In: Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, S. Rauth (eds.)
Recent Advances in Doping Analysis (1). Sport und Buch Strauß, Köln 1994

X. De la Torre*, J. Ortuño*, J. Segura*, J Cabrer+ and E. Vilardell+

STANOZOLOL, METENOLONE AND STEROID PROFILE.

* Departament de Farmacologia i Toxicologia, Institut Municipal d'Investigació Mèdica, Universitat Autònoma de Barcelona, Spain
+ Servei d'Endocrinologia i Nutrició, Hospital Clínic, Barcelona, Spain

1. Introduction

Anabolic steroids are synthetic substances related to male androgenic hormones that are used in medicine in the treatment of male pseudohermaphroditism, different gonadal hormone deficiencies and late stage of breast cancer. For years, androgens with anabolic properties have been also object of misuse among athletes in order to increase weight and improve muscular strength. Since 1974 the use of anabolic steroids has been forbidden by the International Olympic Committee (IOC) due to ethics in sport and health risks.

The use of anabolic steroids during the "out of competition" training period allows that their urinary concentration be very low during competition and their detection in doping control often difficult.

In the other hand, it is well known that the use of anabolic steroids modify the normal excretion profile of endogenous steroids, this giving up another possibility for the detection of their misuse.

The aim of this study is to describe some modifications of the androgenic steroid profile after the abuse of some anabolic steroids.

2. Materials and method

2.1. Samples collection.

Nine male body builders (Table I, users), who routinely used anabolic steroids and who had volunteered for this study were required to abstain from anabolic steroid use for at least three months, while maintaining their training program. After this period, the volunteers could began again their own anabolic schedule. Another eight male body builders (Table I, Control Group) reported non use of anabolic steroid during the full period of the study.
Before each subject entered the study and at months 1 , 3 and 6 a set of physical (virilization and secondary sex characteristics, body and bone composition) and laboratory examinations (LH, FSH, T (pool), SHBG, IGF-I, hypoglycemie insulin test, LHRH and TRH tests) were performed.

In: Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, S. Rauth (eds.)
Recent Advances in Doping Analysis (1). Sport und Buch Strauß, Köln 1994

Table I. Subjects description (mean ± s.d.)

	Anabolic Steroids users	Control Group
Age (years)	28.5±8.2	29.5±7.1
Height (cm)	170.0±26.9	177.1±5.0
Weight (Kg)	86.7±12.4	80.9±7.5
n	9	8

All subjects received during the 6 months study period an hypercaloric diet (3740 Kcal daily approx.) and followed a similar physical training.

Urine samples were collected every 2 weeks during the first 3 months and once a month during the following 3 months. The samples were frozen until analysis.

2.2. *Reference steroids*

Testosterone (T), 17-epitestosterone (E), androsterone (A), etiocholanolone (Et), 11ß-hydroxyandrosterone (11OHA), 11ß-hydroxyetiocholanolone (11OHEt), 5α-androstan-3α,17ß-diol (3α5αDIOL), 5ß-androstan-3α,17ß-diol (3α5ßDIOL), 17α-methyltestosterone (MT), tetrahydrocortisol (THF) and testosterone-[16,16,17]-d_3 (T-d_3) were purchased from Sigma . Etiocholanolone-[2,2,4,4]-d_4 (Et-d_4) and 11ß-OH-androsterone-[2,2,4,4]-d_4 (11OH-d_4) were kindly provided by Prof. M. Donike (Cologne, FRG).

2.3. *Material*

The extraction columns containing XAD-2 as a solid-phase used were from Biochemical Diagnostic Inc. type R. ß-Glucuronidase arylsulfatase (*H. pomatia*,) for hydrolysis was provided by Sigma. Diethylether from Carlo Erba was distilled in glass with calcium hydride before use. The derivatization reagent MSTFA (N-methyl-N-trimethylsilyl-trifluoroacetamide) was provided by Mackerey-Nagel. Ammoniumiodide GR Merck and dithioerythritol 99+% from Aldrich-Chemie were used as supplied. All other reagents were of analytical grade and used as supplied.

2.4. *Sample preparation*

A general flow-chart of the procedure is presented in Figure 1. Some additional details are presented below.

2.4.1. *Isolation of steroids*

An aliquot of 5 mL of urine was applied over the extraction columns (previously washed with

In: Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, S. Rauth (eds.)
Recent Advances in Doping Analysis (1). Sport und Buch Strauß, Köln 1994

2 mL of methanol and 2 mL of water). Columns were washed with 2 mL of distilled water prior to elution with methanol. The methanolic eluate was evaporated to dryness under a nitrogen stream at 50°C.(*Segura* 1993)

2.4.2. Hydrolysis and extraction

The residue was redissolved in 1 mL of acetate buffer 0.2 M pH 5.2, and 50 μL of the enzyme solution (*H. pomatia* ß-glucuronidase aryl sulphatase) were added. The samples were incubated 3 hours at 55°C. After cooling at room temperature, the pH was adjusted to 9-10 with $NaHCO_3/Na_2CO_3$. Extraction was performed with 5 mL of diethylether. Samples were centrifuged, the ethereal phase was transferred and evaporated under a nitrogen stream at 40 °C. Residue was kept in a desiccator under vacuum for at least 30 minutes.

2.4.3. Derivatization for GC/MS analysis

For the trimethylsililation of steroids, the dry residue was dissolved in 50 μL of MSTFA/NH_4I/Dithioerythritol (1000:2:5) and heated at 60°C for 15 minutes. 2 μL of this solution were injected into the GC-MS system.

2.5. GC/MS determination

GC/MS from Hewlett-Packard was used (GC 5890 series II was fitted with a HP7673A autosampler and connected to an MS 5970). The separation was carried out using a methylsilicone fused silica capillary column (HP,Palo Alto,USA) , 25 m, 0.2 mm i.d., film thickness 0,11 μm).
The injector, in split mode (1:10), and the interface were operated at 280°C. The oven temperature program was:initial temperature 200°C 1min , *rate 1:* 3°C/min to 242°C, *rate 2:* 15 °C/min to 300°C, and maintained for 3.15 min at 300°C. Helium was used as a carrier gas at a flow rate of 0,65 mL/min. The mass spectrometer was operated with electron impact ionization (EI 70 eV) and in Selected Ion Monitoring (SIM) acquisition mode.(See Table II for selected ions).

2.6. Quantitation of the endogenous steroids:

In each analytical batch, a calibration methanolic extract (CAL14B) was injected to calculate response factors to quantify each substance. Ions and concentrations of the substances in the calibration mixture are summarized in Table II.
Only the samples found positives to synthetic anabolics were taken into account for the analysis of the steroid profile. For the control group an equal number of random samples was selected.

The following metabolic ratios, based on the estimated concentrations, were studied: androsterone/etiocholanolone (A/Et), 11-hydroxyandrosterone/11-hydroxyetiocholanolone (11OHA/11OHEt) and 5ß-androstandiol/5α-androstandiol (5ß/5α-DIOL). Testosterone/epitestosterone (T/E) concentration ratio was measured because it is relevant for the detection of exogenous testosterone abuse.

In: Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, S. Rauth (eds.)
Recent Advances in Doping Analysis (1). Sport und Buch Strauß, Köln 1994

3. Results

3.1 Screening analysis for anabolic steroids

The results of the presence of anabolic steroids are summarized in Table III. Only the presence of stanozolol metabolite (3'OH-stanozolol), metenolone and its metabolite (1-methylen-3α-hydroxy-5α-androstan-3-one) were detected.
As it can be seen, the protocol reported by users was not followed by the subjects who self-administrated anabolic steroids. Two different groups can be recognized :those who intake only metenolone, and those who sometimes or ever intake metenolone and stanozolol. All the samples from bodybuilders who declared not consuming anabolic steroids were found to be negatives.

3.2 Steroid profile evaluation
(see Tables IV-V and Figures 2-5)

Body builders who intake both stanozolol and metenolone showed a T/E ratio (mean 5.76 ± 0.91) higher than the control group (1.76 ± 0.66) but those who only abused metenolone (0.35 ± 0.05) had a significant lower ratio than controls. For the other two ratios considered, no difference between control group and stanozolol was detected. On the other hand the effect on the 5α/5ß metabolic ratio is remarkable. While the A/Et (5α/5ß) ratio decreased in metenolone group 0.84 ± 0.12 versus 1.54 ± 0.27 in the control, the inverse ratio 5ß/5α-DIOL (5ß/5α) also decreased (1.19 ± 0.02 versus 1.96 ± 0.17) compared to the control group.

Testosterone concentration decreased in the two positive groups about 8-10 folds times times comparing with the control group. While epitestosterone remained similar in the control and metenolone groups, its concentration decreased 20-30 fold times in the stanozolol group, this fact being therefore the main reason for the above mentioned high T/E ratio.
For the 11-hydroxy metabolites ratio, there was no difference between metenolone and control groups while in the stanozolol group the decease on the 11OHEt concentration makes the ratio between the two metabolites higher than in the other groups.

4. Discussion

It is well known that the anabolic steroid abuse causes the inhibition of the hypothalamic-hypofisary axis. (*Alén* (1985), *Fennessey* (1988)). The results here presented are in agreement with this fact but the urinary steroid profile shows that the modifications on the metabolism or excretion are modulated by the specific exogenous steroid abused.
Some of the alterations on the steroid profile can be produced by the 5α-reductase inhibition. (*Hatton* (1988). According to this, the behaviour of the three 5α/5ß ratios studied should be similar. While in the metenolone group the A/ET and 11OHA/11OHET ratios decreased, 5αDIOL/5ßDIOL ratio increased but in the stanozolol group A/ET and 5αDIOL/5ßDIOL ratios were unchanged while 11OHA/11OHET showed a remarkable increase. This results

could be explained if other metabolic pathways are implicated. On the basis of the different behaviour of androstandiols (3α,5α- and 3α,5ß-DIOLS) during the abuse of anabolic steroids, we suggest that the inclusion of more parameters of the endogenous steroids metabolism would be usefull for having a better sight of the alterations of the steroid profile due to the consumption of these substances.

According to *Saartok et al.*(1984), stanozolol has a lack of capacity of binding to the androgen receptor as well as to Sex Hormone-Binding Globulin (SHBG). They postulate a mechanism suggesting the influence of stanozolol on enzymatic activities involved in metabolic pathways regulating the metabolism of endogenous catabolic or anabolic steroids. Consequently, stanozolol would create a general depression of all of the substances of the steroid profile. On the other hand steroids like metenolone,that have a great affinity for androgen receptors, should have a direct action in the target tissues. This different mechanism of action could explain the different behaviour of steroid profile due to different anabolic steroids.

The results of the present study were compared with the reference values and criteria (see Table VI) proposed by the International Weight-lifting Federation in 1990 for non acceptable steroid profiles. According to these criteria none of the samples of the control group had abnormal values (see Table VII), only one positive sample of the metenolone group have abnormal values according to criteria II but none according to criteria I. All the samples of the stanozolol group had abnormal values according to criteria I but none according to criteria II. Following this two criteria, false positive cases (suspect of abuse of androgenic-anabolic steroids) seem not to be probable to appear but positive samples (real abuse of anabolic steroids) could be regarded as "normals" (false negative cases).

5. Conclusions

a. The results here presented suggest that a unique steroid profile is not obtained during the abuse of different anabolic steroids as measured by the conventional concentration or ratios of T, E, A, Et, 11OHA and 11OHEt.

b. The evaluation of concentrations or ratios of additional selected parameters of steroid profile (i.e. 3α,5α/3α,5ß) could be also relevant markers of steroid abuse.

c. Some criteria used to presuppose previous steroid abuse do correctly classify "non users", although some abusers may be misclassified.

d. The results on the stanozolol group, suggest that samples with both a) a ratio testosterone to epitestosterone around 6 and b) a very low epitestosterone concentrations, can appear during stanozolol abuse.

6. Acknowledgements

We acknowledge the financial support from CIRIT (Generalitat de Catalunya) for X. de la Torre and from CICYT (Ministerio de Educación y Ciencia) for part of the project (DEP91-0835).

In: Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, S. Rauth (eds.)
Recent Advances in Doping Analysis (1). Sport und Buch Strauß, Köln 1994

7. References

Alén M., Reinilä M., Vihko R. (1985). *Response of serum hormones to androgen administration in power athletes.* Med. and Sci. in sport and exercise, 17, 354-359.

Donike M. (1992). *Steroid profiling in Cologne.* Presented at the 10th Cologne Workshop on dope analysis, Cologne (FRG).

Fennessey PV., Gotlin RW., Martin D., Smith S., Harrison LM. (1988). *Anabolic steroids in body builders: use, metabolic disposition and physiological effects.* J. Pharm. Biomed. Anal., 6, 999-1002.

Hatton CK., Catlin DH. (1988) . *Urinary steroid profiles in athletes.* 35th ASMS Conference on Mass Spectrometry and Allied Topics, Denver (CO).

Saartok T., Dahlberg E., Gustafsson JÄ. (1984). *Relative binding affinity of anabolic androgenic steroids: comparison of the binding to the androgen receptor in the skeletal muscle and in the prostate, as well as the sex hormone-binding globulin.* Endocrinology, 114, 2100-2106.

Segura J., Pascual J.A., Ventura R., Ustaran J.I., Cuevas A., Gonzalez R. (1993). *International cooperation in analytical chemistry: Experience of antidoping control at the XI Pan American Games.* Clin. Chem., 39, 836-845.

In: Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, S. Rauth (eds.)
Recent Advances in Doping Analysis (1). Sport und Buch Strauß, Köln 1994

URINE EXTRACTION PROCEDURE **Figure 1**

5 ml urine
ISTD (17α-methyltestosterone)
extraction in XAD-2 columns
elution with methanol
take to dryness

|

HYDROLYSIS

redissolve with 1 ml acetate buffer 0.2 M, pH 5.2
50 μl of ß-glucuronidase (H. pomatia)
heat 3 hours at 55°C

|

EXTRACTION

pH 9-10 with $NaHCO_3/Na_2CO_3$ (2:1)
5 ml of diethyl ether
vortex extraction
centrifugation

AQUEOUS PHASE

discard

ORGANIC PHASE

take to dryness
keep in desiccator with KOH/P_2O_5

|

DERIVATIZATION

50 μl MSTFA/NH_4I/Dithioerytrhitol

heat 15 min. at 60°C

|

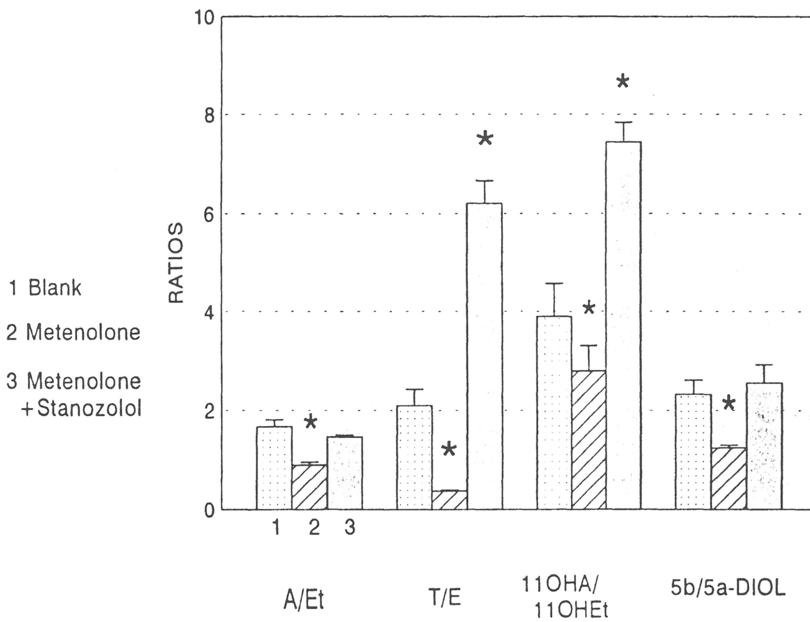
GC/MSD

injection 2 μl

In: Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, S. Rauth (eds.)
Recent Advances in Doping Analysis (1). Sport und Buch Strauß, Köln 1994

Figure 2

STEROID PROFILE IN BODY BUILDERS

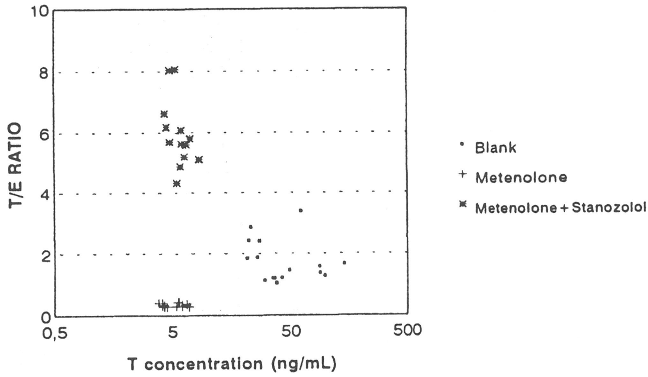


* : p< 0.05

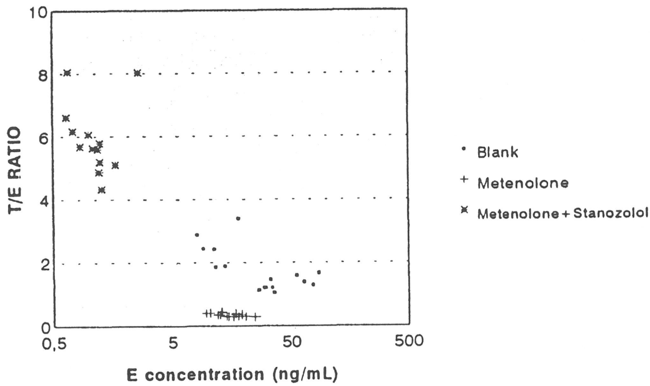
In: Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, S. Rauth (eds.)
Recent Advances in Doping Analysis (1). Sport und Buch Strauß, Köln 1994

Figure 3

STEROID PROFILE IN BODY BUILDERS
T/E vs T concentration



T/E vs E concentration

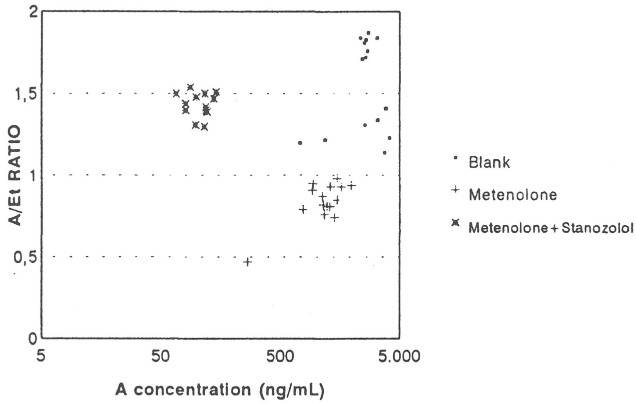


In: Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, S. Rauth (eds.)
Recent Advances in Doping Analysis (1). Sport und Buch Strauß, Köln 1994

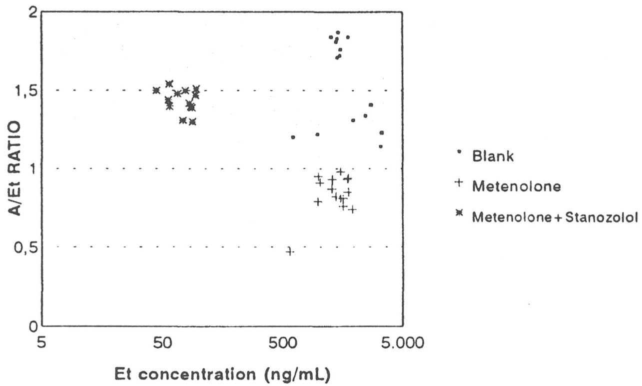
Figure 4

STEROID PROFILE IN BODYBUILDERS

A/Et vs A concentration



A/Et vs Et concentration

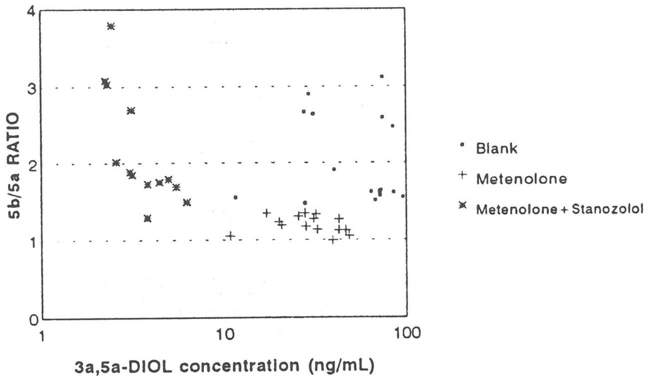


In: Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, S. Rauth (eds.)
Recent Advances in Doping Analysis (1). Sport und Buch Strauß, Köln 1994

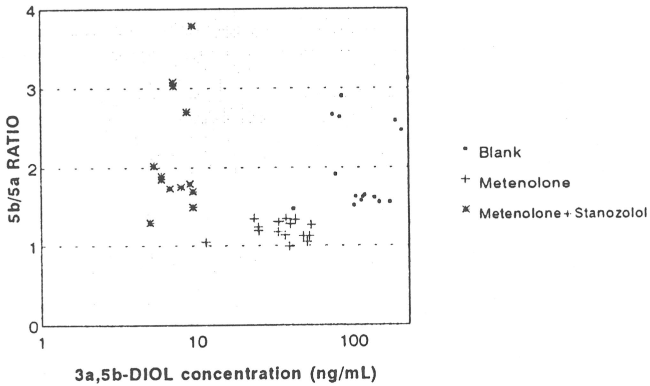
Figure 5

STEROID PROFILE IN BODY BUILDERS

3a,5b-DIOL/3a,5a-DIOL vs 3a,5a-DIOL concentration



3a,5b-DIOL/3a,5a-DIOL vs 3a,5b-DIOL concentration



In: Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, S. Rauth (eds.)
Recent Advances in Doping Analysis (1). Sport und Buch Strauß, Köln 1994

Table II. Calibration mixture CAL14B for endogenous steroids quantitation.

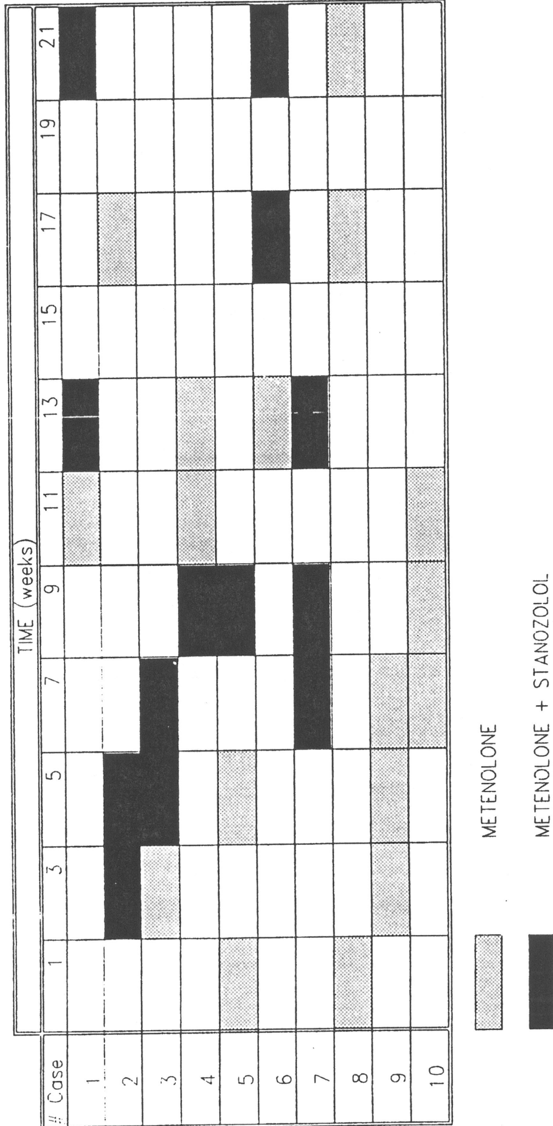
SUBSTANCE	RRT(*)	CONC (ng/mL)	m/z
T (bis-O-TMS)	0.8836	20	432.40
E (bis-O-TMS)	0.8264	120	432.40
T-d_3 (bis-O-TMS) *ISTD1*	0.8813	20	435.40
A (bis-O-TMS)	0.7083	2000	434.40
Et (bis-O-TMS)	0.7173	2000	434.40
Et-d_4 (bis-O-TMS) *ISTD2*	0.7125	500	438.40
11BOHA (tris-O-TMS)	0.8945	400	522.40
11BOHEt (tris-O-TMS)	0.9059	200	522.40
11BOHA-d_4 (tris-O-TMS) *ISTD3*	0.8921	240	526.40
3α5αDIOL (bis-O-TMS)	0.7316	80	241.15
3α5ßDIOL (bis-O-TMS)	0.7362	120	241.15
MT (bis-O-TMS) *ISTD4*	1.0000	500	446.35

(*) RRT are relative to MT (rt= 15.1 ± 0.2 min)

Each substance was quantified by comparing with its deuterated analog but 3α5αDIOL and 3α5ßDIOL by comparing with MT as internal standard (ISTD).

In: Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, S. Rauth (eds.)
Recent Advances in Doping Analysis (1). Sport und Buch Strauß, Köln 1994

Table III



In: Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, S. Rauth (eds.)
Recent Advances in Doping Analysis (1). Sport und Buch Strauß, Köln 1994

Table IV: Endogenous steroids urinary concentrations (ng/mL, standard deviation in brackets)

	ENDOGENOUS STEROIDS							
GROUP	T	E	A	ET	11OHA	11OHET	5α DIOL	5ß DIOL
Control (n=16)	**51.88** (33.76)	**33.87** (23.41)	**2745** (880.76)	**1842** (772.86)	**517.45** (257.23)	**209.16** (173.98)	**64.26** (24.96)	**125.1** (51.28)
Metenolona (n=17)	**5.24** (1.04)	**15.47** (3.90)	**1173** (344.92)	**1381** (355.99)	**388.30** (132.97)	**191.19** (85.99)	**31.03** (10.75)	**36.60** (11.58)
Metenolona+ Stanozolol (n=13)	**5.94** (1.13)	**1.04** (0.30)	**106.82** (22.65)	**74.68** (16.08)	**68.93** (18.70)	**8.23** (4.47)	**3.75** (1.25)	**7.49** (1.56)

(n= number of samples analyzed)

Table V : Urinary metabolites ratios (standard deviation in brackets)

	ENDOGENOUS STEROIDS METABOLITES RATIOS			
GROUP	T/E	A/ET	11OHA/11OHET	5αDIOL/5ßDIOL
Control (n=16)	**1.76** (0.66)	**1.54** (0.27)	**3.22** (1.35)	**0.53** (0.13)
Metenolone (n=17)	**0.35** (0.05)	**0.84** (0.12)	**2.28** (1.02)	**0.85** (0.08)
Metenolone+ stanozolol (n=13)	**5.76** (0.91)	**1.43** (0.07)	**7.05** (0.79)	**0.51** (0.14)

(n= number of samples analyzed)

In: Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, S. Rauth (eds.)
Recent Advances in Doping Analysis (1). Sport und Buch Strauß, Köln 1994

Table VI : Criteria for non acceptable steroid profile according to IWF Rules for Doping Tests at the World Championships 1990. *(Donike 1992)*

The criteria for non acceptable steroid profile are that the parameters 1,2 and 3 are lower than the reference limit given below, or parameter 1,3 and 4 lower than specified:

Parameter	limit
1. Androsterone (ng/mL)	766.4
2. Etiocholanolone (ng/mL)	700.9
3. Epitestosterone (ng/mL)	8.2
4. A/Et	0.45

Table VII : Application of the criteria from Table VI on the positive samples of this study (results are presented as samples with a non acceptable steroid profile/total samples of the group) :

GROUP	CRITERIA I	CRITERIA II
METENOLONE	0/17	1/17
STANOZOLOL	13/13	0/13
CONTROL	0/16	0/16

In: Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, S. Rauth (eds.)
Recent Advances in Doping Analysis (1). Sport und Buch Strauß, Köln 1994