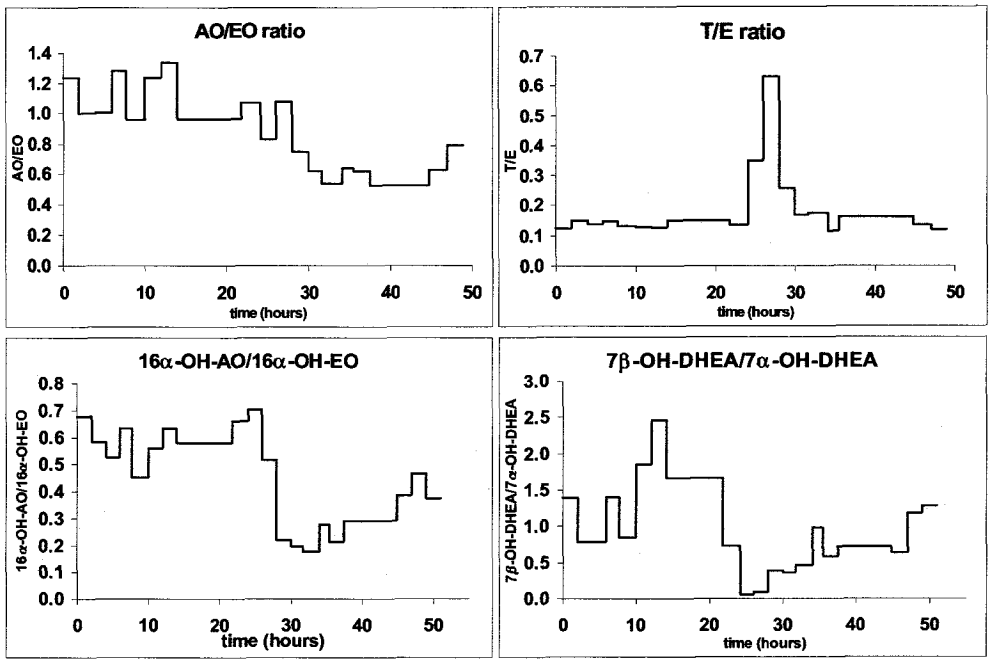


Figure 4: Ratios of excretion rates for the Asian subject. Δ4-AEDIONE was administered at time point 24 hours.

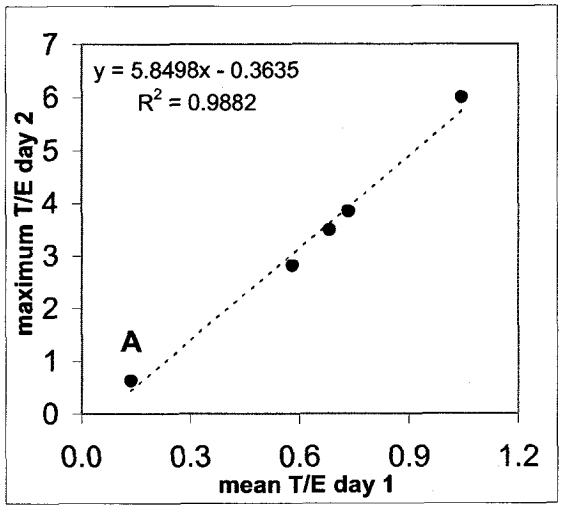


Figure 5: Correlation of the intra-subject mean T/E ratio during the pre-administration day (day 1) with the maximum T/E ratio during the post-administration day (day 2). The subject of Asian origin is indicated with "**A**".

DISCUSSION

Conversion of Δ4-AEDIONE

In this study, a 2-4 times larger dose level of Δ4-AEDIONE was applied than in the case of deuterium labeled Δ4-AEDIONE administration in Chapter 5. However, the calculated conversion was comparable. The total recovery of metabolites after 24 hours was reproducibly around 40%, of which the largest part was accounted for the metabolism to AO and EO (see Table 2). Quantitatively, the conversion to oxygenated metabolites contributed only in minor extent to the total metabolism that was observed. A comparison with the total conversion of DHEA in Chapters 4 and 6 shows that absorption of these orally administered steroids is comparable.

No data on Δ4-AEDIONE concentrations have been provided as concentrations in blank urine samples were always low (estimated as <20-30 ng/ml). Also, the chromatographic separation was often poor due to interference. This resulted in poor reproducibility. Based on estimation, maximum excretion rates were always smaller than 0.6-0.7 μg/hour, whereas baseline excretion rate was estimated as 0.1-0.2 μg/hour. This confirmed the results of Chapter 5 that Δ4-AEDIONE is almost completely metabolized after oral administration.

Sensitivity and specificity

As the conversion of Δ4-AEDIONE is explained for the major part by the metabolism to AO and EO, these steroids have primarily been reported as sensitive parameters for establishing Δ4-AEDIONE abuse [1,5,6]. In this experiment, AO and EO showed around 700% increase in 24-hours excretion rate. The only metabolites that showed a higher sensitivity than AO and EO were 6α/β-OH-Δ4-AEDIONE and 4-OH-Δ4-AEDIONE (see Table 1). This is in agreement with the metabolism of deuterium labeled Δ4-AEDIONE (Chapter 5).

Comparison of the presented data in Table 1 with the excretion data of DHEA administration (Table 2 in Chapter 6) shows that the normalized excretion difference of AO and EO was twice as high in this experiment. This illustrates a limited specificity of AO and EO for Δ4-AEDIONE, which was greatly exceeded by parameters as 4-OH-Δ4-AEDIONE, 6α/β-OH-T and T.

The low sensitivity of 16α-OH-Δ4-AEDIONE was in the same order of magnitude as E (around 50% increase). Therefore, 16α-OH-Δ4-AEDIONE could potentially be used in a steroid ratio analogous to the T/E ratio [7] to correct for intraindividual variability of steroid excretion. A potential steroid ratio with high sensitivity for Δ4-AEDIONE could be 4-OH-Δ4-AEDIONE/16α-OH-Δ4-AEDIONE. However, in this experiment the intraindividual variability of this ratio was not significantly lower than for the individual excretion values (see Table 5). Based on these results a preliminary conclusion should be drawn that the suggested ratio cannot be used to correct for intraindividual excretion differences.

Table 6: *Intra-subject mean and variability[1)] of excretion rates for 4-OH-Δ4-AEDIONE and 16α-OH-Δ4-AEDIONE, and the corresponding ratio. All values were calculated from samples collected during day 1 (pre-administration samples, n=8).*

	4-OH-Δ4-AEDIONE		16α-OH-Δ4-AEDIONE		4-OH-Δ4-AEDIONE /16α-Δ4-AEDIONE	
subject	mean (μg/hour)	CV (%)	mean (μg/hour)	CV (%)	mean (-)	CV (%)
1	1.38	40	2.04	18	0.66	26
2	1.85	18	1.05	16	1.85	35
3	2.06	25	2.10	24	1.00	16
4	1.43	32	0.73	39	2.09	25
5[2)]	0.50	76	1.25	40	0.43	61

1) Expressed as coefficient of variation (CV = SEM/mean*100%)

2) Subject five is the subject of Asian origin.

The Δ5-steroids DHEA, Δ5-AEDIOL, 7α-OH-DHEA, 7β-OH-DHEA, 16α-OH-DHEA and 7-keto-DHEA showed low sensitivity. This confirms the observation made in Chapter 6 that Δ4-AEDIONE does not significantly metabolize to Δ5-steroids. The conversion of Δ5- to Δ4-steroids are considered as irreversible in biosynthesis, which corresponds to a high specificity of these Δ5-steroids for DHEA.

The 7β-OH-DHEA/7α-OH-DHEA ratio is also a specific parameter, because it increased significantly after DHEA administration in Chapter 6 (330 ± 286%; n=8), whereas in the present study the ratio showed an opposite effect (-78 ± 12%; n=4).

T/E ratio

The excretion of both T and E was significantly increased in this experiment. Increased production of T after oral Δ4-AEDIONE administration was reported previously, based on plasma sample analyses [8]. In only one subject a maximum T/E ratio was detected that reached the cutoff level of six. However, this cutoff level is based on the analysis of isolated glucuronides and is therefore not applicable in this experiment.

The presented data of a case study with 3 subjects presented by Uralets *et al.* [1] suggest a positive correlation between the detected maximum T/E ratio after Δ4-AEDIONE administration with the pre-administration mean basal T/E ratio. As illustrated in Figure 5, this suggestion is confirmed by the high correlation for these five subjects ($r^2 = 0.9882$). Comparable results were observed in case of DHEA administration in Chapter 6.

Extrapolation of this linear model to higher basal T/E ratios implies that an estimation can be made for the expected maximum T/E ratio, based on the intraindividual mean basal T/E ratio and the particular dose level. It should, however, also be considered that this estimation could be abused for the frequent administration of relatively high dose levels of Δ4-AEDIONE and at the same time preventing an increase of the T/E ratio to a level higher than six.

Ethnic origin

As was also described previously [3], the mean T/E ratio in blank urine samples of the Asian subject was relatively low (around 0.1). Uralets *et al.* [1] reported a decrease of the T/E ratio in a subject of Asian origin as a result of Δ4-AEDIONE administration. In our study, the effect of Δ4-AEDIONE on the T/E ratio was not essentially different from Caucasians, as based on the correlation presented in Figure 5. Thus, according the results of this case there is no reason to suspect inter-

racial differences in the effect of Δ4-AEDIONE administration on the detected maximum T/E ratio.

SUMMARY

Sensitive metabolites of Δ4-AEDIONE were 4-OH-Δ4-AEDIONE, 6α/β-OH-Δ4-AEDIONE, AO, EO and T. The main specific metabolites of Δ4-AEDIONE were T, 6α/β-OH-T and 4-OH-Δ4-AEDIONE. The 7β-OH-DHEA/7α-OH-DHEA ratio was a specific parameter, because of its opposite effect after DHEA or Δ4-AEDIONE administration.

LITERATURE

1. V.P. Uralets and P.A. Gillette. Over-the-counter anabolic steroids 4-androsten-3,17-dione; 4-androsten-3beta,17beta-diol; and 19-nor-4-androsten-3,17-dione: excretion studies in men. *J. Anal. Toxicol.* **23:** 357-366 (1999)
2. V.P. Uralets and P.A. Gillette. Over-the-counter delta5 anabolic steroids 5-androsten-3,17-dione; 5-androsten-3beta, 17beta-diol; dehydroepiandrosterone; and 19-nor-5-androsten-3,17-dione: excretion studies in men. *J. Anal. Toxicol.* **24:** 188-193 (2000).
3. C. Ayotte, D. Goudreault and A. Charlebois. Testing for natural and synthetic anabolic agents in human urine. *J. Chromatogr. B* **687:** 3-25 (1996).
4. P. van Eenoo, F.T. Delbeke, N. Desmet and P. De Backer. Excretion studies with 4-androstene-3,17-dione. In *Recent advances in doping analysis (6),* W. Schänzer, H. Geyer, A. Gotzmann and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, 1998, pp 171-180.
5. M. Garle and E. Palonek. Androstenedione: Excretion studies frome single and multiple dose experiments. In *Recent advances in doping analysis (6)*, W. Schänzer, H. Geyer, A. Gotzmann and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, 1999, pp 181-184.
6. J.F. Levesque and Ch. Ayotte. Criteria for the detection of androstenedione oral administration. In *Recent advances in doping analysis (7),* W. Schänzer, H. Geyer, A. Gotzmann and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, 1999, pp 169-179.

7. M. Donike, K.-R. Bärwald, K. Klostermann, W. Schänzer and J. Zimmermann. Nachweis von exogenem Testosteron. In *Sport: Leistung und Gesundheit*, H. Heck, W. Hollmann, H. Liesen, R. Rost, Eds. Deutscher Ärzte Verlag, Köln, 1983, pp 293-298.

8. B.Z. Leder, C. Longcope, D.H. Catlin, B. Ahrens, D.A. Schoenfeld and J.S. Finkelstein. Oral androstenedione administration and serum testosterone concentrations in young men. *JAMA* **283:** 779-782 (2000).

PART III:

analytical procedures of steroid profiling

8

TRIMETHYLSILYLATION OF 3-KETO-ANDROST-4-ENE STEROIDS: THERMODYNAMIC VERSUS KINETIC CONTROL OF ENOLIZATION

ABSTRACT

Trimethylsilylation in a mixture of MSTFA/NH_4I/ethanethiol cannot be applied for the selective analysis of the steroids androst-4-ene-6α-ol-3,17-dione and androst-4-ene-6β-ol-3,17-dione, as the stereochemical integrity of C^6 is lost due to 3,5-dienol formation. In contrast, the stereochemical integrity of C^6 is retained upon using MSTFA/KOAc/imidazole, since in that case the 2,4-dienol is formed.

The different routes of product formation were confirmed with androst-4-ene-3,17-dione. In addition to usual gas chromatography-mass spectrometry, ^1H- and ^{13}C-NMR were applied to analyze and characterize the derivatization products. To that purpose derivatization was scaled up from analytical (μg) to preparative (mg) scale.

Fundamental insight in the course of rate limiting enolization followed by trimethylsilylation, was obtained from preparative silylation reactions using the mentioned reagents on the 17-methyl ether of testosterone as a model compound. MSTFA/NH_4I/ethanethiol yielded the 3,5-dienolTMS derivative as the only product, whereas in the case of MSTFA/KOAc/imidazole the 2,4-dienolTMS derivative was the predominant product.

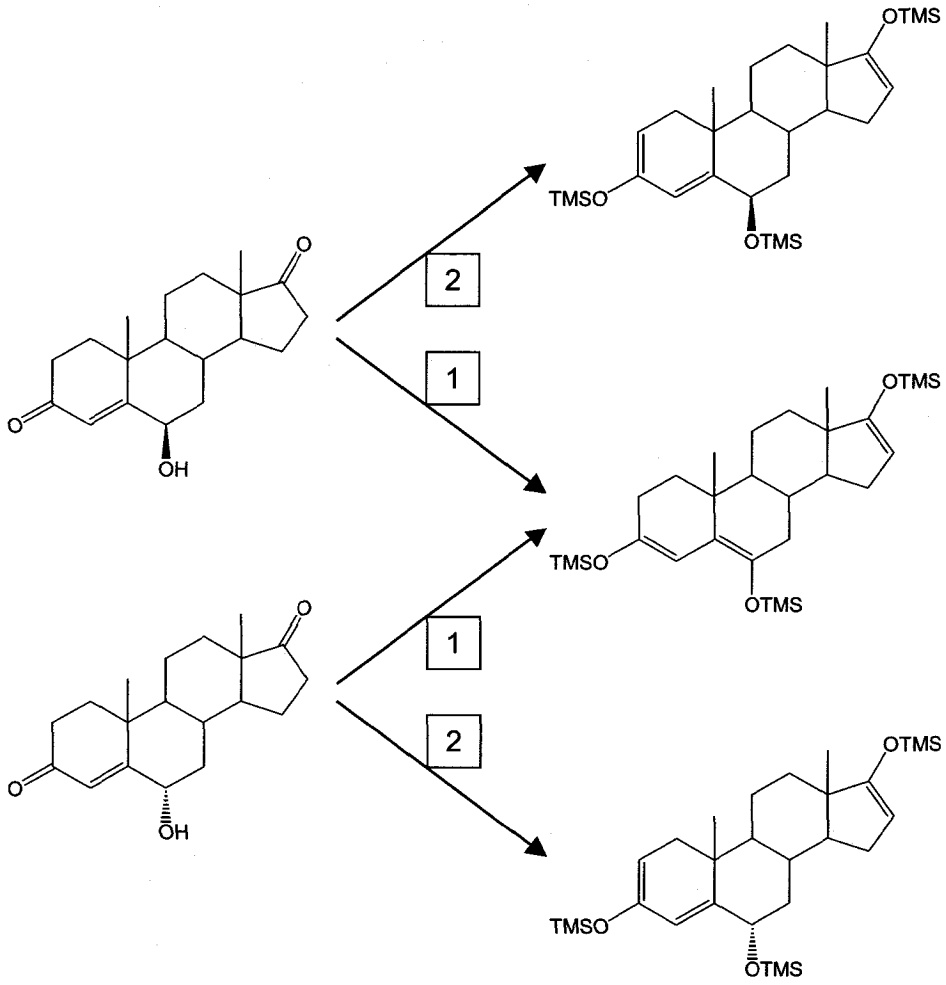
An isomerization experiment that was performed to gain insight into the relative stability of both products revealed that the 3,5-enolTMS derivative is more stable than its 2,4-enolTMS isomer.

Hydrogen-deuterium exchange experiments that were performed upon the model compound to study the role of acidity during trimethylsilylation, proved protons at C^2 to be more acidic than protons at C^6. The latter accounts for predominant formation of the 2,4-dienolTMS derivative during trimethylsilylation under basic conditions (MSTFA/KOAc/imidazole), as proton abstraction occurs preferentially at C^2. The silylation under basic conditions is therefore a kinetically controlled reaction, leading to the least stable enol intermediate and TMS derivative. Silylation under acidic conditions (MSTFA/NH_4I/ethanethiol) is solely determined by the stability of the intermediate enol and endproduct and is therefore under thermodynamic control.

INTRODUCTION

In Chapters 5 and 7, androst-4-ene-6α-ol-3,17-dione (6α-OH-Δ4-AEDIONE) and androst-4-ene-6β-ol-3,17-dione (6β-OH-Δ4-AEDIONE) were studied as metabolites of androst-4-ene-3,17-dione (Δ4-AEDIONE). However, these steroids could not be specifically analyzed with gas chromatography-mass spectrometry (GC-MS), because both compounds reacted to the same 3,5-dienol ether upon derivatization with MSTFA/NH_4I/ethanethiol (1000:2:3; v/w/v).

As noticed before by Schänzer *et al.* [1], steroids hydroxylated at C^6 can retain the stereochemical information on that atom when the derivatization is performed in a mixture of MSTFA/KOAc/imidazole (1000:20:20; v/w/w). Applying this procedure, 2,4-dienol ethers were identified as main products (see Scheme 1).



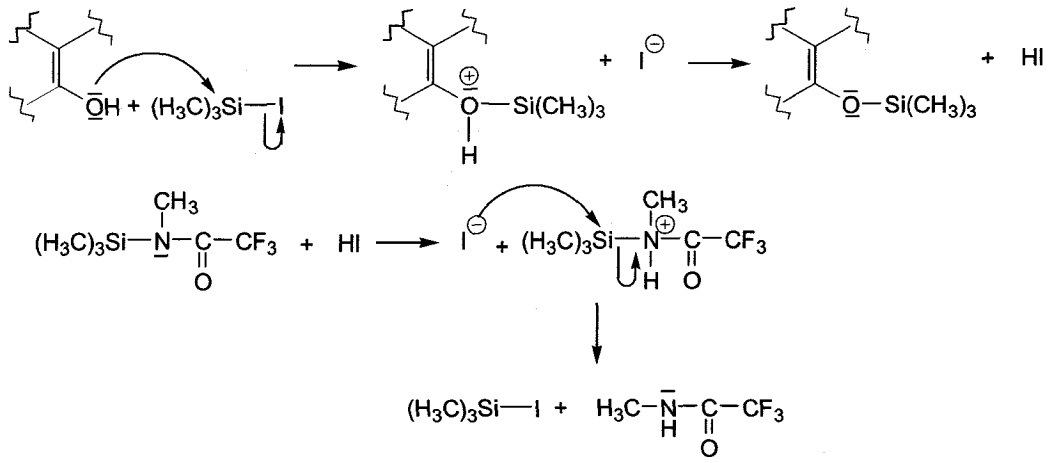
Scheme 1: Formation of 3,5-dienolTMS derivatives (route 1) and 2,4-dienolTMS derivatives (route 2) upon incubation in MSTFA/NH_4I/ethanethiol and MSTFA/KOAc/imidazole, respectively.

MSTFA/NH_4I/ethanethiol (mostly 1000:2:3 v/w/v) is frequently applied in steroid and doping analysis, in particular for trimethylsilylation of hydroxyl as well as enol groups. Derivatization with this reagent or others of comparable constitution has proven to be a robust and efficient method that provides good chromatography and mass spectrometric sensitivity. However, not much information is available about the underlying reaction mechanism.

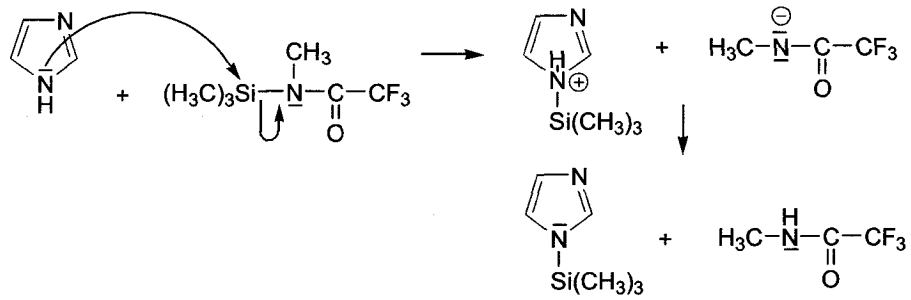
The application of MSTFA/NH_4I/ethanethiol is based on investigations on derivatization by Donike *et al.* [2]. They compared several reagents for derivatization and described trimethylsilyliodide (TMSI) as the most reactive trimethylsilylating reagent.

However, application of TMSI is impractical, as it is highly sensitive to hydrolysis by water and decomposition by oxygen and light. Therefore, TMSI should be kept in the absence from air and light. Decomposition of TMSI would result in extensive iodine formation, that can lead to the formation of non-volatile steroid-iodide adducts. To avoid these side-reactions, ethanethiol, present as S-TMS-ethanethiol in the derivatization medium, is added to reduce iodine, leading to TMSI formation [3].

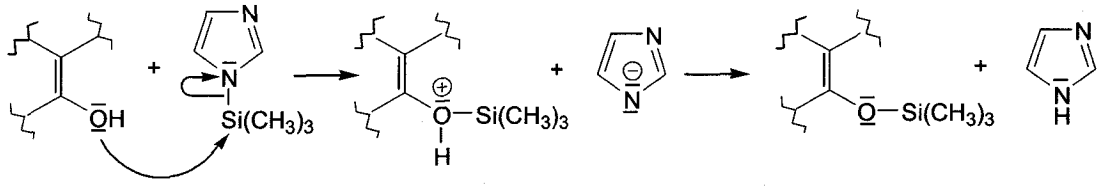
In the MSTFA/NH_4I/ethanethiol mixture, TMSI is formed *in situ* by reaction of iodide with MSTFA. In Scheme 2 a mechanism is suggested for TMSI formation and its subsequent reaction with steroid enols. The reaction with non-enol hydroxyl groups proceeds basically via the same mechanism. According this mechanism also HI is formed, giving rise to acidic conditions in the derivatization medium.



Scheme 2: Mechanisms of the reaction of TMSI with steroid enols and the *in situ* formation of TMSI from MSTFA and iodide.



Scheme 3: *In situ* TMS-imidazole formation from imidazole and MSTFA.

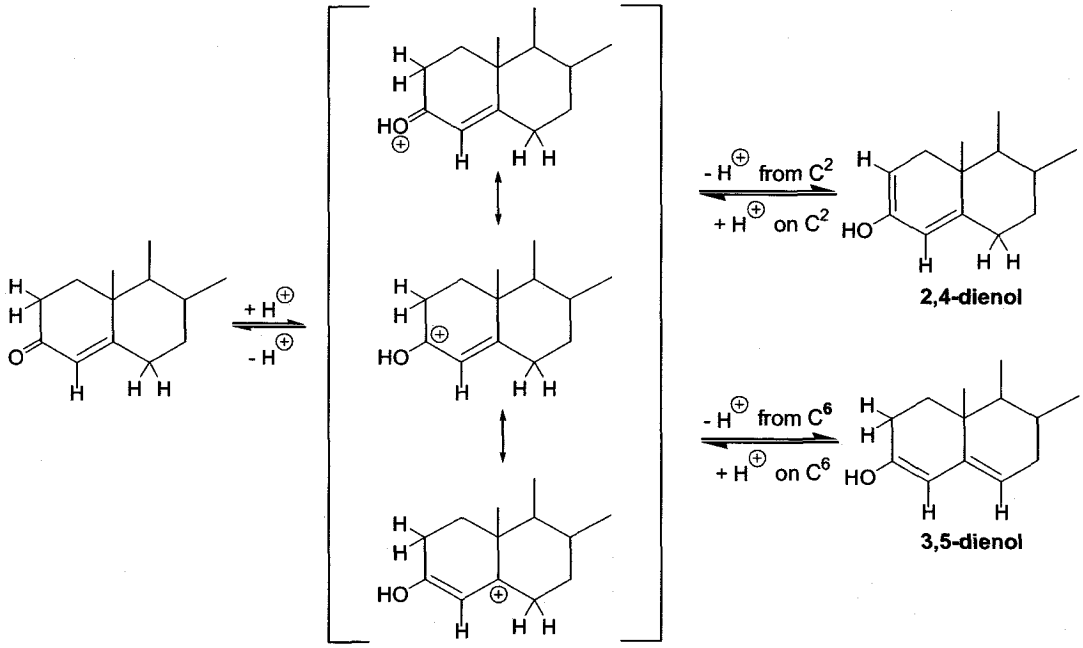


Scheme 4: Trimethylsilylation of steroid enols by TMS-imidazole.

TMSI cannot be used under basic conditions, as it would immediately be decomposed by nucleophilic substitution of the present ions (OH^- or AcO^-) on the trimethylsilylgroup (TMS). However, to apply basic conditions MSTFA/KOAc/imidazole can be used with trimethylsilylimidazole (TMS-imidazole) as *in situ* formed silylating agent from MSTFA and imidazole (see Scheme 3). A mechanism of silylation by TMS-imidazole is presented in Scheme 4.

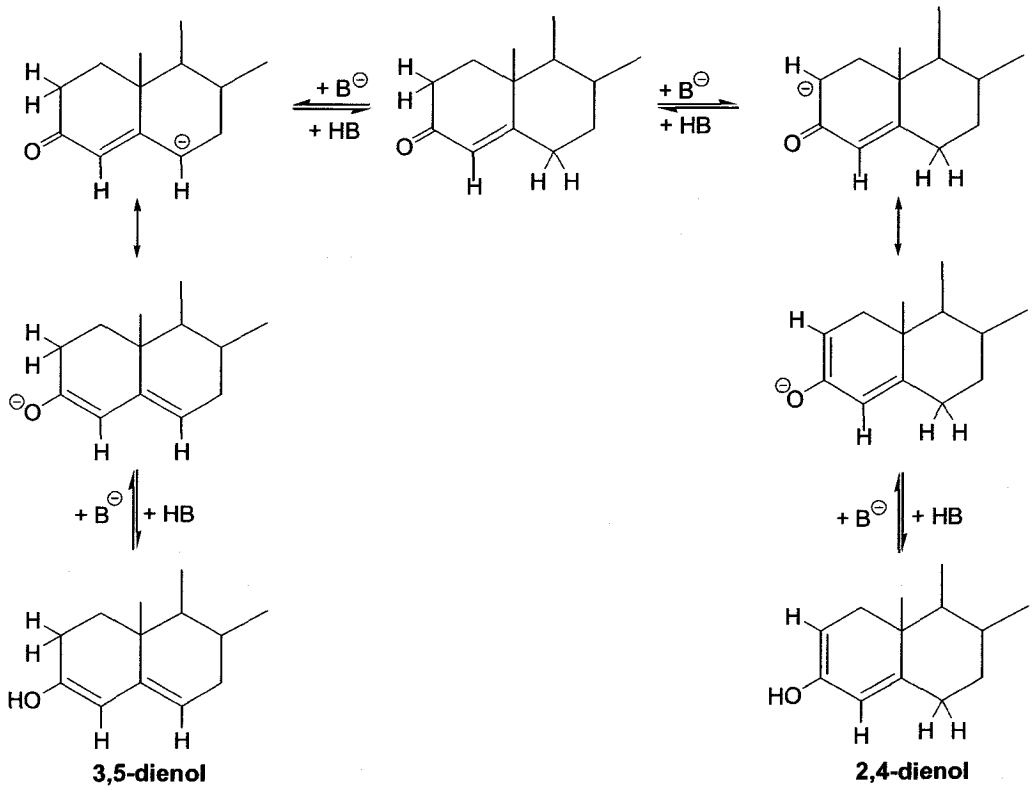
As the way of silylation by the two derivatization reagents is not fundamentally different (compare Schemes 2 and 4), it is unlikely that silylation is responsible for the observed differences in product formation. Therefore, it has to be assumed that the enolization that has to take place prior to silylation of the 3-keto-group, is the critical factor.

It is well established [4] that enolization of ketones can be catalyzed by acid as well as base. Thus, in the case of 3-keto-4,5-ene-steroids enolization under acidic conditions is initiated by protonation of the oxygen atom of the α,β-unsaturated ketone (see Scheme 5). Subsequent deprotonation can occur in 3 evident ways: from the oxygen atom, leading to the starting compound; from C^2, leading to the 2,4-dienol; and from C^6, leading to the 3,5-dienol.



Scheme 5: Mechanism of acid catalyzed enolization of 3-keto-4,5-ene-steroids.

For several reasons it has to be expected that proton-loss from C^6 will occur. Firstly, it is most likely that the mesomeric structure carrying the positive charge on C^5 has the highest relative stability. Secondly, it has to be expected that the 3,5-dienol, which is the result of proton-loss from C^6, is more stable than the 2,4-dienol resulting from proton-loss at C^2. The reason for the last mentioned higher stability is two-fold: the conjugated double bonds in the 3,5-dienol are in the energetically favored *s*-trans configuration and the A-ring is less strained by the presence of only one double bond.



Scheme 6: Mechanism of base catalyzed enolization of 3-keto-4-ene-steroids.

As illustrated in Scheme 6, enolization of 3-keto-androst-4-ene steroids under basic conditions can occur in two ways: proton extraction from either C^2 or C^6 leads to the 2,4-dienol or 3,5-dienol, respectively. Which route is predominant is solely determined by the acidity of the involved protons at C^2 and C^6.

In this study, derivatization of 6α-OH-Δ4-AEDIONE, 6β-OH-Δ4-AEDIONE and Δ4-AEDIONE with both reagents was investigated on an analytical (μg) as well as a preparative (mg) scale. The latter enabled us to analyze the products not only by means of GC-MS, but also with Nuclear Magnetic Resonance (NMR) spectroscopy.

Moreover, the influence of the acidity of both the steroid and the derivatization reagent on the course of the derivatization reaction was investigated. This was achieved by performing hydrogen-deuterium exchange experiments on the model steroid 17-methoxy-testosterone (methoxy-T).

After derivatization of the latter compound on a preparative scale with both reagents, the relative stability of the 2,4-dienolTMS derivative with respect to that of its 3,5-dienolTMS isomer was investigated by performing an isomerization experiment.

EXPERIMENTAL

Chemicals

Steroids: Androst-4-ene-3,17-dione (Δ4-AEDIONE) and testosterone (T) were obtained from Sigma, St. Louis, Missouri, USA. Androst-4-en-6α-ol-3,17-dione (6α-OH-Δ4-AEDIONE), androst-4-en-6β-ol-3,17-dione (6β-OH-Δ4-AEDIONE) were obtained from Steraloids, Newport, Rhode Island, USA.

Chemicals: All other chemicals were of analytical grade, unless indicated otherwise. Solvents were dried using standard procedures. *N*-Methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA), ethanethiol (97%), imidazole, phosphorous pentoxide, potassium acetate, magnesium sulfate and triethylamine hydrochloride were obtained from Sigma-Aldrich Chemie Company, Steinheim, Germany. Ammonium iodide was obtained from Fluka Chemie, Buchs, Switzerland. Sodium hydride (60% dispersion in mineral oil), methyl iodide (99% stabilized) and phenol (99%) were obtained from Acros Organics, Geel, Belgium. Potassium hydroxide, diethyl ether, tetrahydrofuran (THF), hexane, heptane, chloroform, acetone, benzene and methanol were obtained from Merck, Darmstadt, Germany. Diethyl ether, THF, benzene and toluene were of technical quality and were purified by predrying over powdered potassium hydroxide, followed by distillation from the sodium ketyl of benzophenone. Deuterated acetonitrile CD_3CN (^1H: 1.93 ppm; ^{13}C: 1.28 and 118.1 ppm), deuterated chloroform $CDCl_3$ (^1H: 7.27 ppm; ^{13}C: 77.0 ppm), deuterated methanol CD_3OD (^1H: 3.30 ppm; ^{13}C: 49.0 ppm) and deuterated benzene C_6D_6 (^1H: 7.15 ppm; ^{13}C: 128.0 ppm) were obtained from ARC Laboratories, Amsterdam, The Netherlands (isotopic purity ≥99.8%).

Gas chromatography and mass spectrometry

Analysis with gas chromatography-mass spectrometry (GC-MS) was performed in full scan mode with a Hewlett Packard gas chromatograph (Model 5890, Agilent Technologies, Waldbronn, Germany) coupled to a Hewlett Packard quadrupole mass spectrometer (Model 5972A). Ionization was performed in electron ionization (EI) mode at 70 eV.

Gas chromatography was performed with a HP-1 fused silica column (length 18 m, inner diameter 0.2 mm, film thickness 0.11 μm). Through electronic pressure control the column flow (helium) was constantly 1 ml/min. Sample injection of 1 μl was performed in split mode (ratio 1/10). A Hewlett Packard autosampler (Model 7673, Agilent Technologies, Waldbronn, Germany) was used for autoinjection. The injector temperature was set to 250°C. The oven temperature program used was: initial

temperature 180°C, 2°/min to 225°C, 30°/min up to 310°C, constant for 5 min. The interface temperature was set to 280°C.

NMR

All routine NMR spectra were recorded on a Bruker AC 300 spectrometer (^{1}H: 300.13 MHz; ^{13}C: 75.47 MHz) at 298 K in the appropriate solvent to be mentioned in the description of the specific experiment. Chemical shifts are relative to external tetramethylsilane. ^{13}C-^{1}H NMR correlation experiments (HETCOR) were performed at 298 K in appropriate solvents using a Varian Inova 300 MHz spectrometer.

Trimethylsilylations of Δ4-AEDIONE, 6α- and 6β-OH-Δ4-AEDIONE

Silylation for GC-MS analysis was performed by incubation of 1 µg Δ4-AEDIONE, 6α- or 6β-OH-Δ4-AEDIONE in 50 µl of derivatization reagent for 30 min at 80°C in a sealed glass tube. The reagents used were MSTFA/NH_4I/ethanethiol (1000:2:3;v/w/v) [2] and MSTFA/KOAc/imidazole (1000:20:20;v/w/w) [1]. After derivatization reaction mixtures were directly injected for analysis.

For NMR analysis the derivatization reactions were performed by incubation of 10 mg of the respective steroids in 7.25 ml of the derivatization reagents mentioned above. The reactions were performed in an atmosphere of dry nitrogen in a Schlenk vessel. Under continuous stirring the incubation took place at 80°C for 30 min. Besides by NMR analysis, product formation was always checked by GC-MS analysis of the reaction mixture, after dilution with heptane. After completion of the reaction the reaction mixture was evaporated to dryness under reduced pressure of an oil pump. NMR analyses were performed on samples dissolved in C_6D_6.

Synthesis of 17-methoxy-testosterone

17β-Methoxy-androst-4-ene-3-one (methoxy-T) was synthesized based on Floresca *et al.* [5]. However, because of its easier removal from the product, THF was used instead of DMF as the solvent in the following procedure:

To a suspension of 0.264 g (11 mmol) of NaH (freed of oil by washing with hexane) in 50 ml of THF was added 2.88 g (10 mmol) of testosterone (T). The suspension was stirred for an hour at room temperature and subsequently, 14.2 g (100 mmol) of methyl iodide was quickly added dropwise. No noticeable development of heat occurred.

After stirring for 72 hours at room temperature, the initially turbid suspension had changed into an almost homogeneous slightly yellow solution, indicating that almost

all the NaH had been consumed. A sample of approximately 0.5 ml was drawn and processed by respectively diluting with water, extracting the aqueous phase with diethyl ether, drying the ether solution over magnesium sulfate, filtering and evaporating the volatile constituents. Analysis of the semi-solid residue using ^{1}H- and ^{13}C-NMR ($CDCl_3$) revealed the presence of methoxy-T and testosterone in a ratio of approximately 3:2. Furthermore, TLC (silica; chloroform-acetone 10:1 v.v.) showed that small amounts (not detectable with NMR) of at least two other compounds were present.

After adding an additional 0.1 g (4 mmol) of NaH, the mixture was refluxed for 5 hours. Analysis by NMR and TLC of a new sample indicated that almost no T was left and that, together with one main product, small amounts of at least three other products were present.

Water (5 ml) was added dropwise while the reaction mixture was cooled in an ice bath. The volatile constituents were removed at a Rotavap and the slightly yellow solid residue was submitted to an oil pump vacuum until constant weight (5.0 g).

After adding 20 ml of dry diethyl ether the mixture was vigorously stirred for half an hour after which the insoluble white solid was removed by filtration over a glass filter (G3). The solid was treated with diethyl ether in this way two more times. Removal of the solvent from the combined filtrates at a Rotavap afforded 3.42 g of a slightly yellow sticky solid. This was submitted to column chromatography on silica using $CHCl_3$ as eluent to yield pure methoxy-T. NMR analysis was performed on samples dissolved in CD_3OD and CD_3CN. The melting point (uncorrected) was determined with a Mettler FP5/FP51.

Preparative trimethylsilylation of methoxy-T in MSTFA/NH_4I/ethanethiol

In a Schlenk vessel equipped with a magnetic stirring bar were brought: 30.2 mg (0.1 mmol) of methoxy-T and a solution of 14.5 mg (0.1 mmol) of NH_4I and 15 µl of ethane thiol in 3 ml of MSTFA. The vessel, with the vigorously stirred reaction mixture, was immersed in an oil bath of 80°C and heating and stirring were continued for 30 min. Immediately after bringing the colorless reaction mixture to room temperature, a sample was drawn and submitted to GC-MS.

The volatile constituents of the reaction mixture were removed in an oil pump vacuum at room temperature affording a slightly yellow solid. The solid was pure (as established by GC-MS) and was identified as the 3,5-enolTMS ether by means of NMR and GC-MS.

Since the product was expected to be thermally unstable, no effort was made to determine the melting point. Moreover, because of suspected chemical instability

under acid and basic conditions, the use of solvents with an acidic character like chloroform was avoided in all manipulations of the material. NMR analysis was performed with C_6D_6 as solvent.

Preparative trimethylsilylation of methoxy-T in MSTFA/KOAc

This reaction was conducted in the same way as the preparative trimethylsilylation in MSTFA/NH_4I/ethanethiol, but with a solution of 100 mg of potassium acetate in 5 ml of MSTFA as the trimethylsilylating reagent. No imidazole was added in order to prevent problems with the interpretation of NMR spectra. Again GC-MS analysis was performed on a sample drawn immediately after bringing the reaction mixture to room temperature.

Evaporation of the volatile constituents under reduced pressure of an oil pump at room temperature afforded a slightly yellow sticky solid. After adding 3 ml of dry benzene the mixture was vigorously stirred for 30 min. Subsequently, the solution was removed from the solid sticking against the stirring bar and the walls of the Schlenk vessel and filtered through a small plug of glass wool in a Pasteur pipet contained in another Schlenk vessel. The contents of the reaction vessel were treated with benzene two more times followed by evaporation of the solvent under reduced pressure. A white sticky residue was obtained. A sample was dissolved in C_6D_6 and analyzed with NMR.

Isomerization of 2,4-dienolTMS methoxy-T

This reaction was conducted by a modification of a procedure described in the literature [6]. In contrast to the compounds investigated by these authors, the steroidal trimethylsilyl ethers in the present study are solids. Therefore, it was necessary to use a solvent. For reasons of inertness and boiling point the choice was made for toluene, which was applied in the following procedure:

The NMR sample of the preceding MSTFA/KOAc derivatization experiment was recovered in the original Schlenk vessel and after evaporating the NMR solvent C_6D_6 under reduced pressure, 2 ml of dry toluene was added. This resulted in a homogeneous solution to which 5 mg of triethylamine hydrochloride was added.

Subsequently, the reaction mixture was heated in an oil bath of 100°C during half an hour. A sample for GC-MS analysis was drawn immediately after bringing the reaction mixture to room temperature. Afterwards, the solvent was evaporated under an oil pump vacuum at room temperature, leaving an off-white solid residue. This was dissolved in C_6D_6 and analyzed with NMR.

Hydrogen-deuterium exchange (1)

Because KOAc is the basic catalyst used in the preparative derivatizations, the same base dissolved in CD_3OD was used initially in an effort to bring about H-D exchange in methoxy-T. Unfortunately, this experiment showed that no H-D exchange occurred under the influence of KOAc in CD_3OD, even at temperatures up to 50°C.

For that reason also an experiment with a much stronger base, *i.e.* $NaOCD_3$ in CD_3OD, was performed. Therefore, a stock solution of $NaOCD_3$ was prepared in a Schlenk vessel by dissolving approximately 50 mg of sodium in 1 ml of CD_3OD.

In a NMR tube 19.3 mg (0.0639 mmol) of methoxy-T was dissolved in 0.8 ml of CD_3OD and a ^{1}H-NMR spectrum was recorded. Subsequently, stock solution of $NaOCD_3$ was added with a microsyringe, until the solution showed pH 8-9 on moist indicator paper. The occurrence of H-D exchange was monitored by recording ^{1}H-NMR spectra with regular intervals of time and comparing the relative height of the integrals of (clusters of) signals using the integral of the C^{18}-protons as internal standard. Also, GC-MS analyses were performed as a function of time.

Hydrogen-deuterium exchange (2)

H-D exchange was performed with a weaker base, *i.e.* $LiOC_6H_5$ in CD_3OD. To prepare a stock solution of lithium phenolate in CD_3OD, 57 mg (0.61 mmol) of phenol was dissolved in 5 ml of diethyl ether, contained in a Schlenk vessel equipped with a magnetic stirring bar. Subsequently, approximately 20 mg (3 mmol) of lithium powder was added and the mixture was stirred at room temperature. After approximately one hour the mixture became white turbid, presumably by a limited solubility of lithium phenolate in ether. The mixture cleared up slowly after addition of 5 ml of THF.

After stirring during 60 hours the mixture appeared as a colorless, clear solution with small pieces of lithium powder floating in it. The solution was transferred into a syringe and immediately filtered through a small plug of glass wool in a Pasteur pipet contained in a second Schlenk vessel. The solvents were evaporated under reduced pressure and the white solid residue was submitted at room temperature to an oil pump vacuum for 2 hours. Subsequently, 2 ml of CD_3OD was added and after stirring for a few minutes this solvent was also evaporated under reduced pressure, leaving a white solid residue that was submitted to oil pump vacuum for 1 hour. Finally, 1.5 ml of CD_3OD was added.

In a NMR tube 18.7 mg (0.0619 mmol) of methoxy-T was dissolved in 0.8 mL of CD_3OD and a ^{1}H-NMR spectrum was recorded. Subsequently, stock solution of $LiOC_6H_5$ was added with a microsyringe, until the solution showed pH 8-9 on moist indicator paper. The occurrence of H-D exchange was monitored by recording ^{1}H-

NMR spectra with regular intervals of time as indicated above. Also, several GC-MS analyses were performed as a function of time.

RESULTS AND DISCUSSION

MSTFA/NH_4I/ethanethiol derivatization of (hydroxy metabolites of) Δ4-AEDIONE

After silylation of 6α- and 6β-OH-Δ4-AEDIONE with MSTFA/NH_4I/ethanethiol on an analytical scale, full scan GC-MS analysis of the reaction mixture resulted in coeluting peaks, that could not be separated by modification of the temperature program.

Also upon derivatization on a preparative scale, the respective derivatives were identical as shown by GC-MS and NMR analyses (see Figure 1).

Derivatization of Δ4-AEDIONE resulted in one derivative that was detected as a single peak in the chromatogram (see Figure 3).

MSTFA/KOAc/imidazole derivatization of (hydroxy metabolites of) Δ4-AEDIONE

After trimethylsilylation on an analytical scale of 6α- and 6β-OH-Δ4-AEDIONE with MSTFA/KOAc/imidazole, full scan GC-MS analysis resulted in peaks with different retention times and mass spectra (see Figure 2).

The product of the reaction on a preparative scale could not be analyzed by NMR, because imidazole could not be removed from the material.

Derivatization of Δ4-AEDIONE resulted in two chromatographic peaks, of which the minor one showed the same retention time and mass spectrum as the peak that was obtained with MSTFA/NH_4I/ethanethiol (see Figure 3).

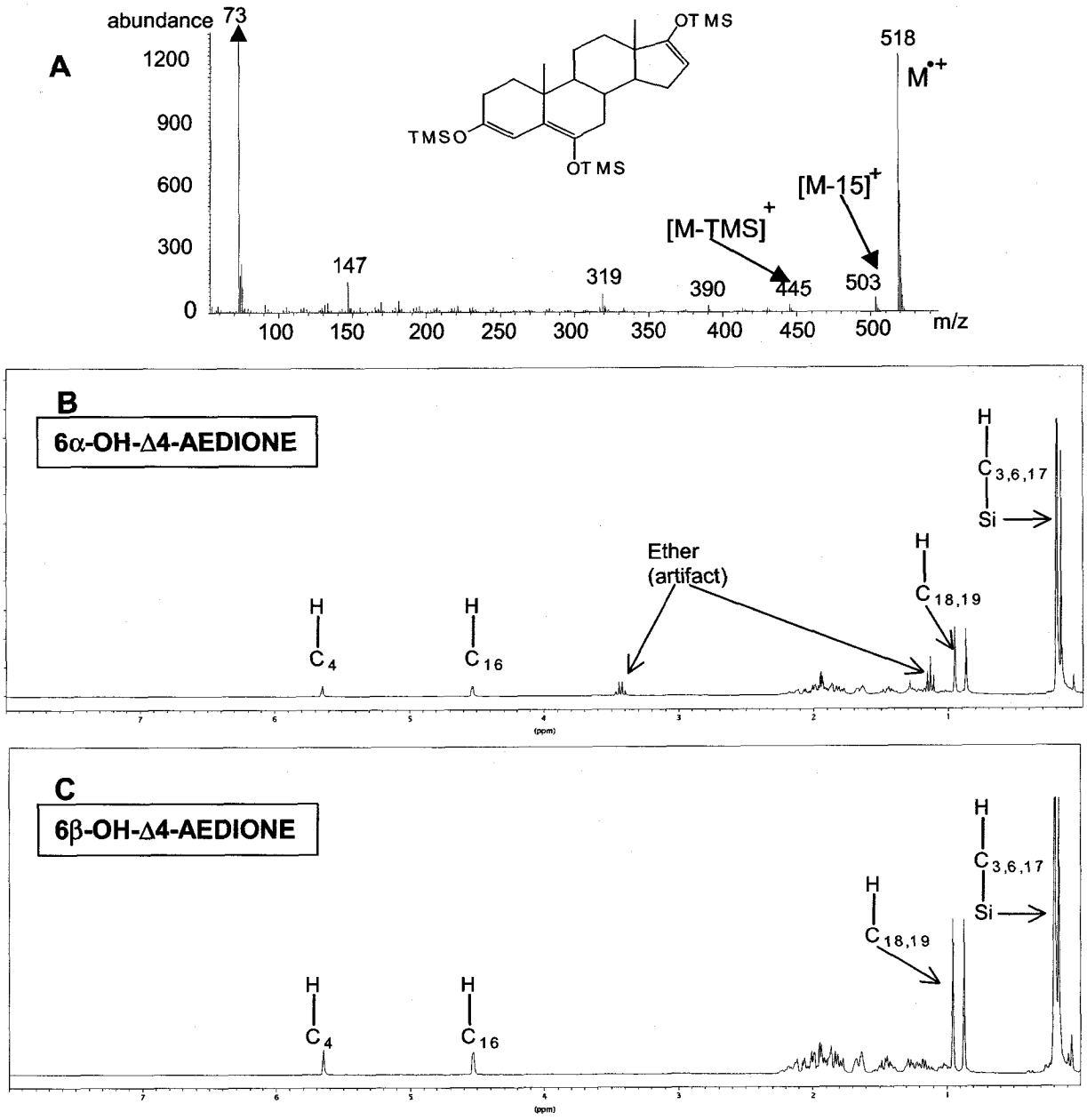
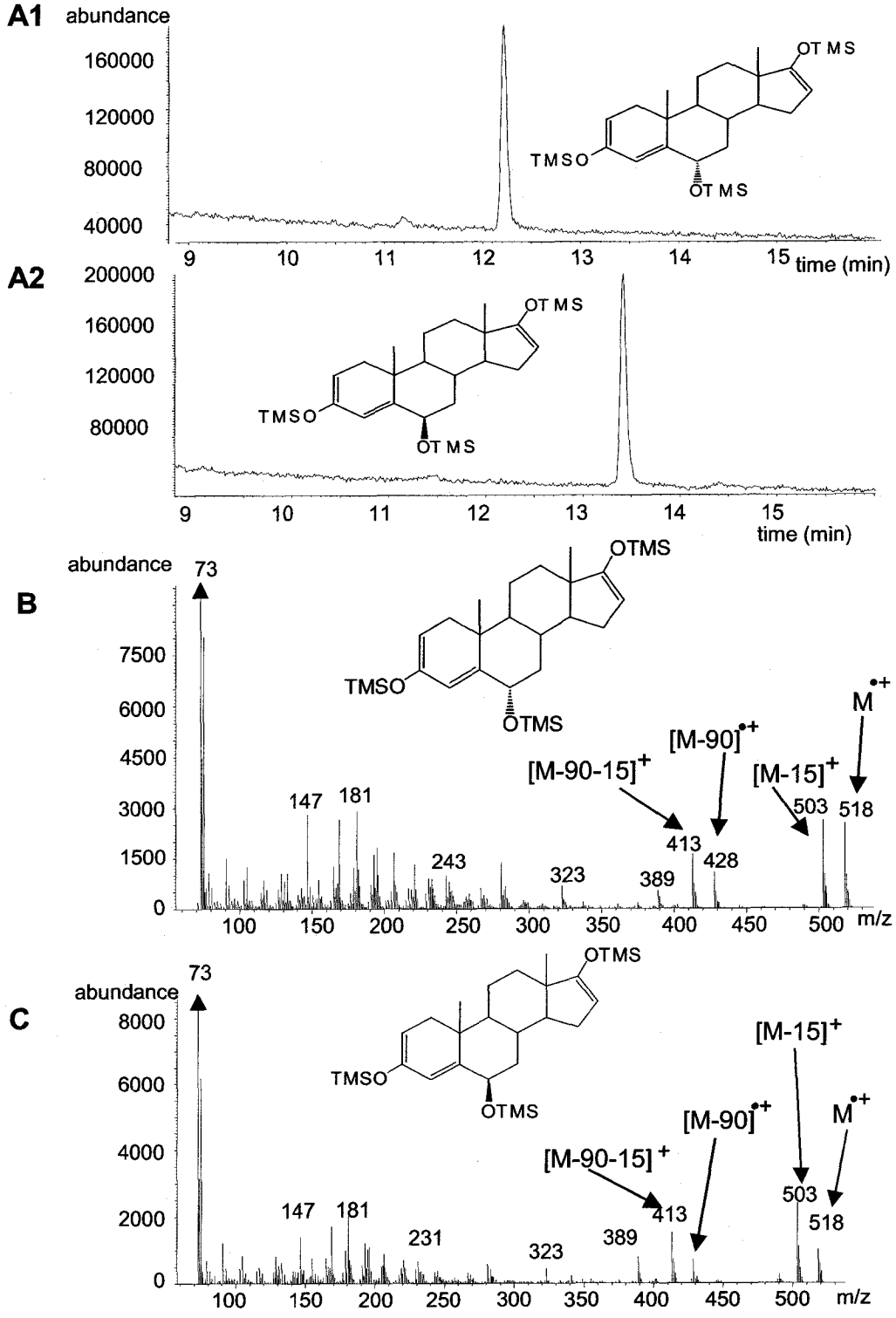


Figure 1: Mass spectrum (**A**) and the ^{1}H NMR spectra (**B** and **C**) of the product obtained upon MSTFA/NH_4I/ethanthiol derivatization of 6α-OH-Δ4-AEDIONE and 6β-OH-Δ4-AEDIONE, respectively.



Figuur 2: Total ion current chromatograms (**A1** and **A2**) and mass spectra of the silylation products of 6α-OH-Δ4-AEDIONE (**B**) and 6β-OH-Δ4-AEDIONE (**C**) using MSTFA/KOAc/imidazole.

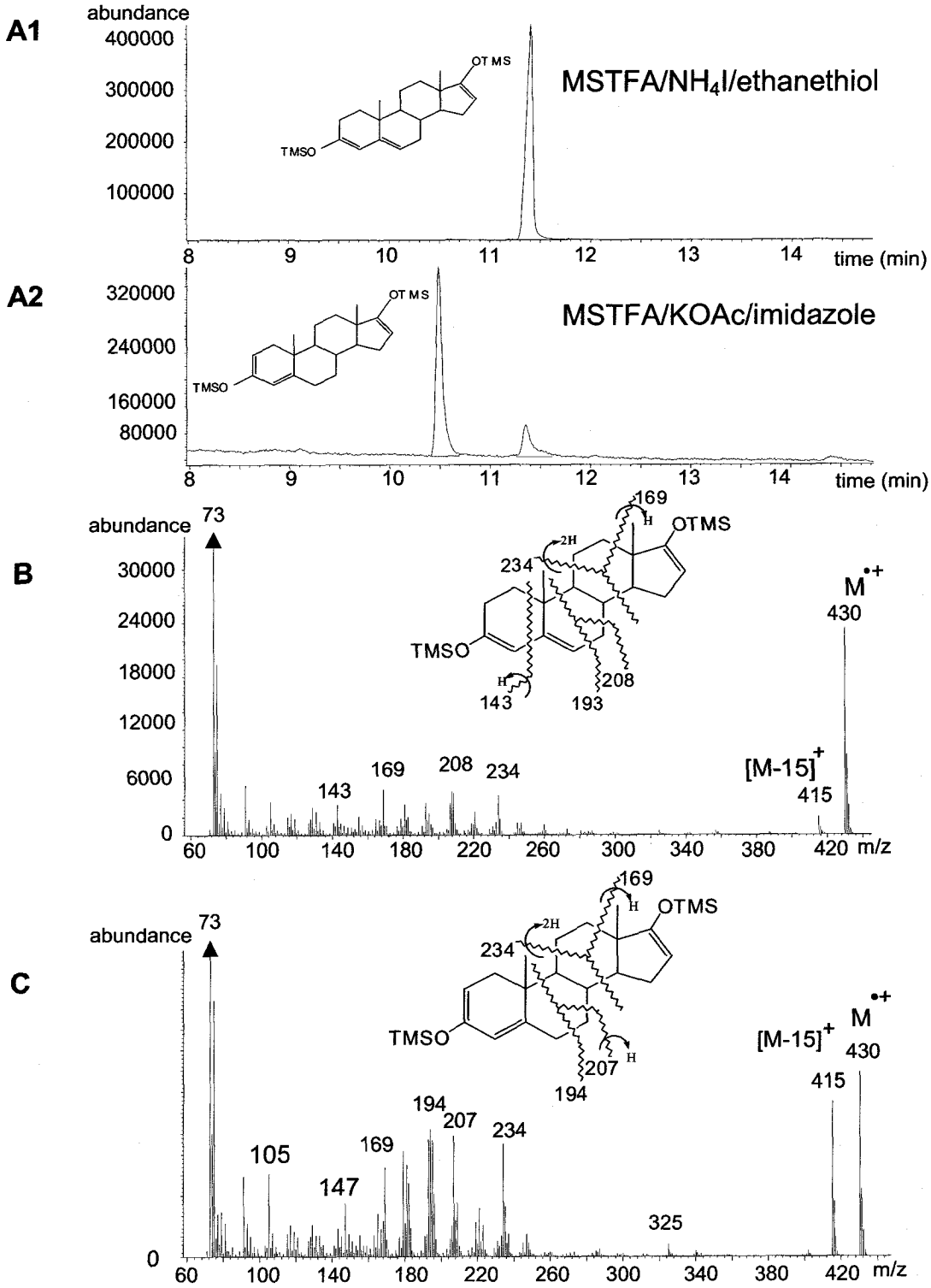


Figure 3: Total ion current chromatograms from silylation products of Δ4-AEDIONE using MSTFA/NH_4I/ethanethiol (**A1**) and MSTFA/imidazole/KOAc (**A2**), as well as the corresponding mass spectra (**B** and **C**).

Synthesis of methoxy-T

A yield of 460 mg (15 %) of pure methoxy-T was obtained: mp 122-123°C (litt. 121-122°C [5]); ^{1}H NMR ($CDCl_3$): δ 0.77 (s, 3, C-18 CH_3), 1.16 (s, 3, C-19 CH_3), 3.20 (t, *J* = 8.4 Hz, 1, C*H*OCH_3), 3.31 (s, 3, OCH_3), 5.69 (s, 1, vinylic H) ppm; ^{13}C NMR ($CDCl_3$): δ 11.5 (C-18), 17.3 (C-19), 20.7 (C-11), 23.2 (C-15), 27.5 (C-16), 31.5 (C-6), 32.7 (C-7), 33.9 (C-2), 35.4 (C-1 or C-12), 35.7 (C-1 or C-12), 37.6 (C-8), 38.6 (C-10), 42.7 (C-13), 50.7 (C-14), 53.8 (C-9), 57.8 (OCH_3), 90.4 (C-17), 123.8 (C-4), 171.1 (C-5), 199.3 (C-3) ppm.

The total ion current chromatogram of the substance, showed one single peak at 9.22 min, with a corresponding full scan mass spectrum as shown in Figure 4. Molecular mass was detected at m/z 302. The main fragments were at m/z 287 ([M-CH_3]$^+$), m/z 260 (A-ring fragmentation). In addition, several m/z values of fragments from other ring fragmentations were observed.

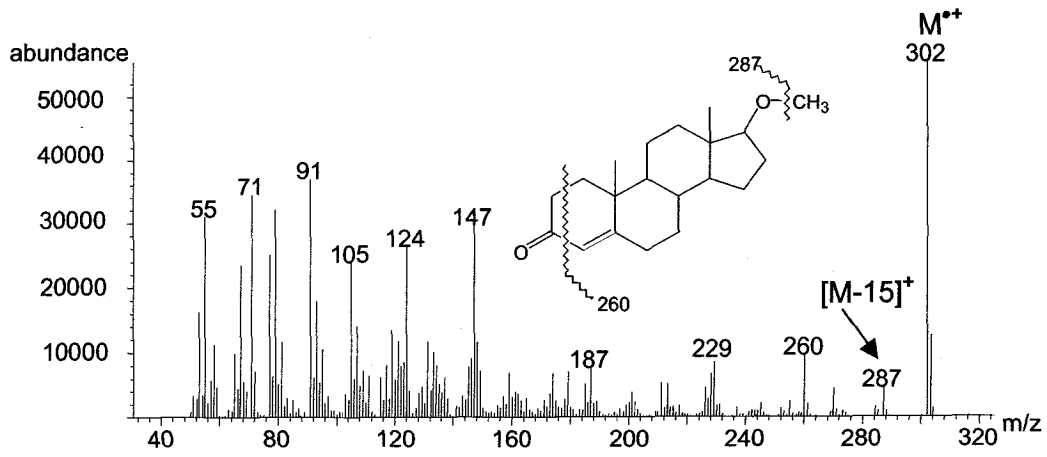


Figure 4: Full scan mass spectrum of methoxy-T.

Preparative trimethylsilylation of methoxy-T in MSTFA/NH_4I/ethanethiol

A single peak was detected in the full scan chromatogram of the reaction mixture. The chromatogram and the corresponding recorded mass spectrum are shown in Figure 5.

^{1}H NMR (CD_3OD): δ 0.18 (s, 9, trimethylsilyl), 0.78 (s, 3, C-18 CH_3), 0.96 (s, 3, C-19 CH_3), 3.29 (t, *J* = 8.4 Hz, 1, C*H*OCH_3), 3.31 (s, 3, OCH_3), 5.13 (m, 1, H^6), 5.23 (d, *J* = 1.9 Hz, 1, H^4) ppm; ^{13}C NMR (CD_3OD): δ 0.3, 11.9, 19.4, 22.0, 24.3, 28.6, 28.7, 32.5, 33.1, 35.3, 35.9, 39.0, 44.0, 49.0 (This signal coincides with the central peak of the CD_3OD signal, as confirmed by a ^{13}C spectrum of the same material in CD_3CN),

53.1, 58.2, 92.2, 110.2, 119.5, 142.2, 151.4 ppm. GC-MS analysis of the NMR sample afforded the same result as the GC-MS of the reaction mixture as depicted in Figure 5.

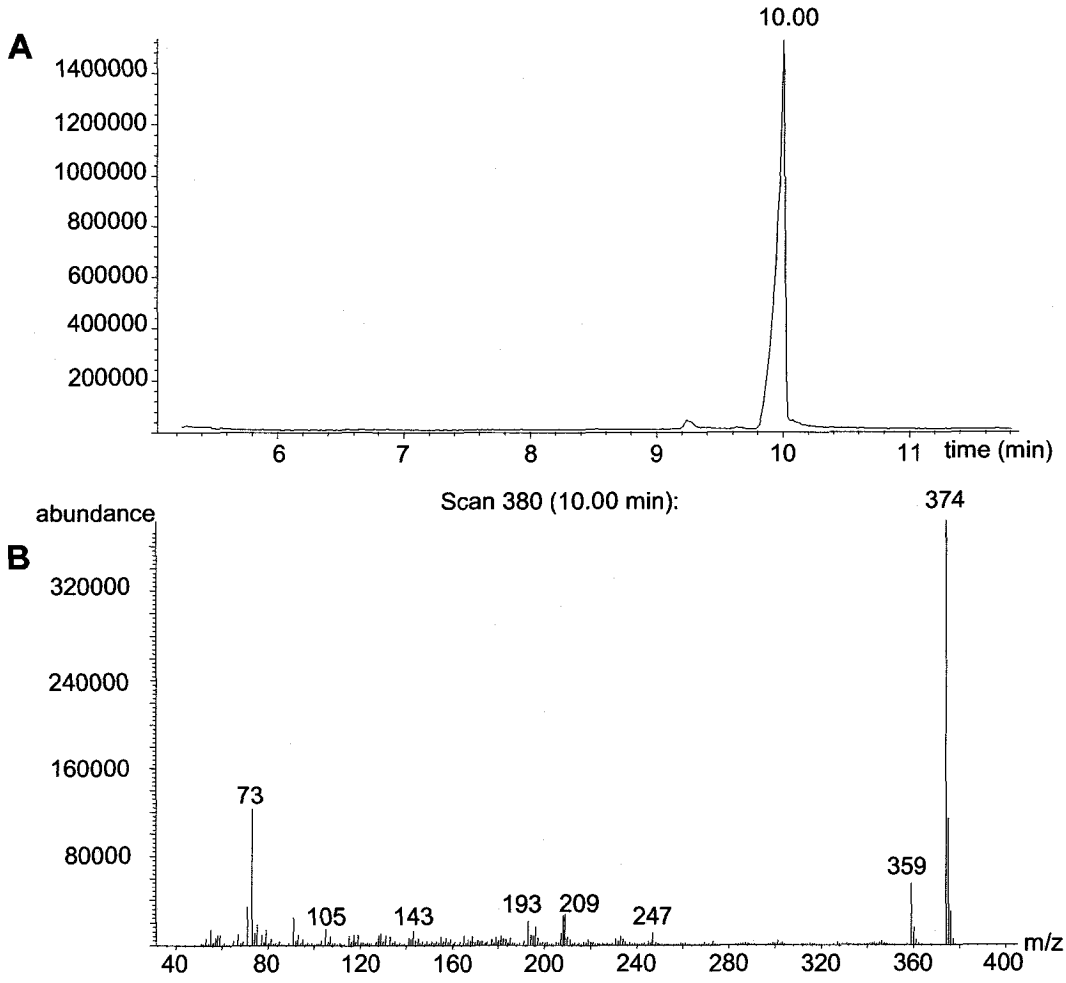


Figure 5: Total ion current chromatogram of the reaction mixture after derivatization on preparative scale of methoxy-T in MSTFA/NH_4I/ethanethiol (**A**) and the corresponding mass spectrum of the main product (**B**).

Preparative trimethylsilylation of methoxy-T in MSTFA/KOAc

The total ion current chromatogram of the sample drawn immediately after the reaction revealed the presence of two peaks with retention times 9.14 min. and 9.92 min, respectively. The peak with retention time 9.92 min afforded a mass spectrum

that was identical with the mass spectrum of the 3,5-dienolTMS ether as depicted in Figure 5.

The peak at 9.14 min represented a mixture of the 2,4-dienolTMS ether with a smaller quantity of non-derivatized methoxy-T. This is illustrated by the extracted ion chromatogram of m/z 374.3 and 302.3 in Figure 6. Based on the same m/z values, the area ratio of methoxy-T/2,4-dienolTMS/3,5-dienolTMS was 0.2 : 0.8 : 1.0.

^{1}H-NMR (C_6D_6) of the residue also revealed that the material was a mixture of methoxy-T, 2,4-dienolTMS ether and 3,5-dienolTMS ether. However, based on the integrals of the corresponding vinylic protons, the ratio was approximately 3.2 : 5.2 : 1.0. ^{1}H-NMR (C_6D_6) 2,4-dienolTMS ether: δ 0.86 (C-18 CH_3), 1.03 (C-19 CH_3), 3.29 (t, J = 8.4 Hz, C*H*OCH_3), 3.20 (s, OCH_3), 4.85 (m, H^2), 5.60 (s, H^4) ppm.

Immediately after recording the NMR spectrum, a sample was drawn from the NMR tube and analyzed by GC-MS. The area ratio of methoxy-T/2,4-dienolTMS/3,5-dienolTMS obtained from the extracted ion chromatogram was 1.0 : 1.9 : 1.0.

This experiment showed that both the 2,4-dienolTMS and 3,5-dienolTMS derivatives of methoxy-T are formed by this derivatization method. The stability of the 2,4-dienol isomer is very low, as was indicated by the relatively increasing concentration of non-silylated methoxy-T upon processing a sample for NMR analysis. Because no methoxy-T formation was detected after processing a sample for NMR analysis of the 3,5-dienolTMS product of MSTFA/NH_4I/ethanethiol derivatization, it has to be concluded that derivative decomposition occurs only with the 2,4-dienol isomer. However, this conclusion could not be confirmed by the change in area ratio of both dienolTMS ethers before and after NMR analysis.

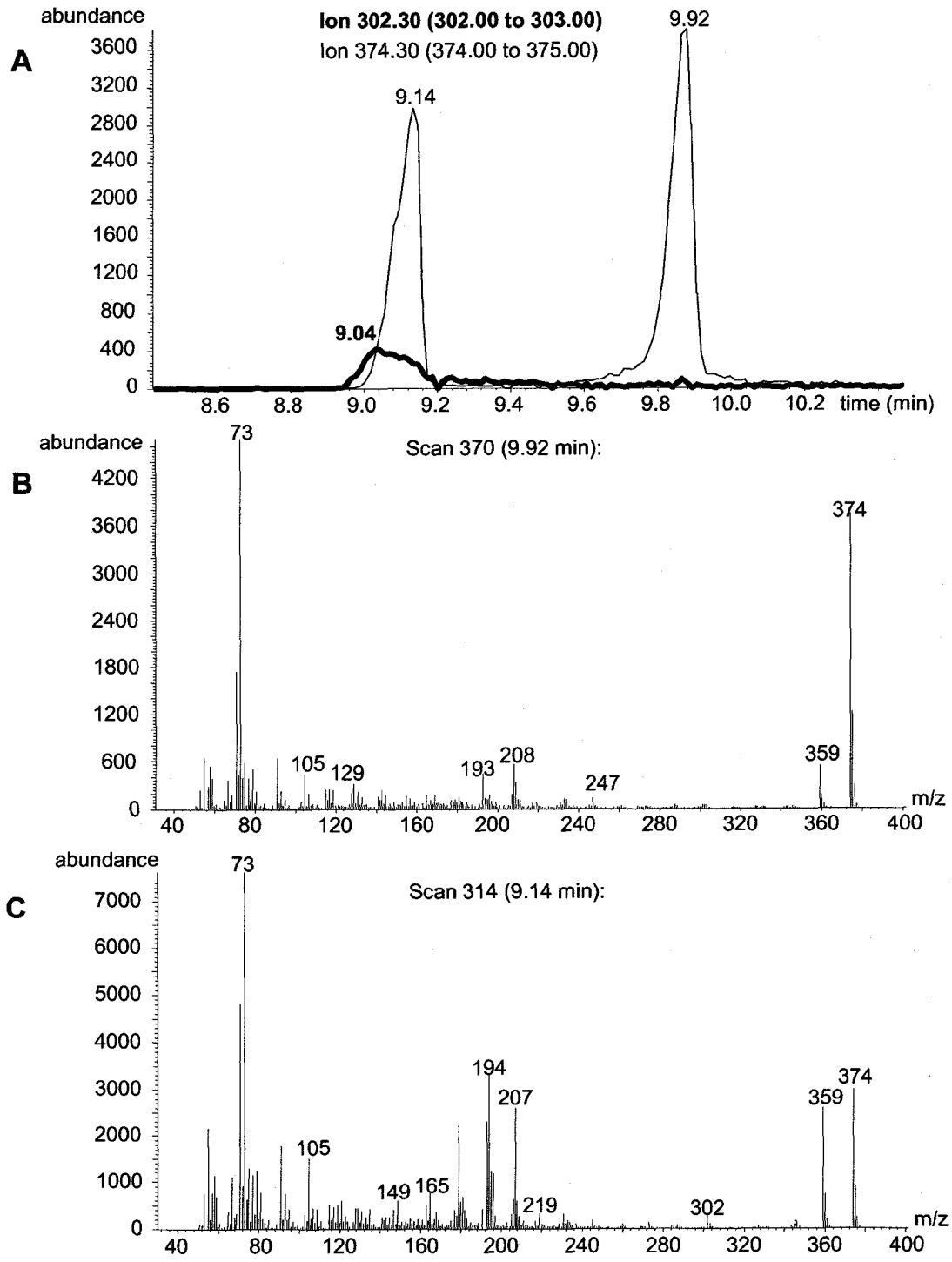


Figure 6: Extracted ion chromatogram (**A**; m/z 302.3 and 374.3) of the reaction mixture after derivatization of methoxy-T in MSTFA/KOAc on preparative scale, and the corresponding full scan mass spectra recorded at 9.14 (**B**) and 9.87 min (**C**), respectively.

Isomerization reaction

Full scan GC-MS analysis immediately performed after the isomerization experiment, revealed the presence of two peaks with retention times 8.99 min and 9.91 min, respectively, with an area ratio of approximately 0.3 : 1.0 in the extracted ion chromatogram (see Figure 7). The peak with retention time 8.98 min showed a mass spectrum fully identical with that of non-derivatized methoxy-T. The mass spectrum of the peak with retention time 9.91 was identical with that of the 3,5-dienolTMS ether. No 2,4-dienolTMS ether could be detected. ^{1}H NMR analysis confirmed the material to be a mixture of methoxy-T and its 3,5-dienolTMS ether in a ratio of approximately 1.7 : 1.0.

This experiment showed that the 2,4-dienolTMS ether was converted to either methoxy-T or the 3,5-dienolTMS ether. As the detected ratio of methoxy-T vs. its 3,5-dienolTMS ether decreased during the isomerization experiment (either established by NMR of GC-MS), it was concluded that the major part of the 2,4-dienolTMS ether was converted to the 3,5-dienol ether.

Hydrogen-deuterium exchange experiment (1)

It appeared that 3 signal clusters decreased as a function of time, *i.e.* the signal of the vinylic proton at 5.69 ppm and two clusters of signals at respectively 2.5 and 2.3 ppm, each representing 2 protons. The results of the measurements are depicted in Figure 8. Protons at C^2 and C^6 could not be assigned to these signal clusters. Therefore, the H-D exchange with $NaOCD_3$ in CD_3OD does not allow a decisive conclusion with respect to the relative acidities of the protons at C^2 and C^6.

This experiment showed that under the applied conditions H-D exchange occurs at three positions in the molecule, including the vinylic proton at C^4. The fact that exchange of the vinylic proton occurred, implies proton abstraction at C^6, which is in an allylic position with respect to the double bond (see Scheme 7).

These observations lead to the conclusion that $NaOCD_3$ is too strong, *i.e.* not selective enough, to differentiate between protons of different acidity. For this reason also an experiment with a weaker base, *i.e.* $LiOC_6H_5$ in CD_3OD, was performed.

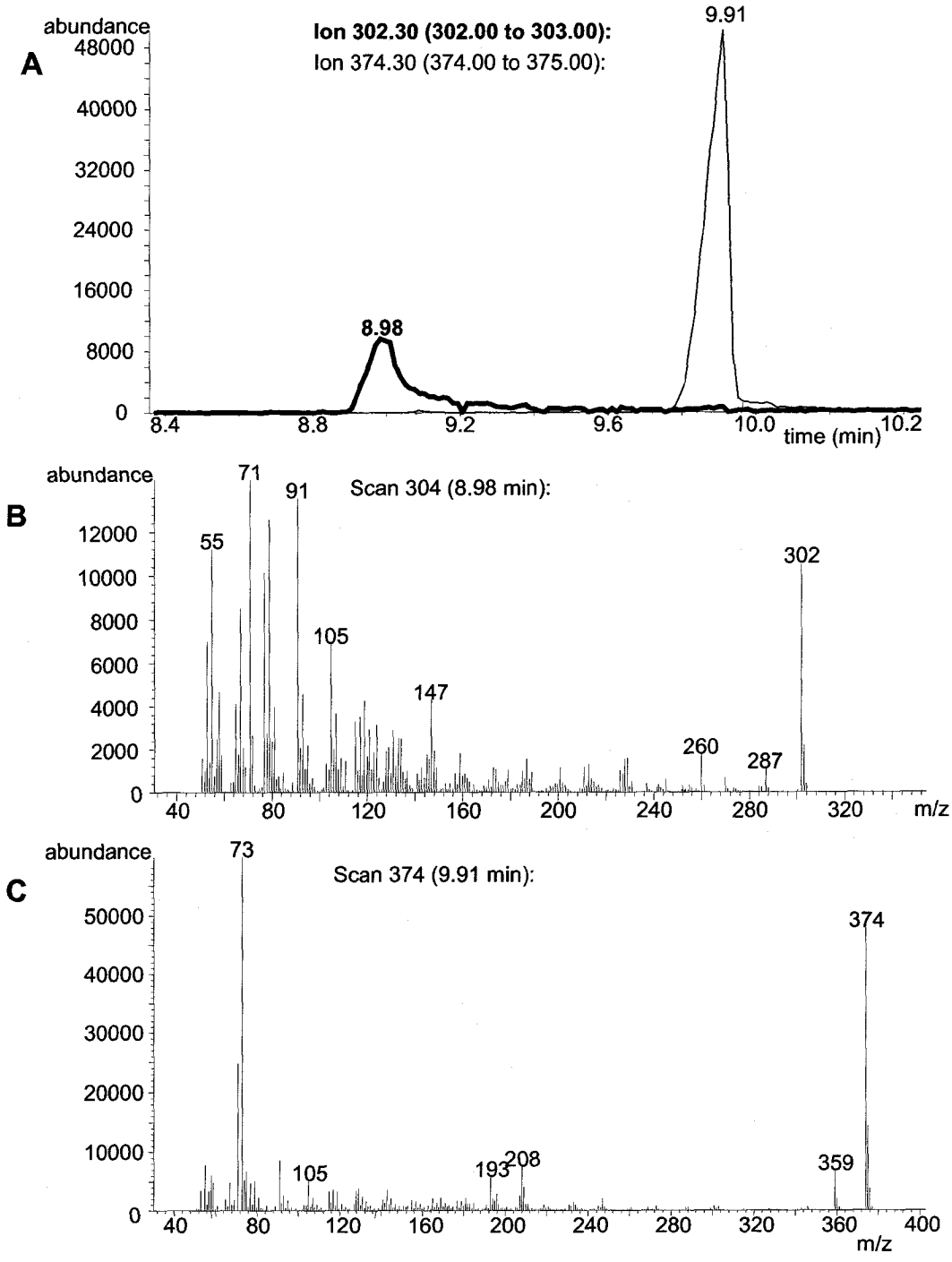


Figure 7: Extracted ion chromatogram (**A**; m/z 374.3 and 302.3) and the corresponding mass spectra (**B** and **C**) of the products of MSTFA/KOAc derivatization of methoxy-T after isomerization by triethylamine.HCl.

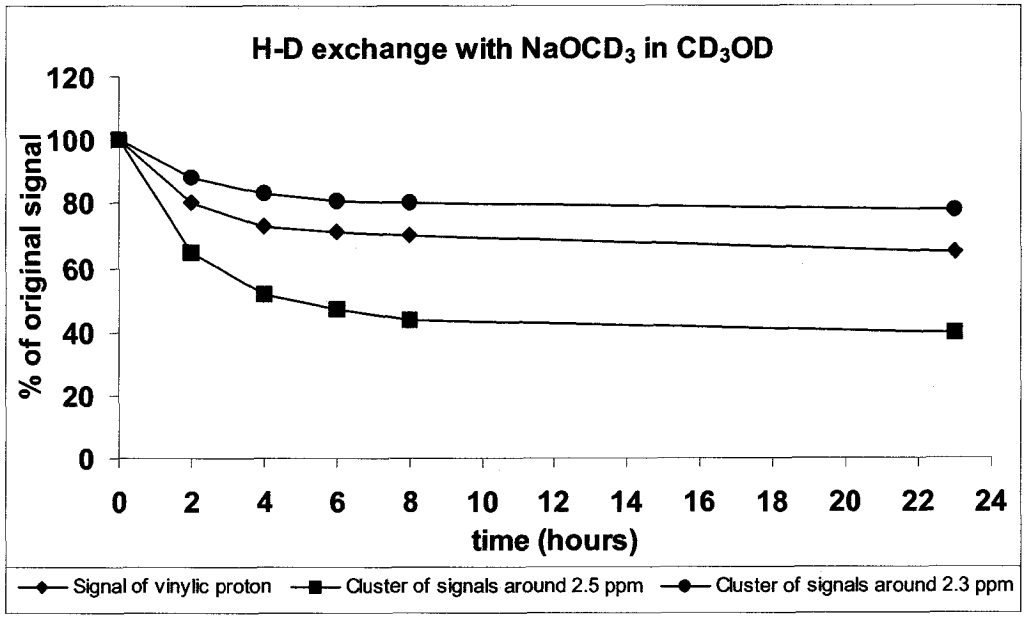
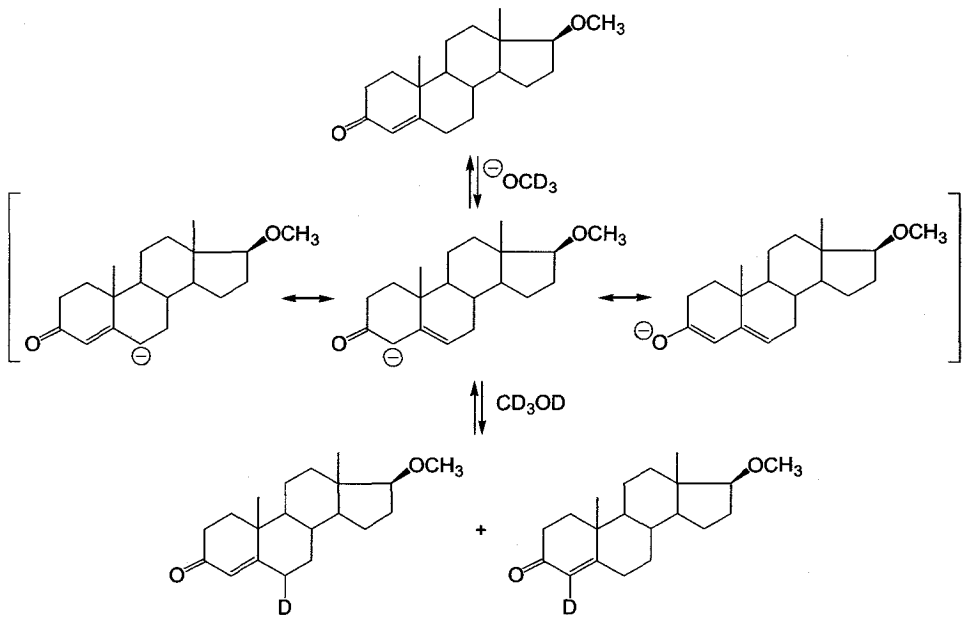


Figure 8: Decay of signals in the ^1H NMR spectrum of methoxy-T upon H-D exchange using $NaOCD_3$ in CD_3OD.



Scheme 7: Deprotonation-redeuteration sequence at C^4 and C^6 of methoxy-T upon H-D exchange with $NaOCD_3$ in CD_3OD.

Hydrogen-deuterium exchange experiment (2)

It appeared that $LiOC_6H_5$ in CD_3OD caused decrease of the same 3 (clusters of) signals as $NaOCD_3$ in CD_3OD, although with a lower velocity. The results of the measurements are displayed in Figure 9. This result implies that also the H-D exchange with $LiOC_6H_5$ in CD_3OD does not allow a decisive conclusion with respect to the relative acidities of the protons at C^2 and C^6.

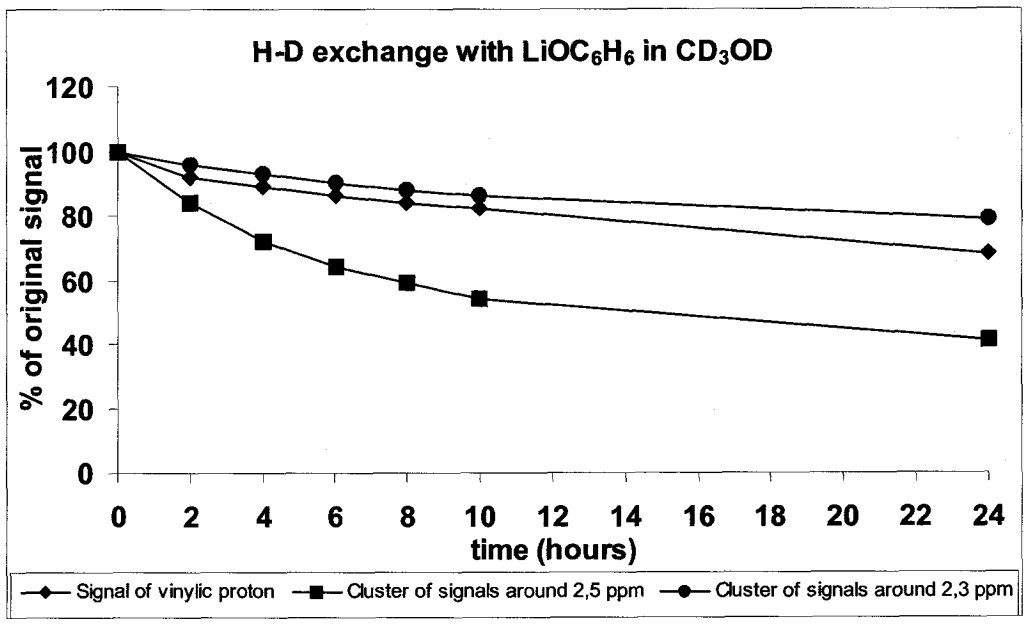


Figure 9: Decay of signals in the ^1H NMR spectrum of methoxy-T upon H-D exchange using $LiOC_6H_5$ in CD_3OD

A C-H correlation was performed to assign the clusters of signals around 2.5 and 2.3 ppm to the respective protons at C^2 and C^6. In addition, help of the ^1H- and ^{13}C NMR spectral data of steroids already available in the literature [7,8] was used. The correlation revealed that the cluster of signals around 2.5 ppm in the ^1H spectrum of methoxy-testosterone originates from 1 proton at C^2 and 1 proton at C^6. The same is the case with the cluster of signals around 2.3 ppm. Therefore, it was concluded that protons corresponding to the cluster of signals around 2.5 ppm are exchanged much faster than other exchangeable protons in the molecule, and are therefore most acidic. However, the C-H correlation does not answer the question whether the most acidic protons reside at C^2 or at C^6.

GC-MS analyses provided decisive information on this question. As was already indicated in Figure 4, the fragmentation pattern of methoxy-T shows a fragmentation of the A-ring corresponding to a m/z value of 260 ($[M-C_2H_2O]^{\bullet+}$). This implies that C^2 and its hydrogen atoms are removed from the remaining fragment.

During the complete H-D exchange period of 7 days, GC-MS analyses showed incorporation of up to 4 deuterium atoms into methoxy-T, as the m/z value corresponding to the molecular mass increased from 302 to 306 (see Figure 10C). After 7 days, 2 deuterium atoms were incorporated into the $[M-C_2H_2O]^{\bullet+}$ fragment. This implies that 2 deuterium atoms had been incorporated at C^2.

After 6 hours one proton was incorporated into the steroid, as the m/z value of $[M]^{\bullet+}$ was completely shifted to 303, while the m/z value of $[M-C_2H_2O]^{\bullet+}$ remained at 260 in the same relative abundance (Figure 10A). This proved that the first deuterium atom that is incorporated into the molecule is situated at C^2. Thus, decisive proof was obtained that one of the protons at C^2 is the most acidic one in the molecule.

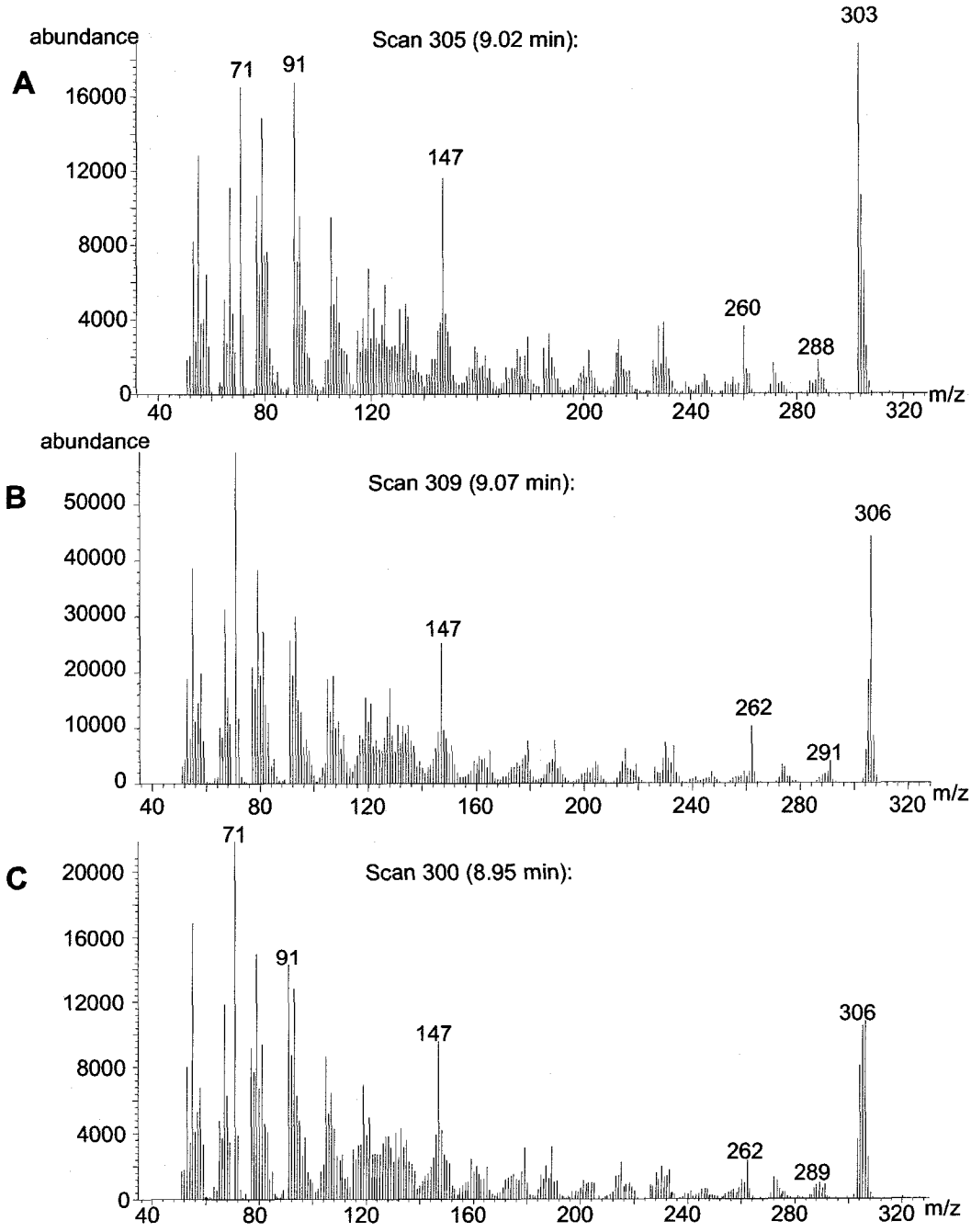


Figure 10: Mass spectra of samples obtained during H-D exchange on methoxy-T after 6 hours (**A**), 70 hours (**B**) and 7 days (**C**).

Kinetically versus thermodynamically controlled derivatization

Donike et al. [2] stated that product formation during derivatization is dependent upon the nature of the solvent, the catalyst, the silylating reagent, and the structure of the steroid. However, to explain the differences in product formation in this study the critical variables should be described as:

1. Stability of the endproduct. Endothermic reactions generally lead to the formation of those products with the highest thermodynamic stability [4], *i.e.* the 3,5-dienolTMS derivative, as shown by the isomerization experiment.
2. Activation energy of enolization. As was shown by the H-D exchange experiments, the protons at C^2 are more acidic than the protons at C^6, explained by the α-position of C^2-protons with respect to the carboxyl group. Rephrased, the activation energy of proton extraction from C^2, leading to 2,4-dienolate anion formation, is lower than proton extraction from C^6 that leads to 3,5-dienolate anion formation. Therefore, product formation under basic conditions, *i.e.* MSTFA/KOAc/imidazole, is kinetically controlled and is independent from (thermodynamic) stability of the reaction products.

The thermodynamic vs. kinetic control of enolization can be explained by a diagram of Gibb's free energy levels (G) of the keto- and enol-structures with the corresponding intermediates (see Figure 11).

The Hammond postulate states that in general the product of highest stability will be produced fastest. This implies that a higher Gibb's free energy of an endproduct corresponds to a higher energy level of the transition-state. However, as is the case in the trimethylsilylation of 3-keto-androst-4-ene steroids, thermodynamics and kinetics do not necessarily need to correspond.

Product formation of kinetic controlled reactions will only be determined by the difference in Gibb's free energy between endproducts (indicated as ΔG^0) in case of equilibrium. In a non-equilibrium situation, the difference in energies of the respective transition states ($\Delta\Delta G^{\ddagger}$) will be the critical factor. This allows the formation of thermodynamically least stable, but kinetically most favorable reaction products, as was proven for the studied base catalyzed enolization.

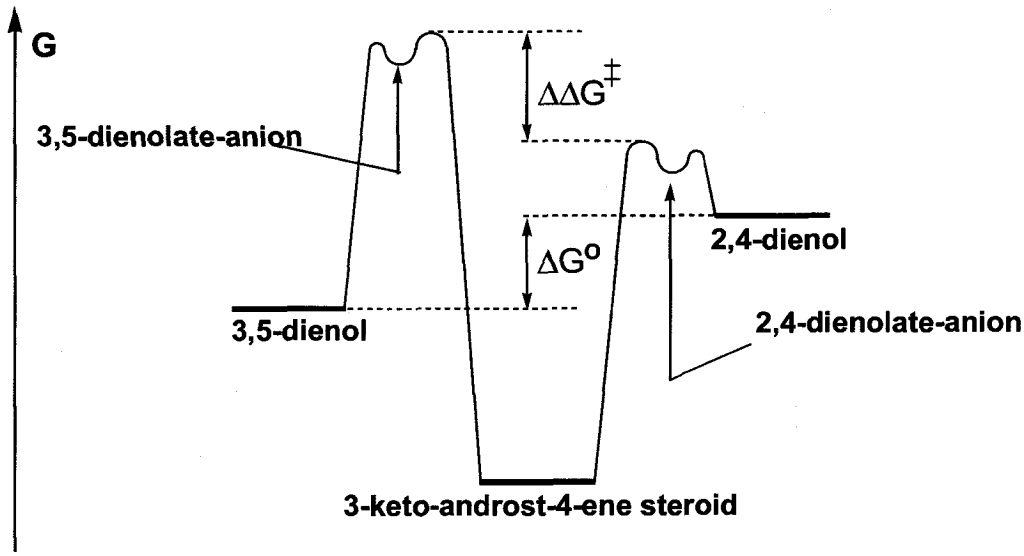


Figure 11: Gibb's free energy profile for base mediated enolization of 3-keto-4,5-ene-steroids with kinetically vs. thermodynamically determined product formation.

Implications for steroid analysis

In Chapters 5-7, 6α- and 6β-OH-Δ4-AEDIONE were analyzed as metabolites of Δ4-AEDIONE. As the applied derivatization reagent was MSTFA/NH_4I/ethanethiol, concentrations of the two metabolites were combined in one value. To obtain a more stereospecific steroid profile a basic derivatization reagent as MSTFA/KOAc/imidazole should be applied. However, as shown above, fragmentation of 2,4-dienolTMS derivatives is extensively higher than of the 3,5-dienolTMS isomers, corresponding to the lower thermodynamic stability. Significantly lower analytical sensitivity will therefore be obtained for the mentioned steroids after MSTFA/KOAc/imidazole derivatization.

REFERENCES

1. W. Schänzer, S. Horning, and M. Donike. Metabolism of anabolic steroids in humans: synthesis of 6 beta-hydroxy metabolites of 4-chloro-1,2-dehydro-17 alpha-methyltestosterone, fluoxymesterone, and metandienone. *Steroids*. **60:** 353-366 (1995).
2. M. Donike and J. Zimmermann. Preparation of trimethylsilyl-, triethylsilyl- and tert-butyldimethylsilyl enol ethers of oxo steroids for gas chromatographic and mass spectrometric studies. *J. Chromatogr.* **202:** 483-486 (1980).
3. G. Opfermann and W. Schänzer. Trimethylsilylation – Aspects for derivatisation. In *Recent Advances in Doping Analysis (4), Proceedings of the 14th Cologne Workshop on Dope Analysis*, W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, 1996, pp 247-252.
4. M. Jones Jr. Organic Chemistry. W.W. Norton & Company, New York, 1997.
5. R. Floresca, M. Kurihara and D.S. Watt. Cleavage of unsaturated α-ketols to ω-oxo-α,β-unsaturated acids. *J. Org. Chem.* **58:** 2196-2200 (1993).
6. A. Deyine, G. Dujardin, M. Mammeri and J-M. Poirier. A facile isomerization procedure for the access to thermodynamic silyl enol ethers. *Synth. Commun.* **28:** 1817-1821 (1998).
7. D.N. Kirk, H.C. Toms, C. Douglas, K.A. White, K.E. Smith, S. Latif, and R.W.P. Hubbard. A survey of the high-field proton NMR spectra of the steroid hormones, their hydroxylated derivatives, and related compounds. *J. Chem. Soc., Perkin Trans.* **2:** 1567-1594 (1990).
8. J.W. Blunt and J.B. Stothers. Carbon-13 NMR studies. Part 69. Carbon-13 NMR spectra of steroids - a survey and commentary. *Org. Magn. Res.* **9:** 439-464 (1977).

9

ARTIFACT FORMATION DUE TO ETHYL THIO-INCORPORATION INTO SILYLATED STEROID STRUCTURES

ABSTRACT

For the application of gas chromatography-mass spectrometry (GC-MS) in steroid analysis, trimethylsilylation of target substances in a mixture of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA), ammonium iodide and ethanethiol is frequently applied. However, using this mixture to silylate the steroids androsterone and etiocholanolone obtained from a urine matrix, the formation of artifacts was established.

The artifacts were identified as ethyl thio-containing products of the respective trimethylsilyl derivatives. The conversion of the studied products increased slowly as a function of time, was dependent on the presence of the urine matrix and was significantly accelerated by adding diethyl disulfide to the reagent before incubation. Also ethyl thio-incorporation into testosterone and epitestosterone was established. A mechanism for ethyl thio-incorporation at the C^{16}-position is proposed.

The conversion that was achieved after 120 hours of sample storage at room temperature was insufficient to significantly influence the analysis of androsterone and etiocholanolone under the studied circumstances. However, the results provide fundamental insight into the mechanism of silylation and the occurring side-reactions. Moreover, when investigating the formation of new metabolites the ethyl thio-incorporation can create false interpretations.

INTRODUCTION

One of the challenging fields in doping analysis has been the mass spectrometric determination of steroids of either exogenous or endogenous origin. Since its introduction in steroid analysis in the eighties by Donike *et al.* [1], *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) has been extensively used as a powerful trimethylsilyl donor in the derivatization procedure. One of the most reported derivatization techniques is the application of a mixture of MSTFA/ammonium iodide/ethanethiol. MSTFA reacts *in situ* with ammonium iodide (NH_4I) to produce trimethyliodosilane (TMSI) that has been reported as the most powerful trimethylsilyl donor available [1]. TMSI reacts with adequate speed to produce both trimethylsilyl (TMS) ether and trimethylsilyl enol (TMS enol) ether derivatives (see Figure 1).

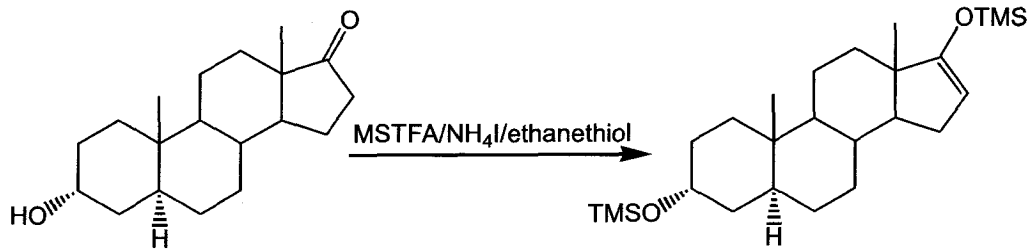


Figure 1: Derivatization of androsterone to its per-TMS ether derivative.

Ethanethiol[1] is added to reduce the formed iodine to hydrogen iodide in order to prevent iodine incorporation into the steroid nucleus. As a result, diethyl disulfide is produced during the derivatization reaction [2]. Diethyl disulfide formation depends on the amount of ammonium iodide and ethanethiol added to the extract and the chosen experimental conditions as reaction time and temperature. Usually a mixture of MSTFA/NH_4I/ethanethiol is used in a ratio of 1000:2:3 (v/w/v).

When applying this procedure, it is assumed that other reactants than TMSI present in the reaction mixture are inert to the steroids to be analyzed. However, as will be described in this paper, incorporation of an ethyl thio-group with steroid structures occurs during the described derivatization procedure.

In this study artifact formation was suspected in derivatized urine sample extracts. An excretion study with [2,2,4,6,6,16,16-$^7H_2$]-androst-4-ene-3,17-dione (d_7-Δ4-AEDIONE) showed that these artifacts were either metabolically or chemically related to Δ4-AEDIONE. Experiments are described to prove incorporation of an ethyl thio-group into the main metabolites of Δ4-AEDIONE: androsterone and etiocholanolone.

[1] Ethanethiol is frequently used as a replacement of dithioerythreitol [2].

As diethyl disulfide is formed as side-product during the described derivatization procedure, an experiment is described to investigate the role of ethanethiol and diethyl disulfide as reagent in ethyl thio-incorporation. Repeated measurements over time give insight into the significance of the side-reactions as compared to the desired derivatization reactions. Also ethyl thio-incorporation into testosterone and epitestosterone was established.

EXPERIMENTAL

Chemicals

Reference steroids: Androst-5-en-3β-ol-17-one (dehydroepiandrosterone), androst-4-en-17α-ol-3-one (epitestosterone), 5α–androstan-3α-ol-17-one (androsterone), 5β–androstan-3α-ol-17-one (etiocholanolone), 5α-androstane-3α,17β-diol, androst-4-ene-3,17-dione and androst-5-ene-3β,17β-diol, 17α-methylandrost-4-ene-11α,17β-diol-3-one (11α-hydroxy-methyltestosterone), and diethyl disulfide were obtained from Sigma, St. Louis, Missouri, USA. [2,2,4,6,6,16,16-$^{7}H_2$]--Androst-4-ene-3,17-dione (purity 98.4%) was obtained from C/D/N Isotopes, Pointe-Claire, Quebec, Canada.

N-Methyl-*N*-trimethylsilyltrifluoroacetamide was obtained from Aldrich Chemical Company, Milwaukee, Wisconsin, USA. Ammonium iodide was obtained from Fluka Chemie, Buchs, Switzerland. Ethanethiol was obtained from Acros Organics, New Jersey, USA. Diethyl ether was obtained from Merck, Darmstadt, Germany.

A crude solution of Helix pomatia (type HP-2, containing 110.000 IU/ml of β–glucuronidase and 1000-5000 IU/ml of arylsulfatase) was obtained from Sigma, St. Louis, Missouri, USA. Columns for solid phase extraction were IST Isolute C_{18} columns (200 mg, non-endcapped) obtained from Sopachem, Lunteren, The Netherlands.

Gas chromatography and mass spectrometry

GC-MS analysis was performed with a Hewlett Packard gas chromatograph (Model 5890, Agilent Technologies, Waldbronn, Germany) coupled to a Hewlett Packard quadrupole mass spectrometer (Model 5972A). Ionization was performed in the electron ionization mode at 70 eV. Gas chromatography was performed with a HP-1 fused silica column (length 18 m, inner diameter 0.2 mm, film thickness 0.11 μm). Through electronic pressure control the column flow (helium) was constant: 1 ml/min.

Sample injection of 1 µl was performed in split mode (ratio 1:10). A Hewlett Packard autosampler (Model 7673) was used for auto-injection. The injector temperature was set at 250°C. The oven temperature program used was: initial temperature 180°C, 2°/min to 225°C, 30°/min up to 310°C, held for 5 minutes. The interface temperature was set at 280°C.

Confirmation of artifact formation

Two products (X, Y) that were suspected to be analytical artifacts, were discovered during the full scan mode analyses of urine samples obtained from an excretion study with d_7-Δ4-AEDIONE. In this study 50 mg of d_7-Δ4-AEDIONE was administered to a healthy male subject (age: 29 years). Urine samples were collected during 24 hours before and 24 hours after administration.

11α-Hydroxy-methyltestosterone (5 µg) was added as internal standard to 4 ml of urine. Solid-phase extraction was performed with C_{18} columns. Before applying urine samples, the column material was preconditioned by washing with 4 ml of methanol and equilibrating with 2 ml of water. Inorganic material was removed from the column by washing with 2 ml of water. The organic material was collected by elution with 4 ml of methanol. After evaporation to dryness, 2 ml of acetate buffer (0.1 M, pH 5.2) was added to the extract. Hydrolysis was performed by adding 100 µl of Helix pomatia and incubating for 1 hour at 55°C. Steroids were isolated from the buffer solution by extracting with 5 ml of diethyl ether (twice). After extraction the phase separation was stimulated by centrifugation (4000 rpm, 5 min). The organic layer was removed and evaporated to dryness under a gentle stream of nitrogen at 40°C. The extract was dried overnight over P_2O_5/KOH under reduced pressure. One µl of the incubation mixture was directly injected.

Screening of the urine extracts was performed in selected ion monitoring (SIM) mode. Δ4-AEDIONE was monitored at m/z 430 representing $[M]^{\bullet+}$. AO and EO were monitored at m/z 434 and 419, representing $[M]^{\bullet+}$ and $[M-15]^+$, respectively. The suspected artifacts X and Y were monitored at m/z 494 and 479. The monitored m/z values corresponding to labeled AO and EO (m/z 440 and 425) and artifacts X and Y (m/z 499 and 484), were chosen on the basis of the maximum area response. These m/z values were established by SIM monitoring of the m/z range 434 to 441 for AO and EO and m/z 494 to 501 for X and Y, respectively.

Derivatization of synthetic steroids

To study the source of formation of X and Y as analytical artifacts, synthetic steroids (25 µg) were derivatized as described above and analyzed for X and Y. Some of the

most relevant endogenous steroids in relation to doping analysis were selected: AO, EO, testosterone (T), epitestosterone (E), Δ4-AEDIONE, dehydroepiandrosterone (DHEA) and androst-5-ene-3β,17β-diol (Δ5-AEDIOL).

Influence of diethyl disulfide on ethyl thio-incorporation with AO and EO

To prove the reactivity of diethyl disulfide in the incorporation of an ethyl thio-group, the derivatization of synthetic AO and EO was performed under the following conditions:

1. 25 μg of AO in 100 μl of MSTFA/NH_4I/ethanethiol (1000:2:3; v/w/v)
2. 25 μg of EO in 100 μl of MSTFA/NH_4I/ethanethiol (1000:2:3; v/w/v)
3. 25 μg of AO in 100 μl of MSTFA/NH_4I/ethanethiol/diethyl disulfide (1000:2:3:3; v/w/v/v)
4. 25 μg of EO in 100 μl of MSTFA/NH_4I/ethanethiol/diethyl disulfide (1000:2:3:3; v/w/v/v)

To every sample 25 μg of 5α-androstane-3α,17β-diol (ADIOL) was added as internal standard. Incubation was performed at 80°C for 30 min. The derivatization mixtures were analyzed on day 0 (immediately after derivatization), day 7 and day 12 (the samples were kept at room temperature between the analyses). The area ratios, Y/AO and X/EO were determined by monitoring ions of the derivatives at m/z 494 (X and Y) and m/z 434 (AO and EO).

Time dependence of ethyl thio-derivative formation

To study the time dependent ethyl thio-derivative production in more detail, the following samples were prepared for analysis and analyzed repeatedly for 120 hours (once every 4 hours):

1. 10 μg of AO and 10 μg of EO in 100 μl of MSTFA/NH_4I/ethanethiol (1000:2:3; v/w/v)
2. 10 μg of AO and 10 μg of EO in 100 μl of MSTFA/NH_4I/ethanethiol/diethyl disulfide (1000:2:3:50; v/w/v/v)
3. Extract of 3 ml of blank urine in 100 μl of MSTFA/NH_4I/ethanethiol (1000:2:3; v/w/v)
4. Extract of 3 ml of blank urine in 100 μl of MSTFA/NH_4I/ethanethiol/diethyl disulfide (1000:2:3:50; v/w/v/v)

ADIOL (10 μg) was used as internal standard. Urine sample cleanup was performed as described above. GC-MS analysis of the per-TMS derivatives was performed by selected ion monitoring of the ions at m/z 434 (AO and EO), 494 (X and Y) and 436

(ADIOL). The area ratios of AO, EO, X and Y vs. ADIOL, respectively, were calculated and graphically presented as a function of time of analysis.

Ethyl thio-incorporation with T and E

T and E (25 µg) were derivatized in 100 µl of MSTFA/NH_4I/ethanethiol/diethyl disulfide (1000:2:3:50; v/w/v/v) for 30 minutes at 80°C. After 12 days of storage in glass vials at room temperature, full scan mass spectra were recorded.

Statistics

Confidence intervals of 95% were calculated for the regression coefficient 'a' (slope of the linear regression line for the area ratio as a function of time). Significance of production of X and Y and loss of AO and EO were tested using the hypothesis H_0: a=0 and H1: a>0 or H_1:a<0. Statistical analysis was performed with SPSS 9.0.

RESULTS

Confirmation of artifact formation

In all urine samples two unknown compounds (X and Y) were detected at m/z 494 and m/z 479 (see Figure 2). The identity and origin of these products were unknown. After administration of d_7-Δ4-AEDIONE and at the retention times of X and Y (16.40 and 16.55, respectively), a signal at m/z 499 and 484 was also detected. These were suspected to be labeled derivatives of X and Y. The signals of X and Y could also be observed in full scan mode, but concentrations of the two products were too low to obtain representative full scan mass spectra.

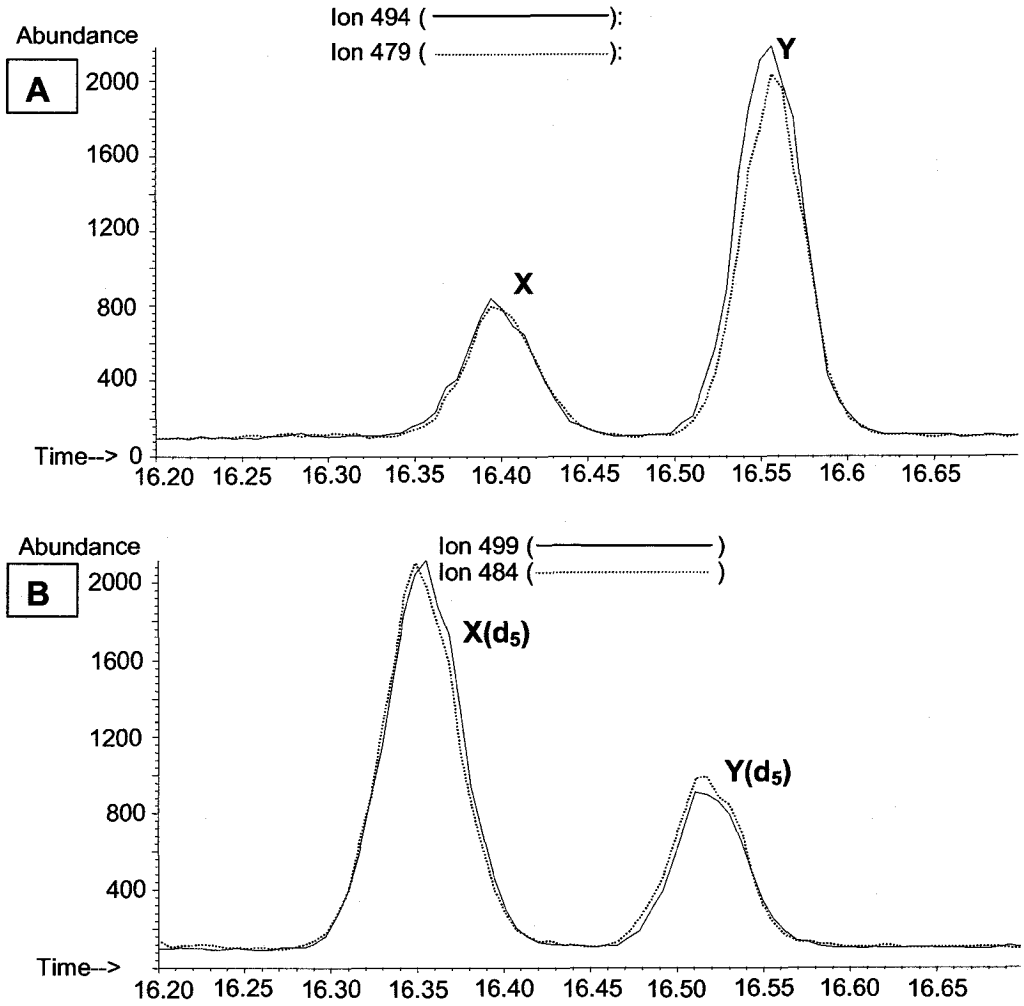


Figure 2: Selected ion chromatograms of non-deuterated (chromatogram A: m/z 494 and 479) and deuterated (chromatogram B: m/z 499 and 484) products X and Y

Derivatization of synthetic steroids

In the derivatization mixtures containing the per-TMS derivatives of AO and EO, minor quantities were detected of respectively Y and X. Other steroids studied did not result in product formation, as determined by the analysis of the selected ions at m/z 494 and 479, respectively. Concentrations of X and Y were too low for obtaining representative full scan mass spectra.

Influence of diethyl disulfide on ethyl thio-incorporation

The results are summarized in Table 1. After 12 days incubation it was possible to obtain representative full scan spectra for the derivatives of X and Y (Figure 3).

Table 1: *Area ratio of ions at m/z 494 and 434 of 4 mixtures over 12 days.*

	Mixture	Day 0	Day 7	Day 12
1	AO in MSTFA/NH_4I/ethanethiol	0.0111	0.0416	NA*
2	EO in MSTFA/NH_4I/ethanethiol	0.0068	0.0357	NA*
3	AO in MSTFA/NH_4I/ethanethiol/diethyl disulfide	0.140	0.927	2.17
4	EO in MSTFA/NH_4I/ethanethiol/diethyl disulfide	0.104	0.694	1.48

* NA = not analyzed

Time dependence of ethyl thio-derivative formation

A significant ($p<0.05$) and approximate constant production rate of X and Y occurred in all four reaction mixtures (Figure 4). However, when MSTFA/NH_4I/ethanethiol was applied, the area of X and Y remained smaller than 1 percent compared to the derivatives of AO and EO (Figure 5) after 120 hours. In case of MSTFA/NH_4I/ethanethiol/diethyl disulfide derivatization of a urine sample extract, the areas of X and Y approached 5 percent of the areas of respectively per-TMS EO and AO after 120 hours incubation at room temperature. Also in this case the concentrations of silylated AO and EO decreased significantly ($p<0.05$).

When the two studied derivatization methods were applied to one urine sample extract that was split into two separate fractions, different recoveries of derivatization were obtained. MSTFA/NH_4I/ethanethiol derivatization of AO and EO led to significantly smaller recoveries of the silylated products compared to the MSTFA/NH_4I/ethanethiol/diethyl disulfide derivatization (approximately 70%). As can be observed in Figure 5, these recoveries showed an approximate linear increase with increasing incubation time, to 85-90% of the recovery obtained with the MSTFA/NH_4I/ethanethiol/diethyl disulfide method. Differences in conversion were not present in case of derivatization of synthetic AO and EO.

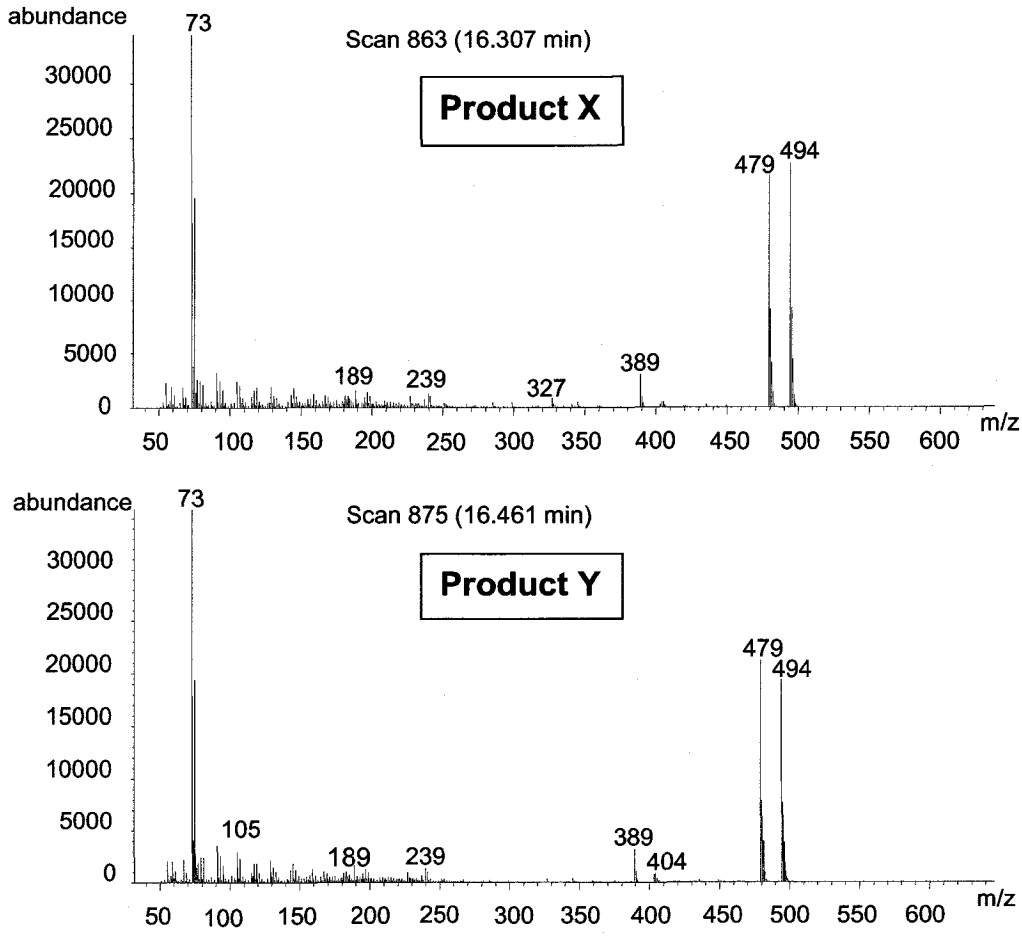


Figure 3: Full scan mass spectra of products X and Y as determined in a urine sample extract incubated in MSTFA/NH_4I/ethanethiol/diethyl disulfide (1000:2:3:50; v/w/v/v).

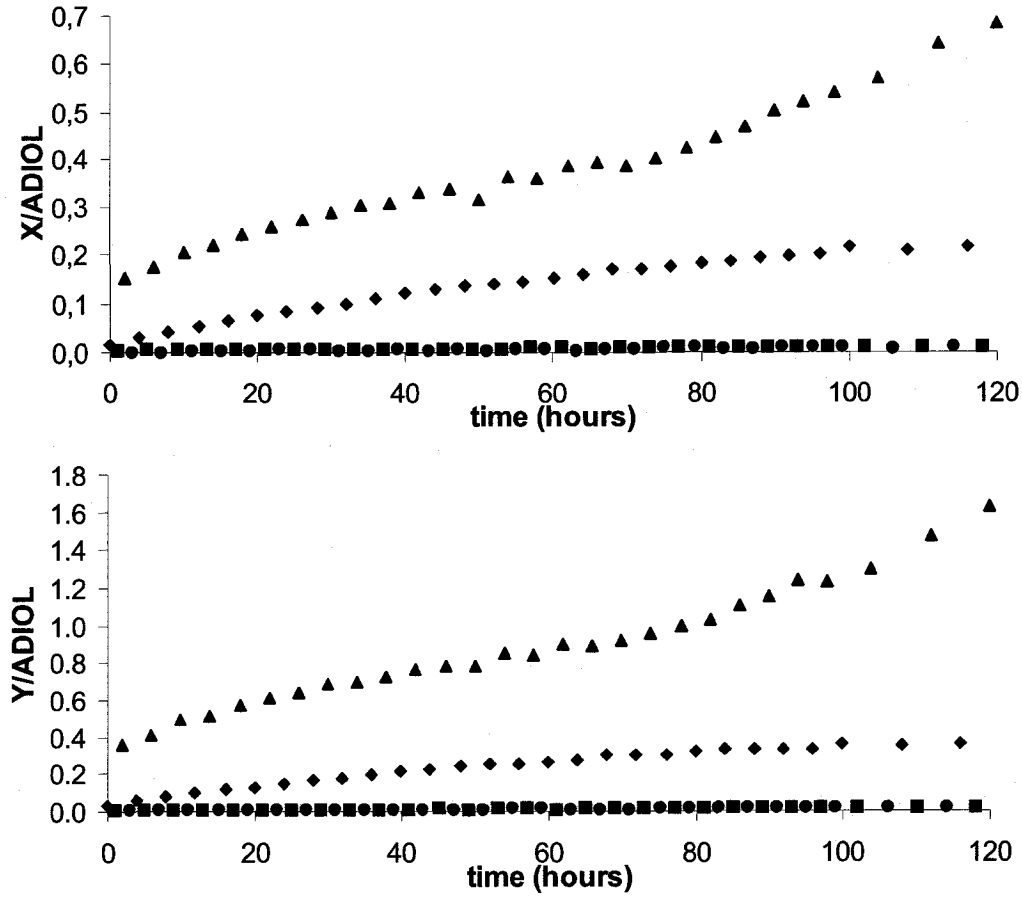


Figure 4: Ratios of X/ADIOL and Y/ADIOL as a function of time after derivatization.

- ▲ - Incubation of a urine extract in MSTFA/NH_4I/ethanethiol/diethyl disulfide (1000:2:3:50; v/w/v/v).
- ● - Incubation of a urine extract in MSTFA/NH_4I/ethanethiol (1000:2:3; v/w/v)).
- ♦ - Incubation of synthetic AO and EO in MSTFA/NH_4I/ethanethiol/diethyl disulfide (1000:2:3:50; v/w/v/v).
- ■ - Incubation of synthetic AO and EO in MSTFA/NH_4I/ethanethiol (1000:2:3; v/w/v).

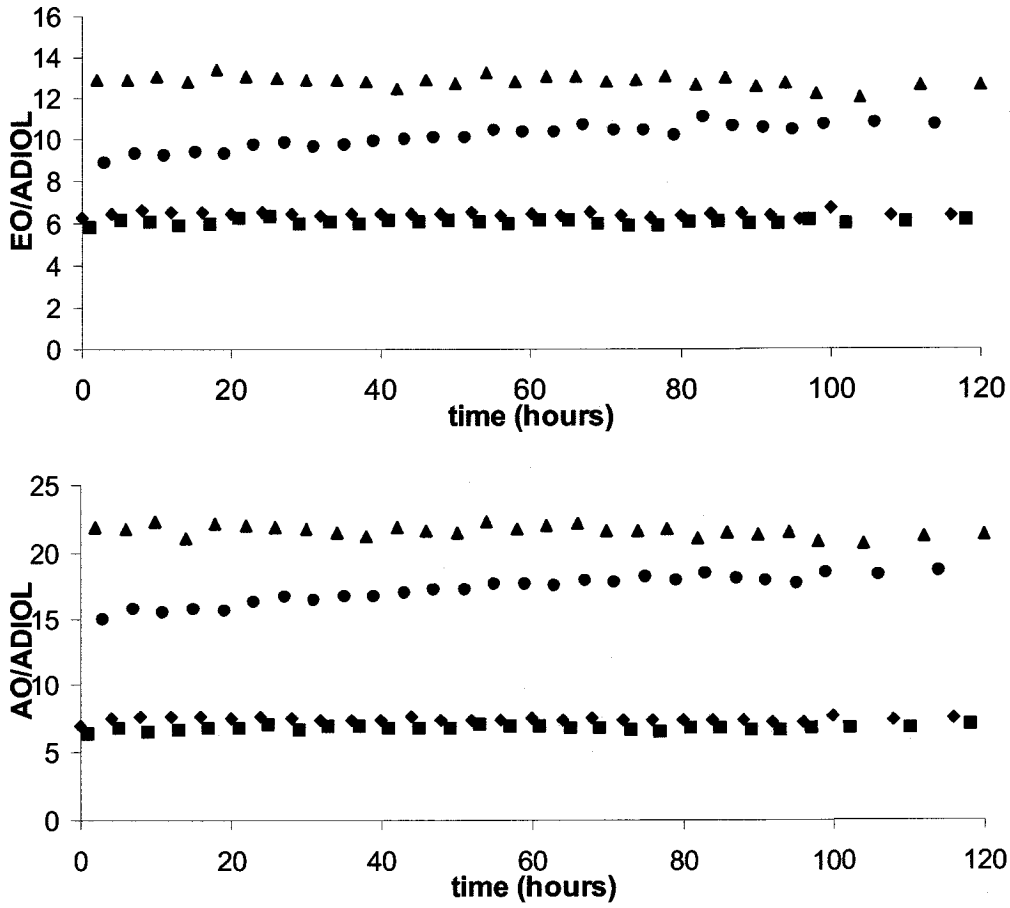


Figure 5: Ratios of AO/ADIOL and EO/ADIOL as a function of time after derivatization.

- ▲ - Incubation of a urine extract in MSTFA/NH_4I/ethanethiol/diethyl disulfide (1000:2:3:50; v/w/v/v).
- ● - Incubation of a urine extract in MSTFA/NH_4I/ethanethiol (1000:2:3; v/w/v)).
- ♦ - Incubation of synthetic AO and EO in MSTFA/NH_4I/ethanethiol/diethyl disulfide (1000:2:3:50; v/w/v/v).
- ■ - Incubation of synthetic AO and EO in MSTFA/NH_4I/ethanethiol (1000:2:3; v/w/v).

Ethyl thio-incorporation with T and E

Total ion current chromatograms of the derivatization mixtures and the mass spectra of the ethyl thio-products of T and E, are shown in Figures 6 and 7.

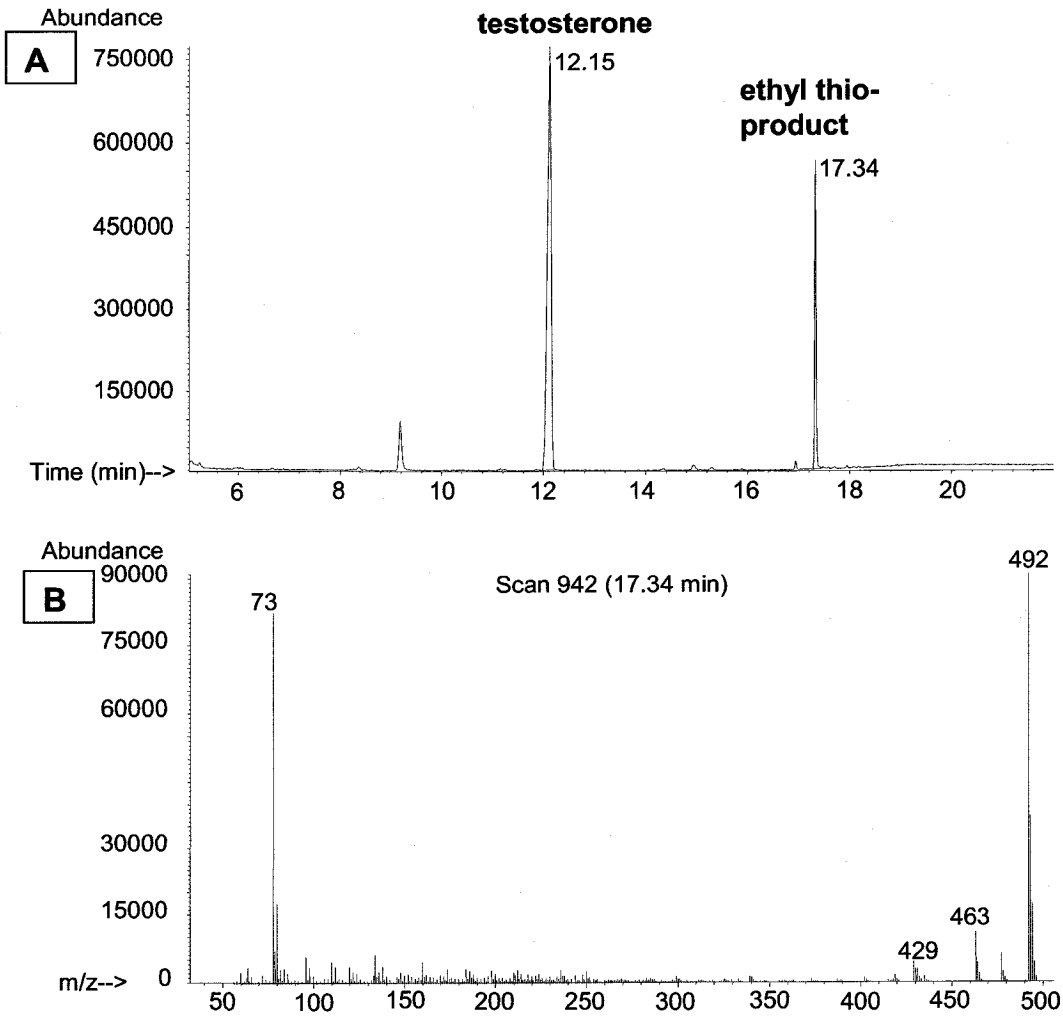


Figure 6: **A**.Total ion current chromatogram of silylated synthetic testosterone (12 days after derivatization). **B**.Full scan mass spectrum of the obtained ethyl thio-derivative.

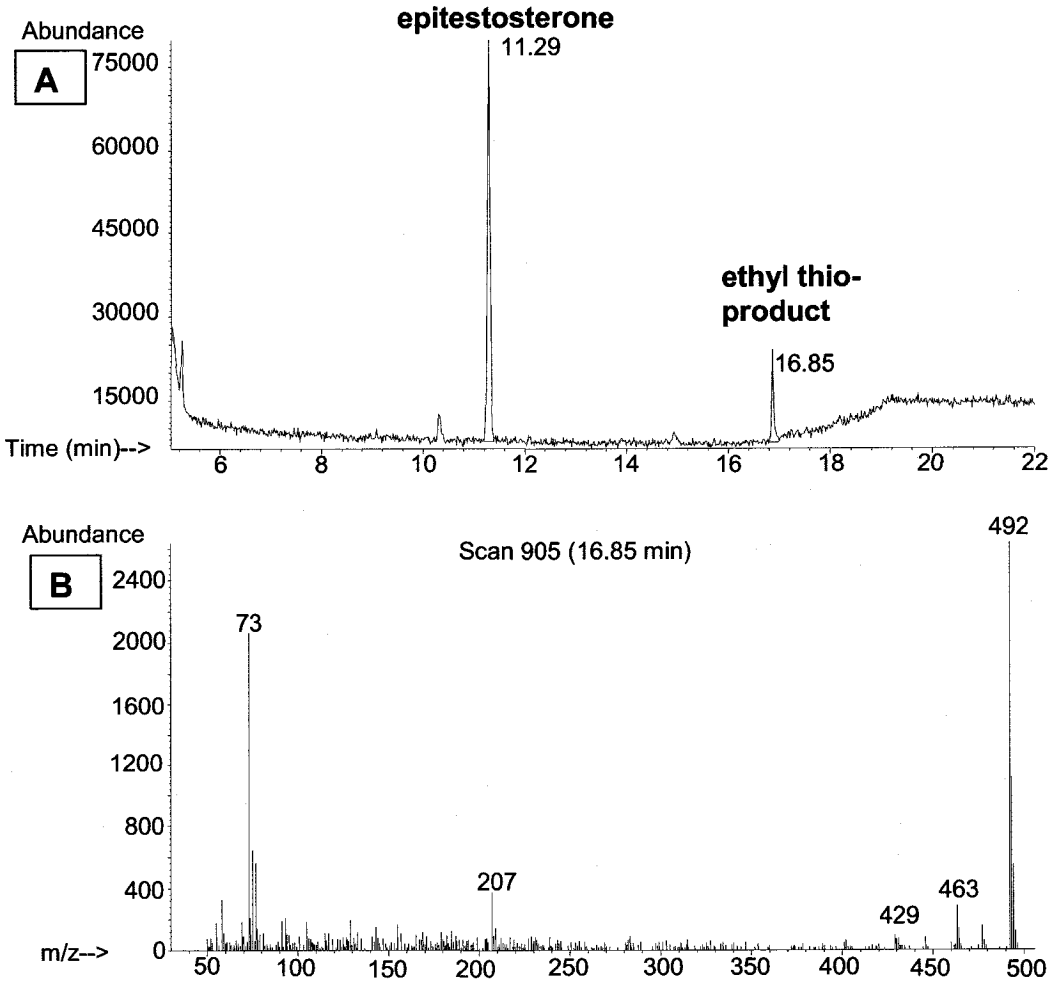


Figure 7: **A.**Total ion current chromatogram of silylated synthetic epitestosterone (12 days after derivatization). **B.**Full scan mass spectrum of the obtained ethyl thio-derivative.

DISCUSSION

X and Y were proven to be analytical artifacts, formed during the derivatization reaction in the MSTFA/NH_4I/ethanethiol (1000:2:3;v/w/v) medium. X and Y were initially observed in blank urine samples. The response of X and Y in the steroid profile increased after administration Δ4-AEDIONE or DHEA (data not shown). When d_7-Δ4-AEDIONE was administered in the described excretion study, besides the signal at m/z 494 and 479 also the signals at m/z 499 and 484 increased. These were suspected to be X and Y containing 5 deuterium atoms, which matched either a metabolically or chemically relation of X and Y to the administered labeled Δ4-AEDIONE. When the observed signal at m/z 494 is assumed to be corresponding to the molecular mass of X and Y, the product formation could not be explained by metabolism, as androgens have a molecular mass range of 430-436 Da and hydroxylated androgen metabolites a range of 518-524 Da.

When chemical side-reactions are considered, a mass of 494 Da could represent the incorporation of an ethyl thio-group into the steroid structure of AO and EO, causing a shift in molecular mass of 60 Da. The increasing signal during repeated GC-MS analysis of one urine sample, illustrated the production of X and Y as a function of time in the derivatization mixture and made the optional source metabolism a less likely explanation. The detection of a significant signal at m/z 494 after derivatization of synthetic AO and EO proved these steroids to be the source of respectively Y and X. Additional proof for ethyl thio-incorporation as the source of the artifact formation was obtained by the extensive acceleration of incorporation after diethyl disulfide was added to the derivatization medium.

The obtained spectra of X and Y show little specific fragmentation. Significant ions in the spectra represent losses usually observed in TMS-derivatization (Table 2).

Table 2: *Suggested fragmentation for X and Y (see Figure 3).*

m/z	Relative signal X/Y (%)	Loss	Fragmentation
494	100.0/91.1	$[M]^{\bullet+}$	$[M]^{\bullet+}$
479	95.0/100.0	$[M-15]^+$	$[M-CH_3]^+$
404	2.2/4.1	$[M-90]^{\bullet+}$	$[M-TMSOH]^{\bullet+}$
389	13.2/15.1	$[M-90-15]^+$	$[M-TMSOH-CH_3]^+$
327	3.9/1.5	$[M-90-15-62]^+$	$[M-TMSOH-CH_3-CH_3CH_2SH]^+$

The reagent MSTFA/NH_4I/dithioerythreitol was established by Donike *et al.* [1] for application in doping analysis of steroids. Nowadays dithioerythreitol is frequently replaced by ethanethiol to prevent chromatographic interference. The essence of

ethanethiol is preventing iodine incorporation into the steroid nucleus [2]. Upon decomposition of TMSI, iodine is formed that can add to the steroid nucleus. To prevent this side-reaction to occur, iodine is reduced by ethanethiol to form hydrogen iodide and diethyl disulfide.

From Table 1 and Figures 4 and 5 can be concluded that diethyl disulfide is more reactive towards ethyl thio-incorporation than ethanethiol. This could explain the relatively extensive ethyl thio-incorporation that occurs during derivatization of urine extracts compared to synthetic substance derivatization, as the total concentration of target compounds for derivatization is much higher in urine extracts than in that case of synthetic substances. That results in higher diethyl disulfide levels and therefore higher ethyl thio-derivative formation. Consequently, a matrix effect can be defined for trimethylsilylation with the described method, corresponding to the scientifically established matrix effect in steroid analysis [3].

As the mass of the molecular ion increases by 60 Da, it must be expected that a proton is replaced by a $-SCH_2CH_3$ group, leaving the double bond at C^{16}-C^{17} intact. A hypothetical mechanism for such incorporation is proposed in Figure 8. According to this mechanism, the TMS-moiety from the derivatized AO or EO is removed from the steroid by nucleophilic attack of diethyl disulfide on the silicon atom under simultaneous addition of an ethyl thio-group with C^{16}. Subsequently, the generated 17-ketogroup is silylated again. Unfortunately, due to the lack of specific fragmentation of X and Y it was impossible to confirm the structure as proposed in Figure 8.

When Δ4-AEDIONE is orally administered, it is rapidly metabolized, showing AO and EO as the main metabolites [4]. Because AO and EO are present in the urine matrix in relatively high concentrations as compared to other androgens as T, E and Δ4-AEDIONE, the studied derivatives were presumable products of those steroids and could easily be detected. This does however not imply that no ethyl thio-incorporation occurs with other steroids present in the matrix. For example, T and E are interesting compounds to study regarding ethyl thio-incorporation for two reasons. First, the α,β–unsaturated 3-keto moiety could result in different quantities of product formation. Second, as the urinary testosterone/epitestosterone ratio (T/E ratio) is applied to establish use of testosterone, this ratio could be affected by significant ethyl thio-incorporation.

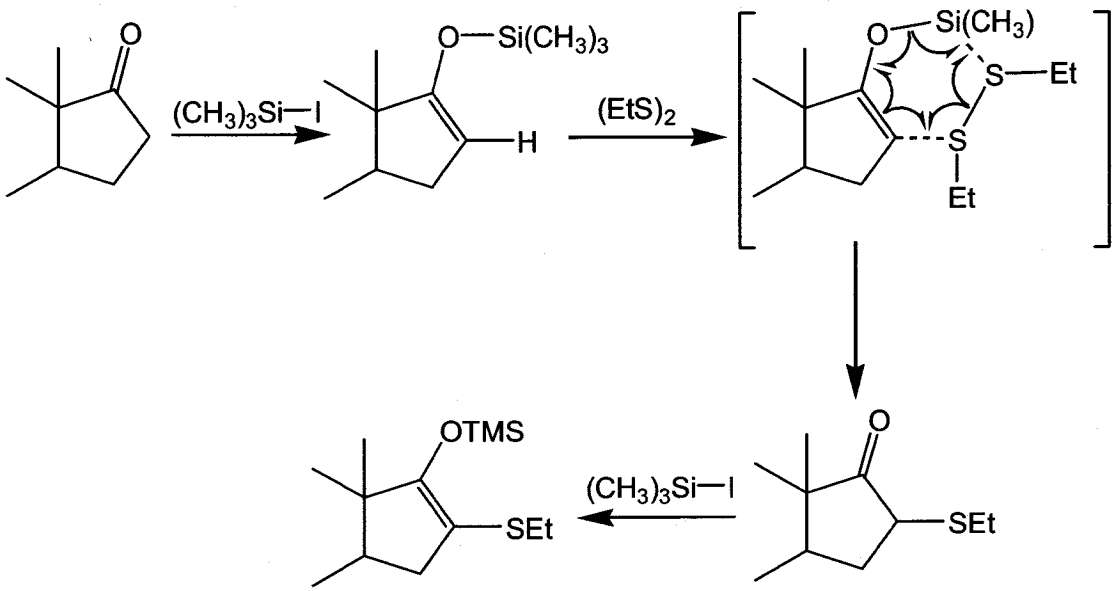


Figure 8: Hypothetical mechanism for ethyl thio-incorporation with AO and EO TMS-derivatives. Step 1: AO/EO are silylated to their per-TMS derivatives. Step 2: The TMS-moiety is lost by nucleophilic attack of EtSSEt on the silicon atom under simultaneous incorporation of the ethyl thio-group with C^{16}. Step 3: The keto group is silylated again by *in situ* formed TMSI.

As shown in Figures 4 and 5, significant ethyl thio-incorporation will only affect the analysis of per-TMS AO and EO, when extra diethyl disulfide is added to accelerate the incorporation. The application of MSTFA/NH_4I/ethanethiol (1000:2:3;v/w/v) did not result in a significant change in the area of AO and EO after 120 hours. This is in agreement with the reported 48 hour stability (at room temperature) or 5-6 days stability (at 4°C) of per-TMS derivatives of steroids in general [5]. The stability estimate of days to weeks reported as by Donike [1] should be considered as an overestimation.

In this case, the role of ethyl thio-incorporation was of purely theoretical interest. However, the presented results give theoretical insight into less accessible aspects of a derivatization procedure that is often applied in steroid analysis. Although the results described in this paper are incomplete to obtain a fully detailed mechanistic overview on the ethyl thio-incorporation in steroid analysis, it illustrates the still insufficient knowledge of the silylation mechanism. In particular, the role of diethyl disulfide has been considered insufficiently. Moreover, when investigating the

formation of new metabolites the ethyl thio-incorporation can create misinterpretations.

The ethyl thio-incorporation could become of practical relevance when derivatized samples are re-analyzed that were stored at room temperature for several days. Also the influence of ethyl thio-incorporation on the quantitative analysis of T and E and the analysis of anabolic steroids at ppb level should be considered. To avoid significant differences in steroid quantification due to artifact formation, deuterated internal standards should be considered as a necessity.

LITERATURE

1. M. Donike and J. Zimmermann. Preparation of trimethylsilyl-, triethylsilyl- and tert-butyldimethylsilyl enol ethers of oxo steroids for gas chromatographic and mass spectrometric studies. *J. Chromatogr.* **202:** 483-486 (1980).
2. G. Opfermann and W. Schänzer. Trimethylsilylation – Aspects for derivatisation. In *Recent Advances in Doping Analysis (4), Proceedings of the 14th Cologne Workshop on Dope Analysis*, W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, 1996, pp 247-252.
3. K. Linnet. Effect of biological matrix on the urinary testosterone/epitestosterone ratio measured by gas chromatography/mass spectrometry in doping analysis. *Biol. Mass Spectrom.* **22:** 412-418 (1993).
4. V.P. Uralets and P.A. Gillette. Over-the-counter anabolic steroids 4-androsten-3,17-dione; 4-androsten-3beta,17beta-diol; and 19-nor-4-androsten-3,17-dione: excretion studies in men. *J. Anal. Toxicol.* **23:** 357-366 (1999).
5. R. Masse, C. Ayotte, and R. Dugal. Studies on anabolic steroids. I. Integrated methodological approach to the gas chromatographic-mass spectrometric analysis of anabolic steroid metabolites in urine. *J. Chromatogr.* **489:** 23-50 (1989).

PART IV:

general discussion

10

DISCUSSION: PERSPECTIVES OF STEROID PROFILING REVIEWED

STEROID PROFILING AND TESTOSTERONE

Initially, the abuse of steroids in sports was focussed on chemically modified derivatives of testosterone. These steroids were of exogenous origin, so detection of the respective substances or the metabolites was sufficient to prove administration. In order to detect the abuse of popular steroids as methandienone (Dianabol®), methenolone (Primobolan®) and stanazolol (Winstrol®) [1] "straightforward analysis" with gas chromatoghraphy-mass spectrometry (GC-MS) was considered to be adequate.

After the abuse of testosterone (T) was suspected during the Olympic Games of Moscow in 1980 more attention was paid to the abuse of steroids of endogenous origin. Therefore, endogenous steroid profiling techniques were developed for the detection of administration of T [2,3] and its metabolite with high anabolic potency 5α-dihydrotestosterone (5α-DHT) [4,5]. As described in Chapter 1 the ratio of testosterone to epitestosterone (T/E ratio) has been used until today for the detection of T abuse. Since 1982 the International Olympic Committee (IOC) has accepted the T/E ratio as method for the detection of T [3].

Besides T and E, usually GC-MS analysis of the main metabolites androsterone (AO), etiocholanolone (EO), stereoisomers of androstanediol, 5α-DHT and main biosynthetic precursors as dehydroepiandrosterone (DHEA), androst-5-ene-3β,17β-diol (Δ5-AEDIOL) and androst-4-ene-3,17-dione (Δ4-AEDIONE) is applied in routine procedures of doping analysis for the purpose of interpretation of an elevated T/E ratio.

Although the T/E ratio was generally and effectively applied in doping analysis, occasional critical comments appeared about the validity of the method by *e.g.*

lawyers involved in doping cases. In particular, the application of the same decision criteria for females as for males was attacked. As discussed in Chapter 1, several variables determine the intra- and intersubject reference values of the T/E ratio. Although amongst doping laboratories there is a general conviction of the validity of the T/E ratio, there is still insufficient published data about the influence of variables as race, menstrual cycle, use of contraceptives, age, exercise, etc. The controversy can therefore mostly be assigned to the availability of scientific data.

An example of one of the variables that effectively influences intra-individual values of the T/E ratio is the consumption of ethanol. As described in Chapter 3, the T/E ratio increases immediately after first alcohol administration. Although the effect is more significant for females compared to males, Chapter 2 shows that both sexes are at risk of an incidental T/E value above 6 after drinking a volume in the order of a bottle of wine (dose of 1.2 g ethanol per kg bodyweight, corresponding to a plasma concentration 1-2‰). The significance of the effect was dependent upon the mean basal intra-subject T/E ratio. Doping laboratories should therefore be equipped with a validated methodology to establish urine alcohol concentrations in case recent alcohol consumption is claimed.

STEROID PROFILING AND FOOD SUPPLEMENTS

Although already during the eighties experiments were performed with nasal administration of Δ4-AEDIONE in former Eastern Germany [6], this steroid did not become widely commercially available until the late nineties. Stimulated by accessibility via the Internet "food supplements" containing steroids became popular marketing products. Unlike Europe, food supplements containing testosterone precursors as DHEA and Δ4-AEDIONE are available as non-prescriptive substances in the United States.

DHEA and Δ4-AEDIONE were the first steroids that became widely available in food supplement formulations. The popularity of these products was illustrated by the case of the baseball player Mark McGwire [7], who publicly admitted and defended the use of Δ4-AEDIONE. In 1997 and 1998 respectively, DHEA and Δ4-AEDIONE were placed on the IOC list of forbidden substances. In 1999 these were followed by androst-5-ene-3β,17β-diol (Δ5-AEDIOL), androst-4-ene-3β,17β-diol (Δ4-AEDIOL), androst-5-ene-3,17-dione (Δ5-AEDIONE), 19-norandrost-4-ene-3,17-dione (19-nor-Δ4-AEDIONE), 19-norandrost-5-ene-3,17-dione (19-nor-Δ5-AEDIONE) and 19-norandrost-4-ene-3β,17β-diol (19-nor-Δ4-AEDIOL).

To detect food supplement steroids in doping analysis, profiling techniques are mostly applied as were originally developed for detection of T administration [2,3], with the T/E ratio as one of the main parameters. However, these profiling techniques were developed in times that T precursors as DHEA and Δ4-AEDIONE were not generally available.

The increased number of abused endogenous steroids has complicated the interpretation of steroid profiles. As shown in Chapters 4-7 both oral DHEA and Δ4-AEDIONE administration result in an increased T/E ratio. As oral administration of Δ5-AEDIOL, Δ4-AEDIOL and Δ5-AEDIONE has a comparable effect [7,8] it is a very unspecific parameter for all these steroids. The same conclusion can be drawn for other previously mentioned parameters as AO, EO, stereoisomers of androstanediol and 5α-DHT, which are main metabolites of all these food supplement steroids. Efficient identification of the abused steroids by the present methodology is therefore not possible.

Regarding 19-norsteroids found in food supplements, applied analytical techniques are limited to the screening of 19-norandrosterone (19-nor-AO) and 19-noretiocholanolone (19-nor-EO). As these are the main metabolites of 19-nor-Δ4-AEDIONE, 19-nor-Δ5-AEDIONE, 19-nor-Δ4-AEDIOL as well as 19-nortestosterone (nandrolone) [1,7,8], also no identification of the administered steroid can be performed by this methodology.

Providing this specific information on the identity can be of particular relevance in case of unintentional steroid administration by polluted food supplement products, as has frequently been claimed in recent years [13,14]. Furthermore, there is increasing scientific acceptance for the concept of natural presence of 19-nor-AO and 19-nor-EO in low concentrations in urine [9,10], as well as the concept of natural excretion of these steroids after consumption of meat [11,12]. To determine the, either endogenous or exogenous, source of 19C- or 19-norsteroids, more specificity of the analytical method is required as is available today.

As explained in Chapter 3, profiling of oxygenated metabolites of food supplement steroids could provide essential information that can aid the identification of the abused substances. To obtain a more specific steroid profile for the detection of food supplement steroids, the metabolism was studied of the model compounds DHEA and Δ4-AEDIONE with a main focus on oxygenated metabolites.

To compare the studied metabolites as parameters in steroid profiling, they were described by means of sensitivity and specificity. For the metabolites, sensitivity was defined as the increase of excretion rate, compared to the mean basal excretion rate (see Table 1, Chapter 6). AO and EO are mostly considered as sensitive parameters for the detection of DHEA and/or Δ4-AEDIONE [7,8]. The steroids that showed a

larger sensitivity for DHEA than AO or EO were (see Chapter 4 and 5): DHEA, 7β-hydroxy-DHEA, Δ5-AEDIOL, 7-keto-AO, 6α/β-OH-Δ4-AEDIONE, 16α-OH-AO and 16α-OH-EO. Higher sensitivity for detection of Δ4-AEDIONE was observed for 4-OH-Δ4-AEDIONE and 6α/β-OH-Δ4-AEDIONE.

To quantitatively describe specificity in this experimental setup (regarding only the administration of two food supplement steroids), the sensitivity of a metabolite for administration of DHEA can divided by the sensitivity for the administration of Δ4-ADIONE (or vice versa). Table 10.1 summarizes quantitative values for specificity of each parameter for respectively DHEA and Δ4-AEDIONE. The best specificity for DHEA administration is supplied by 7β-OH-DHEA, DHEA, 7-keto-AO, Δ5-AEDIOL, 16α-OH-DHEA and 7-keto-DHEA. The best specificity for Δ4-AEDIONE was supplied by 4-OH-Δ4-AEDIONE, T, E and 6α/β-OH-T.

Table 1: *Quantitative values for specificity of the studied parameters for the administration of DHEA and Δ4-AEDIONE. Specificity for Δ4-AEDIONE is the reciprocal value of the specificity for DHEA.*

Parameter	Specificity for DHEA	Specificity for Δ4-AEDIONE
7β-OH-DHEA	36.3	<0.1
DHEA	17.5	0.1
7-keto-AO	15.2	0.1
Δ5-AEDIOL	10.3	0.1
16α-OH-DHEA	8.4	0.1
16α-OH-AO	0.9	1.1
7-keto-DHEA	5.3	0.2
16α-OH-EO	1.7	0.6
AO	0.5	2.0
7α-OH-DHEA	0.5	1.9
EO	0.4	2.7
6α/β-OH-Δ4-AEDIONE	0.3	3.6
16α-OH-Δ4-AEDIONE	1.2	0.8
T	0.2	5.4
E	0.2	5.0
6α/β-OH-T	0.2	5.0
4-OH-Δ4-AEDIONE	<0.1	62.8

As described in the Appendix, administration of one of the most recently available supplement steroids 7-keto-DHEA, or its analogue 3-acetyl-7-keto-DHEA [16], can

also lead to production of oxygenated DHEA metabolites as 7α-OH-DHEA, 7β-OH-DHEA, 7-keto-DHEA and 7-keto-AO. As 7-keto-DHEA is not on the IOC list of forbidden substances, excluding the administration of this steroid is necessary for the analysis of DHEA abuse by means of oxygenated metabolites. As shown in the case study, no non-oxygenated metabolites are formed after oral administration of 7-keto-DHEA-acetate. Therefore, profiling non-oxygenated metabolites still remains necessary, as those show no sensitivity for 7-keto-DHEA.

The described experiments show that measurement of oxygenated and non-oxygenated steroids can provide sensitive and specific information that is needed for identification of the administered steroid.

More research is needed to study the specific metabolism of other mentioned food supplement steroids, leading to additional parameters to evaluate. To facilitate the interpretation of the larger number of parameters in doping analysis, multivariate statistical analysis could be applied as was done previously for profiling non-specific T metabolites [17].

ISOTOPE RATIO MASS SPECTROMETRY AND STEROID PROFILING

Since the Olympic Games of Nagano, Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry (GC-C-IRMS) is applied as confirmation method for abuse of "endogenous" steroids. This technique is able to distinguish between endogenous and exogenous steroids by quantitative analysis of the $^{13}C/^{12}C$ isotope ratio after combustion to CO_2, as explained in Chapter 1.

Analytical procedures for GC-C-IRMS analysis are reported for the analysis of T [18-21], E, DHEA, 5α-DHT [21,22], hydrocortisone and cortisone [24]. The analysis is mostly performed on metabolites of the steroids under investigation as Δ5-AEDIOL [22], AO, EO [25], stereoisomers of androstanediols [19,21,25], tetrahydrocortisol and tetrahydrocortisone [24]. GC-C-IRMS can therefore be considered as a similar technique as regular steroid profiling, with the extra ability to differentiate between metabolites of endogenous and metabolites of exogenous origin. The development of this technique resulted in major advancement in discriminative power of doping analysis regarding steroids of endogenous vs. exogenous origin. However, when specificity is regarded no real innovation was established.

GC-C-IRMS is still mostly used for confirmation purposes, as extensive and laborious sample cleanup is required. For T analysis some methodologies have been reported that are suitable for fast and efficient screening purposes [24,26]. However, the main

contribution of GC-C-IRMS is still the confirmation of T abuse after detecting a high T/E ratio, *e.g.* in cases of an increased T/E ratio caused by alcohol consumption.

Furthermore, could this technique be used to eliminate the ketoconazole test (Chapter 1, Appendix A), in which an athlete is required to self-administer a relatively large dose of ketoconazole. This should be avoided as based on ethics and potential side effects.

GC-C-IRMS can form an excellent combination with profiling of suggested steroid parameters to combine specificity and sensitivity with discrimination between endogenous and exogenous steroids, for the detection of food supplement steroids.

LITERATURE

1. W. Schanzer. Metabolism of anabolic androgenic steroids. *Clin. Chem.* 1996. **42:** 1001-1020 (1996).
2. M. Donike, K.-R. Bärwald, K. Klostermann, W. Schänzer and J. Zimmermann. Nachweis von exogenem Testosteron. In *Sport: Leistung und Gesundheit*, H. Heck, W. Hollmann, H. Liesen, R. Rost, Eds. Deutscher Ärzte Verlag, Köln, 1983, pp 293-298.
3. M. Donike. Steroid profiling in Cologne. In *Proceedings of the 10th Cologne workshop on dope analysis*, M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke and S. Rauth, Eds. Sport und Buch Strauss, Köln, 1993, pp 47-68.
4. A.T. Kicman, S.B. Coutts, C.J. Walker and D.A. Cowan. Proposed confirmatory procedure for detecting 5α-dihydrotestosterone doping in male athletes. *Clin. Chem.* **41:** 1617-1627 (1995).
5. M. Donike, M. Ueki, Y. Kuroda, H. Geyer, E. Nolteernsting, S. Rauth, W. Schanzer, U. Schindler, E. Volker and M. Fujisaki. Detection of dihydrotestosterone (DHT) doping: alterations in the steroid profile and reference ranges for DHT and its 5 alpha-metabolites. *J. Sports Med. Phys. Fitness.* **35:** 235-250 (1995).
6. B. Berendonk. Doping. Von der Forschung zum Betrug. Springer-Verlag, Hamburg, 1992, p. 447.
7. C.E. Yesalis. Medical, legal, and societal implications of androstenedione use. *JAMA*, **281:** 2043-2044 (1999).
8. V.P. Uralets and P.A. Gillette. Over-the-counter delta5 anabolic steroids 5-androsten-3,17-dione; 5-androsten-3beta, 17beta-diol; dehydroepiandrosterone; and 19-nor-5-androsten-3,17-dione: excretion studies in men. *J. Anal. Toxicol.* **24:** 188-193 (2000).

9. V.P. Uralets and P.A. Gillette. Over-the-counter anabolic steroids 4-androsten-3,17-dione; 4-androsten-3beta,17beta-diol; and 19-nor-4-androsten-3,17-dione: excretion studies in men. *J. Anal. Toxicol.* **23:** 357-366 (1999).

10. L. Dehennin, Y. Bonnaire and P. Plou. Urinary excretion of 19-norandrosterone of endogenous origin in man: quantitative analysis by gas chromatography-mass spectrometry. *J. Chromatogr. B.* **721:** 301-307 (1999).

11. Y. Reznik, L. Dehennin, C. Coffin, J. Mahoudeau and P. Leymarie. Urinary nandrolone metabolites of endogenous origin in man: a confirmation by output regulation under human chorionic gonadotropin stimulation. *J. Clin. Endocrinol. Metab.* **86:** 146-150 (2001).

12. B. Le Bizec, I. Gaudin, F. Monteau, F. Andre, S. Impens, K. de Wasch and H. De Brabander. Consequence of boar edible tissue consumption on urinary profiles of nandrolone metabolites. I. Mass spectrometric detection and quantification of 19-norandrosterone and 19-noretiocholanolone in human urine. *Rapid Commun. Mass Spectrom.* **14:** 1058-1065 (2000).

13. K. de Wasch, B.L. Bizec, H. de Brabander, F. Andre and S. Impens. Consequence of boar edible tissue consumption on urinary profiles of nandrolone metabolites. II. Identification and quantification of 19-norsteroids responsible for 19-norandrosterone and 19-noretiocholanolone excretion in human urine. *Rapid Commun. Mass Spectrom.* **15:** 1442-1447 (2001).

14. D.H. Catlin, B.Z. Leder, B. Ahrens, B. Starcevic, C.K. Hatton, G.A. Green, J.S. Finkelstein. Trace contamination of over-the-counter androstenedione and positive urine test results for a nandrolone metabolite. *Jama* **284:** 2618-2621 (2000).

15. H. Geyer, U. Mareck-Engelke, U. Reinhart, M. Thevis and W.Schanzer. The analysis of nutritional supplements for anabolic-androgenic steroids. In *Recent advances in doping analysis (8),* W. Schänzer, H. Geyer, A. Gotzmann and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, 2000, pp 23-32.

16. M. Davidson, A. Marwah, R.J. Sawchuk, K. Maki, P. Marwah, C. Weeks and H. Lardy. Safety and pharmacokinetic study with escalating doses of 3-acetyl-7-oxo-dehydroepiandrosterone in healthy male volunteers. *Clin. Invest. Med.* **23:** 300-310 (2000).

17. H.R. Norli, K. Esbensen, F. Westad, K.I. Birkeland, P. Hemmersbach, and R. Aguilera. Detection of testosterone misuse: comparison of two chromatographic sample preparation methods for gas chromatographic-combustion/isotope ratio mass spectrometric analysis. *J. Chromatogr. B.* **687:** 43-53 (1996).

19. R. Aguilera, M. Becchi, H. Casabianca, C.K. Hatton, D.H. Catlin, B. Starcevic and H.G. Pope Jr. Improved method for detection of testosterone abuse by gas chromatography/combustion/isotope ratio mass spectrometry analysis of urinary steroids. *J. Mass Spectrom.* **31:** 169-176 (1996).

20. C.H.L. Shackleton, A. Phillips, T. Chang and Y. Li. Confirming testosterone administration by isotope ratio mass spectrometric analysis of urinary androstanediols. *Steroids* **62:** 379-387 (1997).

21. R. Aguilera, T. E. Chapman, B. Starcevic, C.K. Hatton and D.H. Catlin. Performance characteristics of a carbon isotope ratio method for detecting doping with testosterone based on urine diols: controls and athletes with elevated testosterone/epitestosterone ratios. *Clin. Chem.* **47:** 292-300 (2001).

22. C.H.L. Shackleton, E. Roitman, A. Phillips and T. Chang. Androstanediol and 5-androstenediol profiling for detecting exogenously administered dihydrotestosterone, epitestosterone, and dehydroepiandrosterone: Potential use in gas chromatography isotope ratio mass spectrometry. *Steroids* **62:** 665-673 (1997).

23. M. Ueki and M. Okano. Analysis of exogenous dehydroepiandrosterone excretion in urine by gas chromatography/combustion/isotope ratio mass spectrometry. *Rapid Commun. Mass Spectrom.* **13:** 2237-2243 (1999).

24. E. Bourgogne, V. Herrou, J.C. Mathurin, M. Becchi, J. de Ceaurriz, Detection of exogenous intake of natural corticosteroids by gas chromatography/combustion/isotope ratio mass spectrometry: application to misuse in sport. *Rapid Commun. Mass Spectrom.* **14:** 2343-2347 (2000).

25. R. Aguilera, T.E. Chapman and D.H. Catlin. A rapid screening assay for measuring urinary androsterone and etiocholanolone delta(13)C (per thousand) values by gas chromatography/combustion/isotope ratio mass spectrometry. *Rapid Commun. Mass Spectrom.* **14:** 2294-2299 (2000).

26. R. Aguilera, D.H. Catlin, M. Becchi, A. Philips, C. Wang, R.S. Swerdloff, H.G. Pope and C.K. Hatton. Screening urine for exogenous testosterone by isotope ratio mass spectrometric analysis of one pregnanediol and two androstanediols. *J. Chromatogr. B* **727:** 95-105 (1999).

PART IV:

appendix

APPENDIX

CASE STUDY: METABOLISM OF 3-ACETYL-7-KETO-DHEA

INTRODUCTION

Examples of steroids that recently have appeared on the food supplement market are 7-keto-dehydroepiandrosterone (7-keto-DHEA) and its analogue 3-acetyl-7-keto-DHEA. As 7-keto-DHEA is one of the metabolites of DHEA (see Chapters 4 and 6), an overlapping metabolic profile can be expected after oral administration of these steroids.

In relation to doping analysis (3-acetyl-7)-keto-DHEA can be of relevance, when the analysis of DHEA is concerned. DHEA has been declared as doping agent by the International Olympic Committee (IOC) since 1997. As described in Chapter 3, insufficient analytical methodology is available for specific determination of DHEA abuse. When profiling oxygenated metabolites is considered for the detection of DHEA abuse, selective analysis of DHEA and (3-acetyl-)7-keto-DHEA is required, because the latter are not on the IOC list of forbidden substances.

In this study 3-acetyl-7-keto-acetate was orally administered to one male volunteer. Timed urine samples were collected previous and after administration, which were analyzed for androgens and oxygenated metabolites of DHEA (see Chapter 3).

EXPERIMENTAL

DHEA administration

Urine samples of one male (age: 30 years) were collected every 2 hours and overnight during two successive days. On the second day at 9 a.m. two capsules each containing 50 mg 3-acetyl-7-oxo-DHEA (7-Keto Fuel, Twinlab®, New York, USA) were orally administered. For each collected urine sample the exact void time,

volume and specific gravity (Urine Specific Gravity Refractometer, Atago, Japan) was recorded. Samples were stored at -20°C until time of analysis.

Analytical procedure

The analytical procedure is described in detail in Chapter 4. The following parameters were quantified: dehydroepianderosterone (DHEA), androst-5-ene-3β,17β-diol (Δ5-AEDIOL), testosterone (T), epitestosterone (E), androsterone (AO), etiocholanolone (EO), 7-keto-DHEA, 7α-hydroxy-dehydroepiandrosterone (7α-OH-DHEA), 7β-hydroxy-dehydroepiandrosterone (7β-OH-DHEA), 7-keto-androsterone (7-keto-AO), 16α-hydroxy-dehydroepiandrosterone (16α-OH-DHEA), 16α-hydroxy-androsterone (16α-OH-AO) and 16α-hydroxy-etiocholanolone (16α-OH-EO).

RESULTS

The calculated excretion rates (in μg/hour) of the analyzed non-oxygenated and oxygenated steroids are illustrated as a function of time in Figures 1 and 2, respectively. Of the oxygenated steroids, only those that were oxygenated at C^7 (7α-OH-DHEA, 7β-OH-DHEA, 7-keto-DHEA and 7-keto-AO) showed an increased excretion rate during 15-20 hours after time of administration. 7-Keto-AO excretion was increased during 30 hours. No increased excretion rate of non-oxygenated steroids was observed. Relevant steroid ratios are shown in Figure 3. The T/E, AO/EO and 16α-OH-AO/16α-OH-EO ratio were not significantly affected. The 7β-OH-DHEA/7α-OH-DHEA ratio was increased during 20 hours.

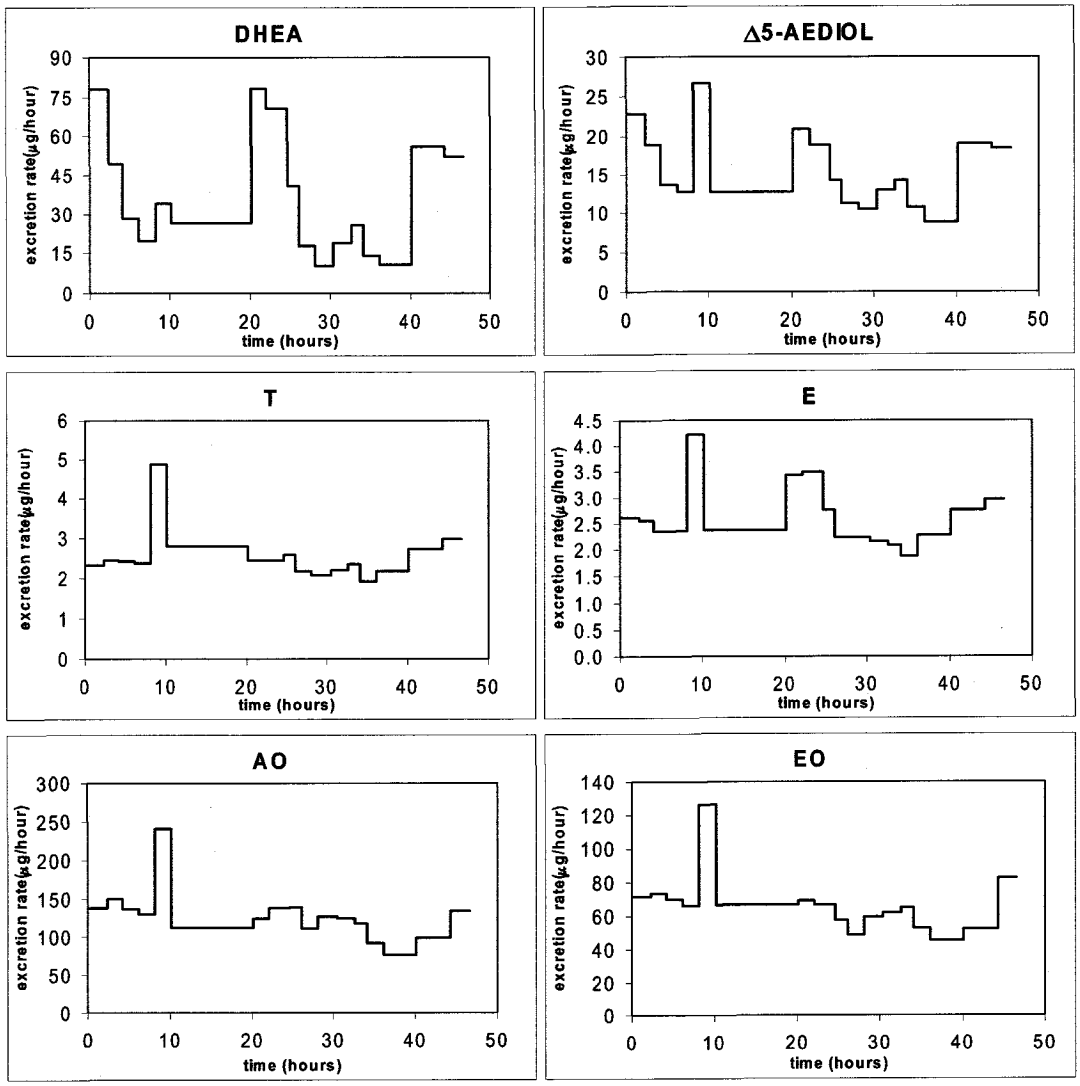


Figure 1: Excretion rates of non-oxygenated steroids as a function of time. At time point 20 hours 3-acetyl-7-keto-DHEA was orally administered.

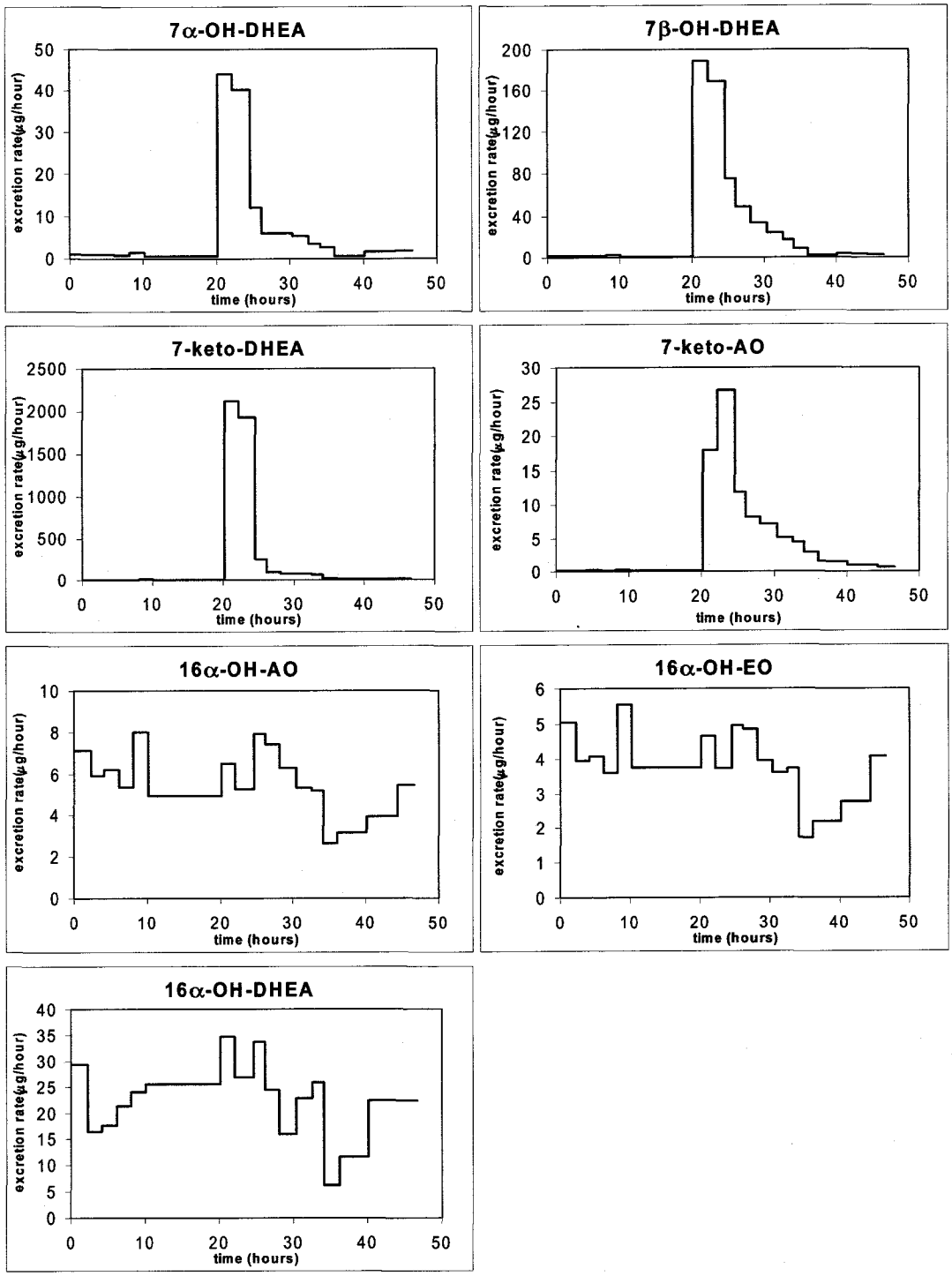


Figure 2: Excretion rates of oxygenated steroids as a function of time. At time point 20 hours 3-acetyl-7-keto-DHEA was orally administered.

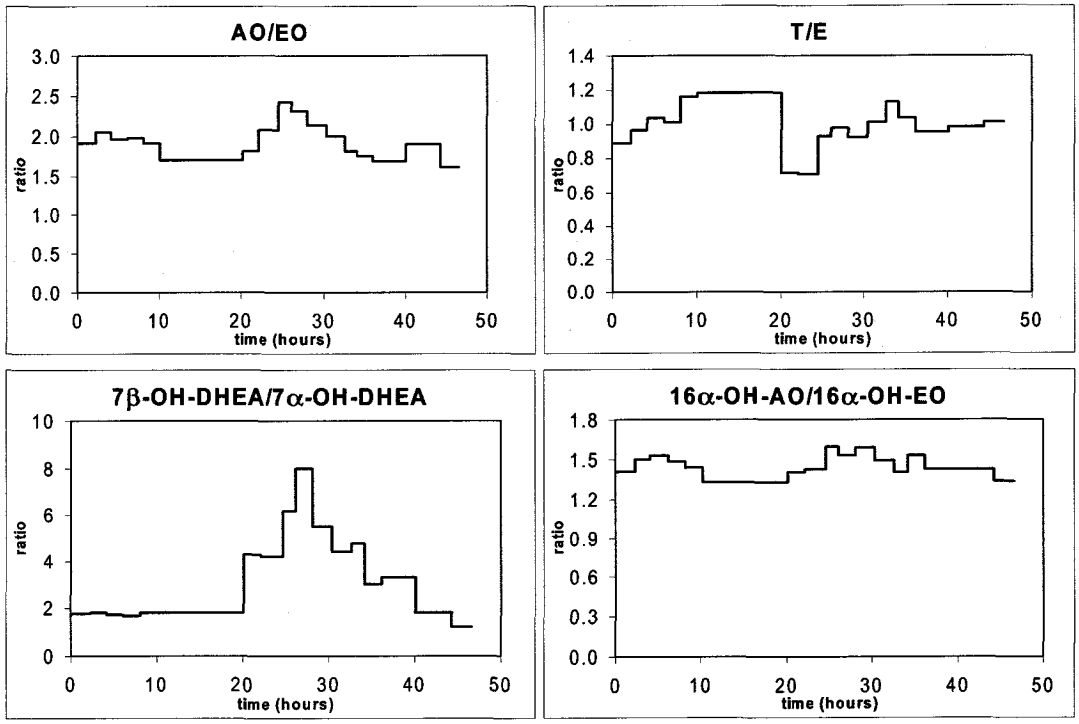


Figure 3: Ratios of excretion rates as a function of time. At time point 20 hours 3-acetyl-7-keto-DHEA was orally administered.

DISCUSSION

Application of (3-acetyl-)7-keto-DHEA

Claims for the pharmacological effect of 7-keto-DHEA and 3-acetyl-7-keto-DHEA are basically similar as for DHEA. It is still unclear what the precise function of DHEA is within the human body, except for being a biosynthetic precursor for androgens as T. Maximum DHEA plasma concentration can be detected around the age of 20, followed by a steady decrease during aging [1,2]. Several studies have presented evidence that replacement therapy of 25-50 mg/day increases androgen concentrations and improves the feeling of well being in males and females of advanced age [3,4] and in females with adrenal insufficiency [5].

The detection of 7-keto-DHEA in human urine samples was first reported by Fukushima et al. [6]. Suggestive evidence is reported that oxygenated metabolites as 7-keto-DHEA, show physiological effects explaining some of the effects of DHEA, as antiglucocorticosteroid [7] and thermogenic activity [8,9]. There is a lack of knowledge about the significance of claimed effects of 7-keto-DHEA on *e.g.* memory

function and the immune system. Therefore, therapeutic potential of 7-keto-DHEA as drug or its usefulness as food supplement to replace DHEA, are highly suggestive [10].

A pharmacokinetic study has been reported with escalating dose levels of 3-acetyl-7-keto-DHEA in 22 male subjects in a randomized, double blind, placebo-controlled design [10]. It was shown that oral administration of 3-acetyl-7-keto-DHEA was safe up to dose levels of 200 mg/day for 4 weeks. The main metabolic route led to the production of 7-keto-DHEA sulfate and limited androgen production occurred. This supported the claim that 7-keto-DHEA does not result in activation of androgen or estrogen receptors.

(3-Acetyl-)7-keto-DHEA in doping analysis

Administration of 100 mg (3-acetyl-)7-keto-DHEA leads to significantly increased excretion of several oxygenated steroids, that were previously classified as DHEA metabolites (see Chapters 4 and 7). Therefore, a potential risk for false positive results is introduced.

However, clear differences were observed as compared to the excretion data after DHEA administration. In the present experiment the excretion of androgens and 16α-hydroxy metabolites was not significantly affected. The latter even showed a limited decrease in excretion rate between 15-25 hours after time of administration.

Regarding the steroid ratios, only the 7β-OH-DHEA/7α-OH-DHEA ratio showed a comparable effect as was observed for DHEA administration. The AO/EO, 16α-OH-AO/16α-OH-EO and the T/E ratio were not significantly affected in the present study.

Concluding, this case study clearly illustrates that interpretation of a combined profile of androgens, oxygenated DHEA metabolites and corresponding steroid ratios, can provide a clear distinction between the administration of DHEA and (3-acetyl-)7-keto-DHEA.

LITERATURE

1. F. Labrie, A. Belanger, L. Cusan, J.L. Gomez and B. Candas. Marked decline in serum concentrations of adrenal C19 sex steroid precursors and conjugated androgen metabolites during aging. *J. Clin. Endocrinol. Metab.* **82:** 2396-2402 (1997).
2. C.R. Parker Jr. Dehydroepiandrosterone and dehydroepiandrosterone sulfate production in the human adrenal during development and aging. *Steroids.* **64:** 640-647 (1999).
3. F. Labrie, A. Belanger, L.T. Van, C. Labrie, J. Simard, L. Cusan, J.L. Gomez and B. Candas. DHEA and the intracrine formation of androgens and estrogens in peripheral target tissues: its role during aging. *Steroids.* **63:** 322-328 (1998).
4. A.J. Morales, J.J. Nolan, J.C. Nelson and S.S. Yen. Effects of replacement dose of dehydroepiandrosterone in men and women of advancing age. *J. Clin. Endocrinol. Metab.* **78:** 1360-1367 (1994).
5. W. Arlt, F. Callies, J.C. van Vlijmen, I. Koehler, M. Reincke, M. Bidlingmaier, D. Huebler, M. Oettel, M. Ernst, H.M. Schulte and B. Allolio. Dehydroepiandrosterone replacement in women with adrenal insufficiency. *N. Engl. J. Med.* **341:** 1013-1020 (1999).
6. D.K. Fukushima, A.D. Kemp, R. Schneider, M.B. Stokem and T.F. Gallagher. Steroid metabolism. XXV. Isolation and characterization of new urinary steroids. *J. Biol. Chem.* **210:** 129-137 (1954).
7. R. Hampl, R. Morfin and L. Starka. Minireview 7-Hydroxylated Derivatives of Dehydroepiandrosterone: What Are They Good For? *Endocr. Regul.* **31:** 211-218 (1997).
8. H. Lardy, N. Kneer, Y. Wei, B. Partridge and P. Marwah. Ergosteroids. II: Biologically active metabolites and synthetic derivatives of dehydroepiandrosterone. *Steroids* **63:** 158-165 (1998).
9. H. Lardy, B. Partridge, N. Kneer and Y. Wei. Ergosteroids: induction of thermogenic enzymes in liver of rats treated with steroids derived from dehydroepiandrosterone. *Proc. Natl. Acad. Sci.* **92:** 6617-6619 (1995).
10. M. Davidson, A. Marwah, R.J. Sawchuk, K. Maki, P. Marwah, C. Weeks and H. Lardy. Safety and pharmacokinetic study with escalating doses of 3-acetyl-7-oxo-dehydroepiandrosterone in healthy male volunteers. *Clin. Invest. Med.* **23:** 300-310 (2000).

Summary

Quantitative analysis of endogenous steroids (referred to as steroid profiling) is applied in doping analysis to detect the abuse of steroids that also have an endogenous origin, *e.g.* testosterone. The concentration of testosterone versus epitestosterone (T/E ratio) in urine is considered as the most sensitive parameter for detection of testosterone abuse. In Chapter 1 the validity of the T/E ratio is discussed by reviewing several factors that can influence this ratio, possibly leading to false-positive results. Despite these influential factors, the T/E ratio has been applied since the early eighties and can still not be displaced by the more recently developed isotope ratio mass spectrometry.

Chapter 2 describes an experiment in which the relation was investigated between the metabolism of alcohol and the increase of the T/E ratio. Male and female subjects consumed an average dose of alcohol (1.2 g/kg bodyweight). Urine and plasma samples were analyzed for testosterone, epitestosterone and for several precursors and metabolites of testosterone. All subjects showed an increase of the T/E ratio, during 10-12 hours after the start of consumption. However, the effect was significantly higher in females than in males. The chance of a T/E ratio increasing above the criterion of the International Olympic Committee (T/E = 6) by recreational alcohol consumption is realistic and should be taken into account, especially in out-of-competition doping control. Although the cause of T/E increase is not completely understood, simultaneous utilization of cofactors for both steroid biosynthesis and ethanol metabolism seems to play a predominant role.

Since steroid containing food supplements have become easily available, a frequently occurring abuse of steroids as dehydroepiandrosterone (DHEA) and androst-4-ene-3,17-dione (Δ4-AEDIONE) in sports is suspected. As described in Chapter 3, detection of these steroids in doping analysis is mostly performed by profiling techniques originally developed for detection of testosterone administration.

After oral administration of DHEA or Δ4-AEDIONE the T/E ratio and the excretion rate of metabolites as androsterone and etiocholanolone increases (Chapters 4-7). Profiling of these parameters can therefore not lead to specific information about the identity of the administered substance.

Chapters 4 and 5 describe excretion studies with respectively deuterium-labeled DHEA and Δ4-AEDIONE. Several oxygenated metabolites of these steroids were identified. The relative increase of the excretion rates after administration showed that the analysis of oxygenated steroids provides information of equal sensitivity as profiling the "regular" non-oxygenated metabolites.

Chapters 6 and 7 describe excretion studies with non-labeled DHEA and Δ4-AEDIONE. Urine samples were analyzed for oxygenated and non-oxygenated metabolites of both steroids. Sensitivity and specificity of each studied parameter were established. The most sensitive parameters for DHEA were 7β-hydroxy-DHEA, androst-5-ene-3β,17β-diol, 7-keto-androsterone, 6α/β-hydroxy-Δ4-AEDIONE, 16α-hydroxy-androsterone, 16α-hydroxy-etiocholanolone, androsterone, etiocholanolone and DHEA itself. High sensitivity for detection of Δ4-AEDIONE was observed for 4- and 6α/β-hydroxy-Δ4-AEDIONE, androsterone, etiocholanolone and testosterone. The most specific metabolites for DHEA (corresponding to a high increase in excretion rate after administration of DHEA and no or a low increase after administration of Δ4-AEDIONE) were 7β- and 16α-hydroxy-DHEA, 7-keto-DHEA, 7-keto-androsterone, androst-5-ene-3β,17β-diol and DHEA itself. The most specific metabolites for Δ4-AEDIONE were 4-hydroxy-Δ4-AEDIONE, testosterone, epitestosterone and 6α/β-hydroxy-testosterone. Concluding, the analysis of oxygenated metabolites can lead to additional information about the identity of the administered steroid, despite the relatively low conversion to these metabolites compared to the conversion to non-oxygenated metabolites.

Chapter 8 describes the derivatization of 3-keto-4-ene steroids. The trimethylsilylation of these steroids in a reagent of MSTFA/NH_4I/ethanethiol results in 3,5-dienolTMS derivatives. This is in contrast to the basic reagent MSTFA/KOAc/imidazole that mainly leads to the corresponding 2,4-dienolTMS isomers. An isomerization experiment proved that the 3,5-dienolTMS derivative of the model steroid 17-methoxy testosterone is more stable than the 2,4-dienolTMS isomer. The formation of thermodynamically least favorable 2,4-dienolTMS products was explained by hydrogen-deuterium exchange experiments. These showed that the protons at C^2 are more acidic than the protons at C^6. This leads to the formation of kinetically favorable 2,4-dienol products under the basic conditions of the MSTFA/KOAc/imidazole reagent.

Chapter 9 describes artifact formation that occurs during the derivatization of androsterone and etiocholanolone in MSTFA/NH_4I/ethanethiol. These artifacts were identified as ethyl thio-containing products of the respective trimethylsilyl derivatives. The conversion was significantly accelerated by addition of diethyl disulfide to the reagent prior to incubation. A mechanism is proposed for ethyl thio-incorporation at

the C^{16}-position. In this experiment the conversion to the artifacts was insufficient to significantly influence the analysis of androsterone and etiocholanolone. However, when the formation of new metabolites is investigated, the ethyl thio-incorporation can lead to false interpretations.

Samenvatting

Kwantitatieve analyse van endogene steroïden, ofwel steroïd profilering, wordt toegepast in doping analyse voor de detectie van steroïden die tevens een endogene oorsprong hebben, bijv. testosteron. De concentratie van testosteron versus die van epitestosteron (T/E ratio) in urine wordt hierbij beschouwd als de meest gevoelige parameter voor de detectie van testosteron misbruik. In hoofdstuk 1 wordt de validiteit van de T/E ratio besproken middels een overzicht van de factoren die deze ratio beïnvloeden en mogelijk leiden tot vals-positieve resultaten. Ondanks deze beïnvloedende factoren wordt de T/E ratio toch sinds begin tachtiger jaren gebruikt en is nog steeds niet verdrongen door de meer recentelijk ontwikkelde isotopen ratio massaspectrometrie.

Hoofdstuk 2 beschrijft een experiment waarbij de relatie onderzocht werd tussen het metabolisme van alcohol en de toename van de T/E ratio. Mannelijke en vrouwelijke proefpersonen consumeerden een gemiddelde dosis alcohol (1,2 g/kg lichaamsgewicht). Urine- en plasmamonsters werden geanalyseerd op testosteron, epitestosteron en op verschillende precursors en metabolieten van testosteron. Alle proefpersonen vertoonden een verhoging van de T/E ratio, gedurende ongeveer 10-12 uur na start van consumptie. Echter, het effect bij vrouwen was groter dan bij mannen. De kans dat de T/E ratio stijgt tot boven het criterium van het Internationale Olympische Comité (T/E = 6) door recreatieve alcoholconsumptie is daarom realistisch. Met name bij "out-of-competition" doping controles moet men hiermee rekening houden. Hoewel de oorzaak van de T/E ratio toename niet volledig is opgehelderd, lijkt het gelijktijdig gebruik van co-factoren tijdens de biosynthese van steroïden en alcohol metabolisme een overheersende rol te spelen.

Nadat in de negentiger jaren steroïden bevattende voedingssupplementen gemakkelijk verkrijgbaar werden, vermoedde men een frequent voorkomend gebruik van steroïden als dehydroepiandrosteron (DHEA) en androst-4-een-3,17-dion (Δ4-AEDIONE) in de sport. Zoals beschreven in hoofdstuk 3, wordt de detectie van deze steroïden in doping analyse meestal uitgevoerd met profileringstechnieken zoals die zijn ontwikkeld voor de detectie van testosteron.

Na orale toediening van DHEA of Δ4-AEDIONE verhoogt de T/E ratio en de excretiesnelheid van metabolieten als androsteron en etiocholanolon (hoofdstukken

4-7). Profilering van deze parameters kan daarom niet leiden tot specifieke informatie over de identiteit van de toegediende verbinding.

Hoofdstukken 4 en 5 beschrijven excretiestudies met respectievelijk gedeutereerd DHEA en Δ4-AEDIONE. Verschillende geoxygeneerde metabolieten van deze steroïden werden geïdentificeerd. De toename van de excretiesnelheden na toediening toonde aan dat de analyse van geoxygeneerde steroïden informatie van vergelijkbare gevoeligheid oplevert als de profilering van "reguliere" niet-geoxygeneerde metabolieten.

Hoofdstukken 6 en 7 beschrijven excretiestudies met respectievelijk niet-gedeutereerd DHEA en Δ4-AEDIONE. Urinemonsters werden geanalyseerd op geoxygeneerde en niet-geoxygeneerde metabolieten van beide steroïden. Gevoeligheid en specificiteit werden bepaald van iedere bestudeerde parameter. De meest gevoelige parameters voor DHEA waren 7β-hydroxy-DHEA, androst-5-een-3β,17β-diol, 7-keto-androsteron, 6α/β-hydroxy-Δ4-AEDIONE, 16α-hydroxy-androsteron, 16α-hydroxy-etiocholanolon, androsteron, etiocholanolon en DHEA zelf. Hoge gevoeligheid voor de detectie van Δ4-AEDIONE werd waargenomen voor 4- en 6α/β-hydroxy-Δ4-AEDIONE, androsteron, etiocholanolon en testosteron. De meest specifieke metabolieten voor DHEA (corresponderend met een sterke toename van de excretiesnelheid na toediening van DHEA en geen of een beperkte toename na toediening van Δ4-AEDIONE) waren 7β- en 16α-hydroxy-DHEA, 7-keto-DHEA, 7-keto-androsteron, androst-5-een-3β,17β-diol en DHEA zelf. De meest specifieke metabolieten voor Δ4-AEDIONE waren 4-hydroxy-Δ4-AEDIONE, testosteron, epitestosteron en 6α/β-hydroxy-testosteron. Concluderend, de analyse van geoxygeneerde metabolieten kan leiden tot additionele informatie over de identiteit van het toegediende steroïd, ondanks de relatief lage conversie naar deze metabolieten vergeleken met de conversie naar niet-geoxygeneerde metabolieten.

Hoofdstuk 8 beschrijft de derivatisering van 3-keto-4-een-steroïden. De trimethylsilylering van deze steroïden in het reagens MSTFA/NH_4I/ethaanthiol resulteert in 3,5-dienolTMS derivaten. Daarentegen resulteert derivatisering in het reagens MSTFA/KOAc/imidazol voornamelijk in de corresponderende 2,4-dienolTMS isomeren. Een isomerisatie-experiment toonde aan dat het 3,5-dienolTMS derivaat van het modelsteroïd 17-methoxytestosteron stabieler is dan de 2,4-dienolTMS isomeer. De vorming van het thermodynamisch minst voordelige 2,4-dienolTMS produkt werd verklaard door waterstof-deuterium uitwisselingsexperimenten. Deze toonden aan dat de protonen op C^2 zuurder zijn dan de protonen op C^6. Onder basische condities van MSTFA/KOAc/imidazol leidt dit tot de vorming van het kinetisch voordelige 2,4-dienol produkt.

Hoofdstuk 9 beschrijft de vorming van artefacten, die gevormd worden tijdens de derivatisering van androsteron en etiocholanolon in MSTFA/NH_4I/ethaanthiol. Deze artefacten werden geïdentificeerd als ethylthio-bevattende produkten van de respectievelijke trimethylsilyl derivaten. De conversie werd aanzienlijk versneld door het toevoegen van diethyl disulfide aan het reagens voor incubatie. Een mechanisme wordt voorgesteld voor ethylthio-invoeging op de C^{16}-positie. In dit experiment was de conversie naar de bestudeerde artefacten onvoldoende om de analyse van androsteron en etiocholanolon significant te beïnvloeden. Wanneer de vorming van nieuwe metabolieten echter bestudeerd wordt kan de vorming van de ethylthio-artefacten tot verkeerde interpretaties leiden.

Dankwoord

Het volbrengen van dit promotiewerk is een flinke klus geweest. Er zijn verschillende mensen geweest die mij gedurende het traject bijgestaan hebben en die ik hierbij graag wil bedanken.

Als eerste bedank ik mijn collega's Ingrid Bosman, Ronald van Ooijen en Frits Dost. In het bijzonder noem ik daarbij Ronald omdat hij met name in de slotfase veel analytische ondersteuning gegeven heeft. Nu we allemaal onze eigen weg zijn ingeslagen, wens ik jullie succes en hoop ik jullie nog vaak tegen te komen. Ook noem ik hierbij Nel Annen en Anneke Warnaar vanwege de steun die zij me onder andere tijdens de afronding gegeven hebben.

Douwe de Boer wil ik als begeleider en leermeester erg bedanken. Hoewel hij tussentijds Utrecht verliet voor een groot avontuur in Lissabon, heeft dit onze samenwerking niet kunnen schaden. Ik ben dankbaar voor de goede discussies en zijn correcties van de manuscripten.

Jan Zwikker bedank ik voor zijn leiding van het project dat is beschreven in hoofdstuk 8. Zijn inspiratie, kennis en experimentele inzet hebben dit hoofdstuk vorm gegeven. Hierbij is ook het werk van Vera Kaats-Richters en Katja Bouwman onmisbaar geweest.

De promotores Robert Maes en Jos Thijsen bedank ik voor de discussies, de begeleiding en de vrijheid die ze mij gegeven hebben om mijn onderzoek uit te voeren.

Ook zijn er een aantal studenten geweest die meegewerkt hebben aan projecten binnen en buiten het kader van dit proefschrift. Dit zijn: Annemieke Verburg (alcohol), Katja Bouwman (androsteendion), Lia Hakvoort (DHEA), Maarten Hoogveld (bromantaan), Pauline van der Voort (boldenon), Rik Deurloo en Rob van Riel (beiden alcohol).

Graag noem ik onze Vlaamse collega's Frans Delbeke, Peter van Eenoo en Noël Desmet. Mede dankzij hen was de jaarlijkse "Workshop on Dope Analysis" in Keulen behalve leerzaam ook enorm gezellig. Nu ik reeds geen groentje meer ben in dit gezelschap durf ik bij deze te bekennen dat ik nog steeds geen Hämschen gegeten heb. Misschien gebeurt dat ooit nog eens in hun aanwezigheid.

Last but not least bedank ik het thuisfront. Zonder Esther, familie en vrienden was dit alles niet tot stand gekomen. Daarbij moet ook het Promotie Team genoemd worden: André, Andy, Els, Esther, Frank, Hans, Jan-Hein, Jara, Manon, Mariken, Ricky en Tomas. Allemaal ontzettend bedankt.

Curriculum vitae

The author of this thesis was born on April 16, 1970 in Helmond, The Netherlands. In 1990 he graduated from VWO and started his study Chemical Technology at the University of Technology in Eindhoven. He specialized in bioanalytical chemistry by means of a one year research project that was focussed on the analysis of markers for oxidative damage by free radicals. In 1996 he started his pHD study that is described in this thesis. During this period he further specialized in steroid analysis and was responsible for routine doping control analysis and screening of anabolic steroid formulations circulating on the black market. Several educational activities were employed until 2001. In the beginning of 2001 he joined the contract research organization NOTOX in 's-Hertogenbosch as study director.

List of publications

1. Application of the ketoconazole test to exclude testosterone abuse in doping analysis [Abstract]. D.H. van de Kerkhof, D. de Boer, J.H.H. Thijssen and R.A.A. Maes. Presented at the Xth International Congress on Hormonal steroids, Québec City, 1998.
2. Survey of anabolic formulations on the Dutch black market [abstract]. D.H. van de Kerkhof, M.C. Hoogveld, D. de Boer and R.A.A. Maes. Presented at the Workshop on Dope Analysis, 1998.
3. The position of the hydroxyl group in the main bromantane metabolite. D.H. van de Kerkhof, D. de Boer and R.A.A. Maes. In: *Recent advances in doping analysis (6)*, W. Schänzer, H. Geyer, A. Gotzmann and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, 1999, pp. 361-374.
4. Confirmation of endogenous boldenone production: A procedure for an *in vitro* experiment. D.H. van de Kerkhof, P.M. van der Voort, D. de Boer and R.A.A. Maes. In: *Recent advances in doping analysis (7)*, W. Schänzer, H. Geyer, A. Gotzmann and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, 2000, pp. 161-168.
5. Evaluation of testosterone/epitestosterone ratio influential factors as determined in doping analysis [review]. D.H. van de Kerkhof, D. de Boer, J.H.H. Thijssen and R.A.A. Maes. *J. Anal. Toxicol.* **24:** 102-115 (2000).
6. Derivatization aspects of specific metabolites of androst-4-ene-3,17-dione: 6α-hydroxy-androst-4-ene-3,17-dione and 6β-hydroxy-androst-4-ene-3,17-dione. D.H. van de Kerkhof, K. Bouwman, J.W. Zwikker, V.E.M. Kaats-Richters, R.D. van Ooijen, D. de Boer and R.A.A. Maes. In: *Recent advances in doping analysys (8)*, W. Schänzer, H. Geyer, A. Gotzmann and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, 2001, pp. 129-140.
7. Artifact formation due to ethyl thio-incorporation into silylated steroid structures. Accepted by J. Chromatogr. A.
8. Grenzen van doping en tests. J.H.H. Thijssen and D.H. van de Kerkhof. Accepted by Nederlands Tijdschrift voor Geneeskunde.
9. Trimethylsilylation of 3-keto-androst-4-ene steroids: thermodynamic versus kinetic control of enolization. Manuscript in preparation.
10. Evaluation of IMx® microparticle-enzyme immunoassays for detecting human chorionic gonadotrophin in urine for doping control purposes. R. Verkaik, D. de Boer, E.A. Dubois, H. de Munnik, D.H. van de Kerkhof, M.A. Blankenstein and R.A.A. Maes. Manuscript in preparation.