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Steroid Profiling in Cologne

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1. Introduction

When it became known to the athletic community that more and more synthetic anabolic steroids could be detected by the comprehensive and sensitive gas-chromatographic / mass-spectrometric screening procedure presented in previous Cologne Workshops and published in 1984 [1], their reaction was to apply endogenous substances, e.g. testosterone and/or human chorionic gonadotropin (HCG). Testosterone was the first endogenous substance used in place of anabolic androgenic steroids. By applying short acting testosterone preparations the time period between final application of anabolic steroids and the time of competition where doping control was expected could also be covered.

The search for a method to detect testosterone misuse was the beginning of steroid profiling in doping analysis. The starting point was the observation [2] that after oral, rectal or intramuscular testosterone application the rate of excretion of testosterone glucuronide increased more than other testosterone metabolites. In excretion studies with testosterone and deuterated testosterone, no change in the excretion rate of epitestosterone was observed. Thus epitestosterone was shown not to be a metabolite of testosterone, and the ratio of testosterone to epitestosterone could thus be used as an indicator for testosterone application.

In 1982, the Medical Commission of the International Olympic Committee (IOC) banned the use of testosterone and defined a ratio of testosterone- to epitestosterone-glucuronide above 6 as an offence of the doping regulations. Since then steroid profiling has been part of the screening procedure for anabolic steroids in Cologne. Androsterone and etiocholanolone are also included in this profile procedure as well as other endogenous steroids [3], so that the quality of the analytical procedure, especially the hydrolysis step, can be monitored.

A decrease in the amount of endogenous steroids excreted following the application of anabolic androgenic steroids was first observed in samples [4] originating from weightlifters and track and field athletes. This phenomenon was also observed, when in 1985 the International Federation of Bodybuilding (IFBB) banned the use of anabolic steroids and introduced dope controls at major competitions. The extreme misuse of anabolic steroids in this sport revealed the influence of long-term application of anabolic steroids on the endocrine system [5]. It was concluded that monitoring the steroid profile of the athlete may be a valuable method to detect long-term misuse of anabolic steroids. Several approaches to profile monitoring have been explored and results from some of these projects are summarized in this paper.

2. Modifications of the analytical procedure

a) Samples are prepared as described in the protocol published previously [1,3]; however, the aliquot of urine was reduced from 5 ml to 2 ml and the volume of sample injected for analysis was increased from 1 to 3 μl. The advantage is a reduction of the sample preparation time.

b) Further, the urine sample volume prepared is oriented based on its specific gravity. A multiple aliquot of urine is prepared depending on the specific gravity, as shown in Table 1, so that the sensitivity in detecting anabolic steroids is increased. In addition, the quantitation of endogenous steroids, and especially testosterone and epitestosterone, in diluted urine samples is more accurate.

Table 1: Volume of urine prepared based on its specific gravity

urine specific gravity	urine volume of aliquot
> 1.010	2 ml
< 1.010	4 ml
< 1.005	8 ml
< 1.0025	16 ml
< 1.001	20 ml

c) Because of the complex chemistry of the oxidation of dithioerythritol which results in additional peaks in the GC chromatogram, ethanthiol is now used to stabilize the derivatization mixture.

Table 2: Screening procedure IV protocol for analysis of combined fractions of conjugated and free steroids.

Sample preparation

2 ml of urine and a mixture of internal standards (see Table 2) are added to an Amberlite XAD-2 column. The column (pasteur pipette, closed with a glass pearl, bed height 2 cm) is wahsed with 2 ml of bidistilled water and eluted with 2 ml of methanol.

The methanolic eluate is evaporated to dryness and the residue is dissolved in 1 ml of 0.2 M sodium phosphate buffer pH 7.

50 μl of ß-glucuronidase from E. coli (K12, Boehringer Mannheim) are added to the buffer solution and the mixture is hydrolysed for 1 h at 50°C. The solution is alkalized with 250 μl of 7% potassium carbonate solution to pH 9-10 and the steroids are extracted with 5 ml of diethylether on a mechanical shaker for 5 minutes. After centrifugation the etheral layer is transferred to a glass tube and evaporated to dryness under vacuo.

Derivatization

The dry residue is derivatized with 100 μl of MSTFA/NH_4I/ethanthiol 1000:2:3 (v:w:w) and heated for 15 minutes at 60°C.

GC/MS parameters

GC/MS HP 5996
carrier gas: helium, 1 ml/min at 180°C, split 1:10
column: HP Ultra-1 fused silica, crosslinked methyl silicone (OV 1), 17 m, 0.2 mm i.D., 0.11 μm film thickness
temperature program: 180°C; 3°C/min - 231°C; 30°C/min - 310°C

2.3 Quantitation

Methyltestosterone was first introduced as an internal standard when at that time the main interest was to quantify the amount of testosterone and epitestosterone excreted. In extending the steroids of interest to include androsterone, etiocholanolone, 11ß-hydroxyandrosterone and 11ß-hydroxyetiocholanolone it was found that the stability of the calibration factors with methyltestosterone as internal standard was not satisfying. Figure 1 shows the shift in the calibration factors for the target steroids in 35 calibration mixtures measured under routine conditions from January through May 1992 calculated with methyltestosterone as an internal standard. It is obvious that most changes in the

calibration factors are parallel and can be attributed to the variation of the peak area of methyltestosterone.

A logical approach to reduce the variation in calibration factors for the target steroids was to introduce deuterated internal standards at concentration in the range of the respective steroids. The deuterated standards in Table 2 were synthesized [6] and introduced into the profiling procedure.

Table 2: Internal standards used in the quantification of endogenous steroids.

internal standard	concentration [ng/ml]	target compound
methyltestosterone	500	all registered endogenous steroids
[2,2,4,4-$^2H_4$]-etiocholanolone	500	androsterone, etiocholanolone
[16,16,17-$^2H_3$]-testosterone	40	testosterone, epitestosterone
[2,2,4,4-$^2H_4$]-11ß-hydroxyandrosterone	240	11ß-hydroxy-androsterone, 11ß-hydroxy-etiocholanolone

Figure 2 shows the calibration factors calculated with the respective deuterated substance as internal standards. As can be seen from Table 3, the coefficient of variation is minimized when using the respective deuterated steroid as an internal standard for calculation of the calibration factors.

Table 3: Calibration factors for the quantification of characteristic endogenous androgenic steroids calculated with different internal standards: -M = methyltestosterone -E = d4-etiocholanolone -T = d3-testosterone -O = d4-11ß-hydroxy-androsterone

	N	Min	Max	Mean	St. Dev.	CV %
ANDR-M	35	.68	1.11	.838	.112	13.37
ETIO-M	35	.72	1.18	.893	.120	13.44
EPIT-M	35	1.75	3.00	2.439	.353	14.47
TEST-M	35	1.79	3.16	2.537	.359	14.15
OHA-M	35	.42	.78	.565	.101	17.88
OHE-M	35	.33	.63	.460	.081	17.61
ANDR-E	35	1.03	1.38	1.213	.097	**8.00**
ETIO-E	35	1.13	1.46	1.293	.100	**7.73**
EPIT-E	35	2.71	4.67	3.540	.462	13.05
TEST-E	35	2.76	4.85	3.684	.486	13.19
OHA-E	35	.66	1.12	.820	.143	17.44
OHE-E	35	.51	.91	.669	.119	17.79
ANDR-T	35	.35	.56	.440	.062	14.09
ETIO-T	35	.37	.61	.470	.067	14.26
EPIT-T	35	1.02	1.49	1.276	.143	**11.21**
TEST-T	35	1.08	1.54	1.325	.131	**9.89**
OHA-T	35	.24	.44	.295	.052	17.63
OHE-T	35	.20	.34	.241	.038	15.77
ANDR-O	35	1.67	3.32	2.387	.482	20.19
ETIO-O	35	1.84	3.55	2.549	.533	20.91
EPIT-O	35	4.85	9.49	6.938	1.399	20.16
TEST-O	35	5.09	9.66	7.203	1.354	18.80
OHA-O	35	1.25	1.74	1.575	.157	**9.97**
OHE-O	35	1.03	1.44	1.283	.123	**9.57**

3. Testosterone / epitestosterone ratio

A prominent change in the steroid profile can be observed after testosterone application. The reason is that the amount of testosterone-17-glucuronide excreted after testosterone application increases by at least an order of magnitude more than any of the testosterone metabolites (Figure 3 and Table 4).

To examine the metabolic pathways, an excretion study with 50 mg 1,2-dideutero-testosterone [7] was performed. In Table 4 the results of this excretion study are compared with excretion rates of some testosterone metabolites from a reference population [8,9].

The excretion rate for 1,2-dideutero-testosterone increased in the first 7 hours by a factor of 147 whereas epitestosterone only increased by a factor of 2. This result with labelled testosterone confirmed the observation that the epitestosterone concentration was not influenced by testosterone application. In addition it established the selection of epitestosterone as an "endogenous internal standard" to detect application of testosterone.

Smaller compared to the increase in excreted testosterone were the relative increases of androsterone and etiocholanolone, about 8 and 10 respectively. A consequence of this increase is that the ratio of androsterone to testosterone (A/T) decreases and this can be used as additional parameter to detect application of testosterone (see 4.1). In the above described experiment the A/T ratio following application of testosterone was only 3.4 while in the reference population the A/T ratio was 63..

Table 4: Metabolism of testosterone, 50 mg 1,2-dideutero-testosterone, applied orally.

Steroid	median of the reference population* µg/h (0-7 h p.a.)	dideutero-metabolites µg/h (0-7 h p.a.)	factor exogenous/endogenous
Androsterone	144	1140	7.9
Etiocholanolone	106	1106	10.4
Epitestosterone	2.1	4.1	2.1
Testosterone	2.3	339	147.4

* 105 sport students [8,9]

4. Steroid profile in doping analysis

Until now, doping analysis has been limited to the detection of banned substances or their metabolites. The use of the steroid profile as a tool to detect the misuse of anabolic steroids has been difficult since one must first define standard or "normal" values. Nevertheless, steroid profiling can serve to examine the endogenous status of an athlete. The following examples will be discussed:

1. development of the steroid profile in weightlifting.
2. steroid profile atlas for the German decathlon team.
3. longitudinal monitoring of elevated T/E ratios.

4.1 Weightlifting World Championships 1989 and 1990

After the disaster of the 1988 Olympic Games in Seoul with 5 positive athletes, two of which were suspended gold medalists, the International Weightlifting Federation (IWF) took strict measures to fight against the misuse of anabolic steroids. The number of samples taken in and out of competition was increased and all samples were analysed in Cologne in order to compare results with an existing data base and to establish a data base with the steroid profiles of weightlifters.

After evaluation of the results of 1989, the IWF Executive Board and the IWF Congress decided to take appropriate sanctions when the steroid profile showed deviations from the "norm" in order to extend the deterrent effects of doping controls at and out of competition. This regulation became effective for the 1990 World Championships in Budapest. Table 5 shows the text defining a non-acceptable steroid profile. The reference limits were calculated using routine doping control samples from cyclists measured in 1988 and 1989 in Cologne [9].

Table 5: Excerpt from regulations of the IWF regarding the steroid profile.

The quantitative determination will be performed on 3 aliquots of the urine sample, each aliquot injected at least twice into the gas-chromatograph / mass spectrometer.

Criteria for a non acceptable steroid profile will be that the first three values of the following parameters are lower than the reference limits given in the table below, or the first, the third and the fourth lower than specified:

Parameter	limit
1. Androsterone [ng/ml]	766,4
2. Etiocholanolone [ng/ml]	700,9
3. Epitestosterone [ng/ml]	8,2
4. Androsterone/Etiocholanolone	0,45

Table 6 shows the application of these reference limits on the samples obtained during the Weightlifting World Championships in 1989 and 1990. In 1989, 15 athletes fulfilled the criteria and would have been banned, whereas in 1990, none of the athletes fulfilled the criteria. Of special (biochemical) interest is that not only the concentrations of the endogenous steroids normalized, but also the androsterone / etiocholanolone ratio (A/E), which means that the activity of the 5α-reductase returned to normal.

Table 6: Application of the reference limits on the samples of the Weightlifting World Championships (WCH) 1989 (n=84) and 1990 (n=96).

	WCH 1989 n	WCH 1990 n
Androsterone < 766.4	19	0
Etiocholanolone < 700.9	22	4
Epitestosterone < 8.2	23	5
Androsterone/Etiocholanolone < 0.45	1	0
three criteria fulfilled	15	0

The improvement of the steroid profiles of the weightlifter population can be statistically demonstrated as a significant increase in the concentrations as shown in Figure 4.

4.2 German decathlon team

The German decathlon team decided to underline their attitude against doping by agreeing to give a urine sample every two weeks as an out-of-competition doping control with the goal of establishing a steroid profile atlas for each athlete. Figures 5 and 6 show the variability of the androsterone/etiocholanolone (A/E) and the testosterone/epitestosterone (T/E) ratios as results of a preliminary evaluation. None of the steroid profiles was suppressed or showed changes in the ratios T/E or A/E over the time period investigated.

4.3. Elevated T/E-ratios: longitudinal monitoring and suppression studies

During the past years, it occurred on several occasions that an athlete with a testosterone/epitestosterone ratio above 6 claimed not to have misused testosterone, its precursors, HCG, or anabolic steroids. The high T/E ratio in these cases is often caused by a low epitestosterone concentration which may be endogenous or induced. One possible approach to follow up such cases is a longitudinal monitoring of the steroid profile of this athlete, best under quarantaine conditions, second best by out-of-competition controls on short notice.

A second approach was described by Oftebro [10]. Ketoconazol, a cytochrome P_{450} inhibitor, is administered to suppress the testicular testosterone production. Based on our experience, a single dose of ketoconazol, 6 mg/kg bodyweight, allows one to differentiate between a high T/E ratio due to the application of testosterone, or a naturally elevated T/E ratio caused by a relative low epitestosterone concentration.

A case study with a cyclist who on several occasions showed a T/E ratio of about 6 or higher is presented. Figure 7 shows the T/E ratio, testosterone and epitestosterone concentration of this athlete throughout the year 1991. The T/E ratio lies within the range of 5.1 to 8.4 (mean = 6.9, stdv = 1.2) and a coefficient of variation of 17%. This coefficient of variation lies within the order of magnitude of individual variations, a first hint to a "normally" elevated T/E ratio. A further cause is the low epitestosterone concentration.

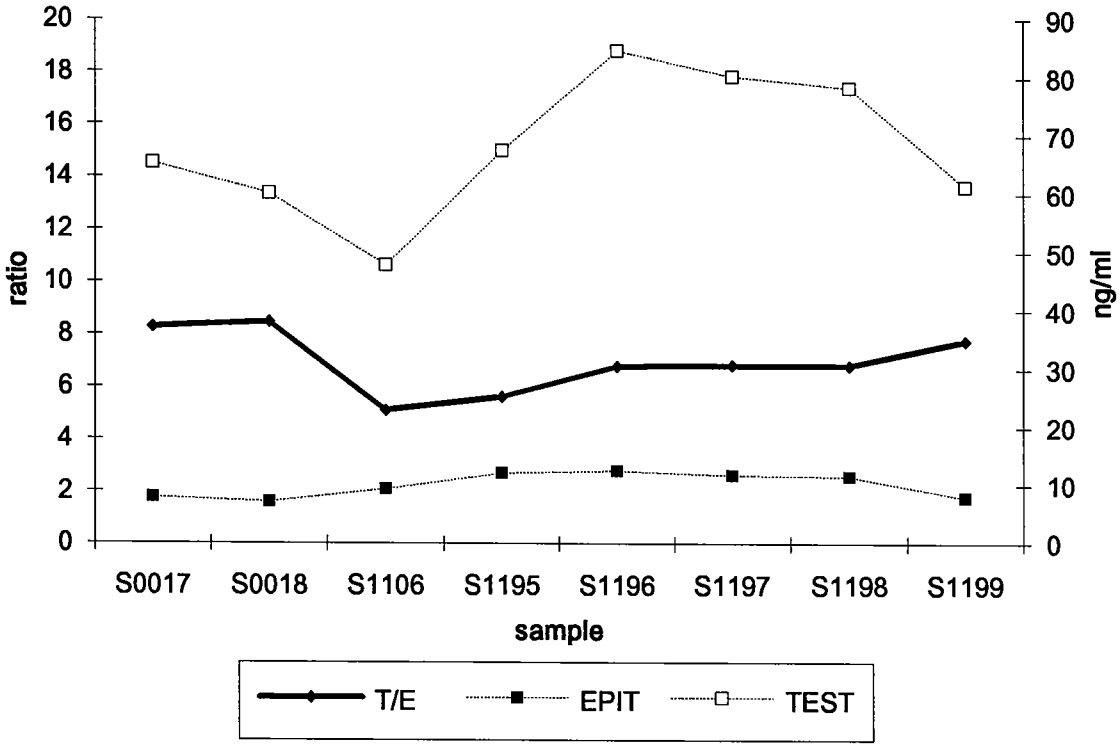

Figure 7: T/E ratio, and urinary testosterone and epitestosterone concentrations in several samples from a cyclist throughout the year 1991.

In order to evaluate the steroid profile, three experiments were performed:

1. urine collection over 60 hours, 10 spot samples plus 2 pooled samples overnight
2. a depression study of the testis with ketoconazol
3. a depression study of the adrenal gland with dexamethasone.

Figures 8 shows the results of the samples collected over the 60 hour period. The epitestosterone concentration is fairly constant, but low, wherease the testosterone concentration is much higher and fluctuates to a larger extent. The T/E ratio varies between 6 and 14 (mean=8.89, stdv=1.97). The coefficient of variation of the T/E ratio is 22% and lies well within the range found in a study on the longterm stability of the steroid profile [11] and the coefficient of variation observed on this subject previously (compare Figure 7).

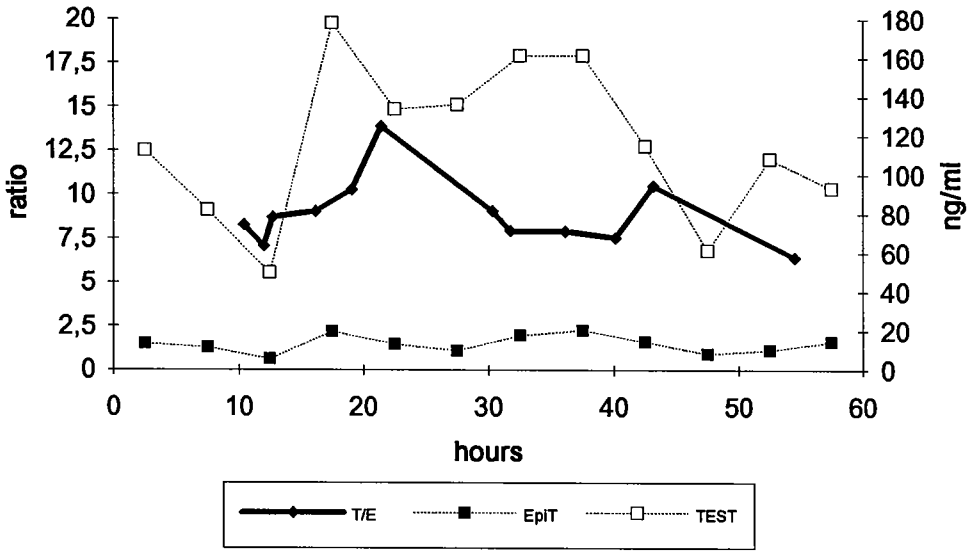

Figure 8: T/E ratio, and testosterone and epitestosterone concentations of 12 samples from a cyclist provided consecutively over 3 days.

In the testicular testosterone suppression study, four hours after application of ketoconazol, both the testosterone and epitestosterone concentrations were significantly lower than the starting values. The testosterone excretion was suppressed to a higher extent than the epitestosterone excretion resulting in a decrease of the T/E ratio from about 6 to about 2 (Figure 9). The depression of the testosterone excretion would not be observed in the case of exogenously applied testosterone.

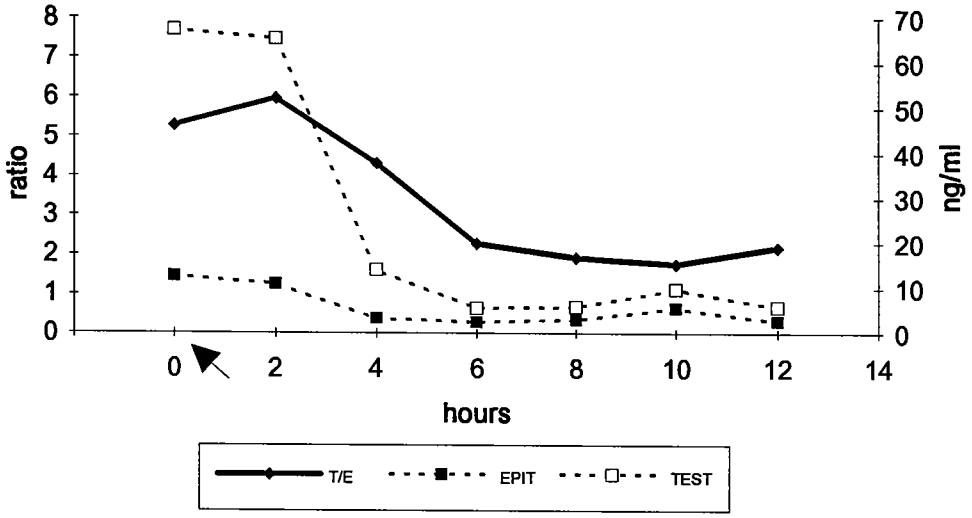

Figure 9: T/E ratio, and testosterone and epitestosterone concentrations in samples delivered from a cyclist after application of ketoconazol, 2 x 200 mg orally at t=0.

Depression of the adrenal gland by application of a synthetic corticosteroid, dexamethasone, did not influence the rate of testosterone excretion, but it did seem to depress slightly the epitestosterone excretion rate. The T/E ratio increased to a small extent, but the fluctuation paralleled the testosterone concentration.

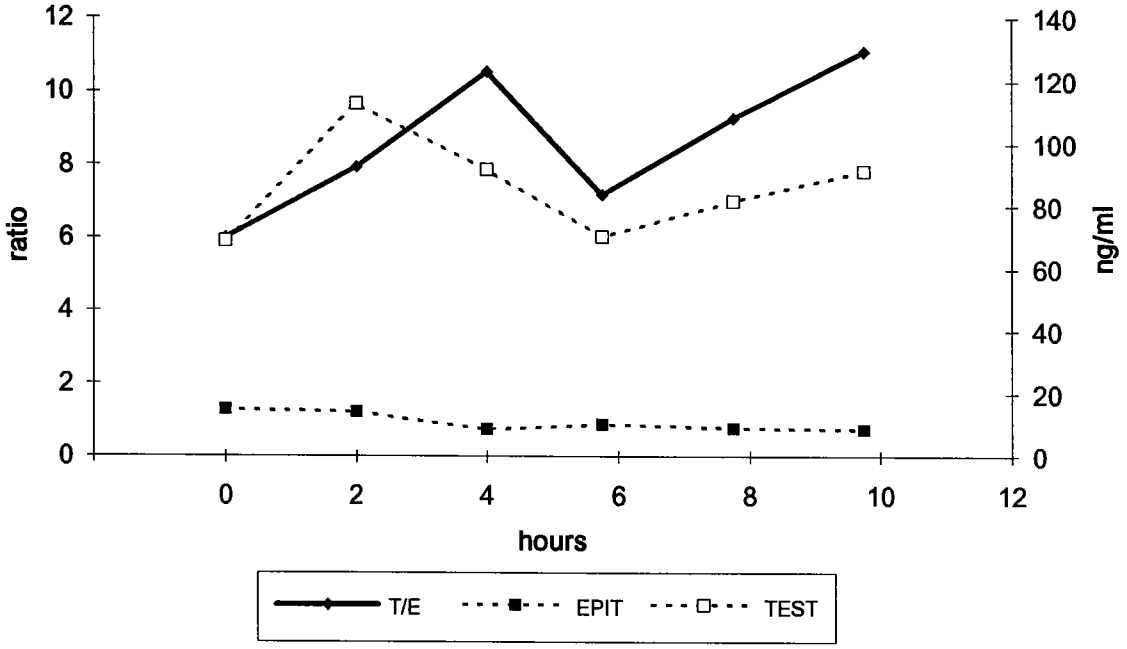

Figure 10: T/E ratio, and urinary testosterone and epitestosterone concentrations in samples from a cyclist delivered after the application of dexamethasone, 3 mg orally at t=0.

In discussion of the origin of epitestosterone, the ketoconazol and dexamethasone experiments indicate that the testis secretes epitestosterone or its precursors to a larger extent than the adrenal gland.

Summary

Steroid profiling is a valuable tool when performed routinely on urinary doping control samples under carefully controlled analytical conditions. The details of the analytical procedure especially the quantitation using four different internal standards, three of them deuterated, are presented.

The steroid profiles of an individual without interference from anabolic androgenic steroids or inhibitors of enzymes are relative stable. The most prominent change in the steroid profile is observed after testosterone application, which will lead to a high dose dependent increase of the testosterone-glucuronide. The suppression of the endogenous production of androgenic steroids by anabolic steroids is well known. Therefore the application of lower reference limits by the International Weightlifting Federation is presented as an example. Further examples for a meaningful application of steroid profiling are longitudinal studies on German decathlon athletes, and the differentiation between high T/E ratios caused by endogenous low epitestosterone concentrations or by testosterone administration.

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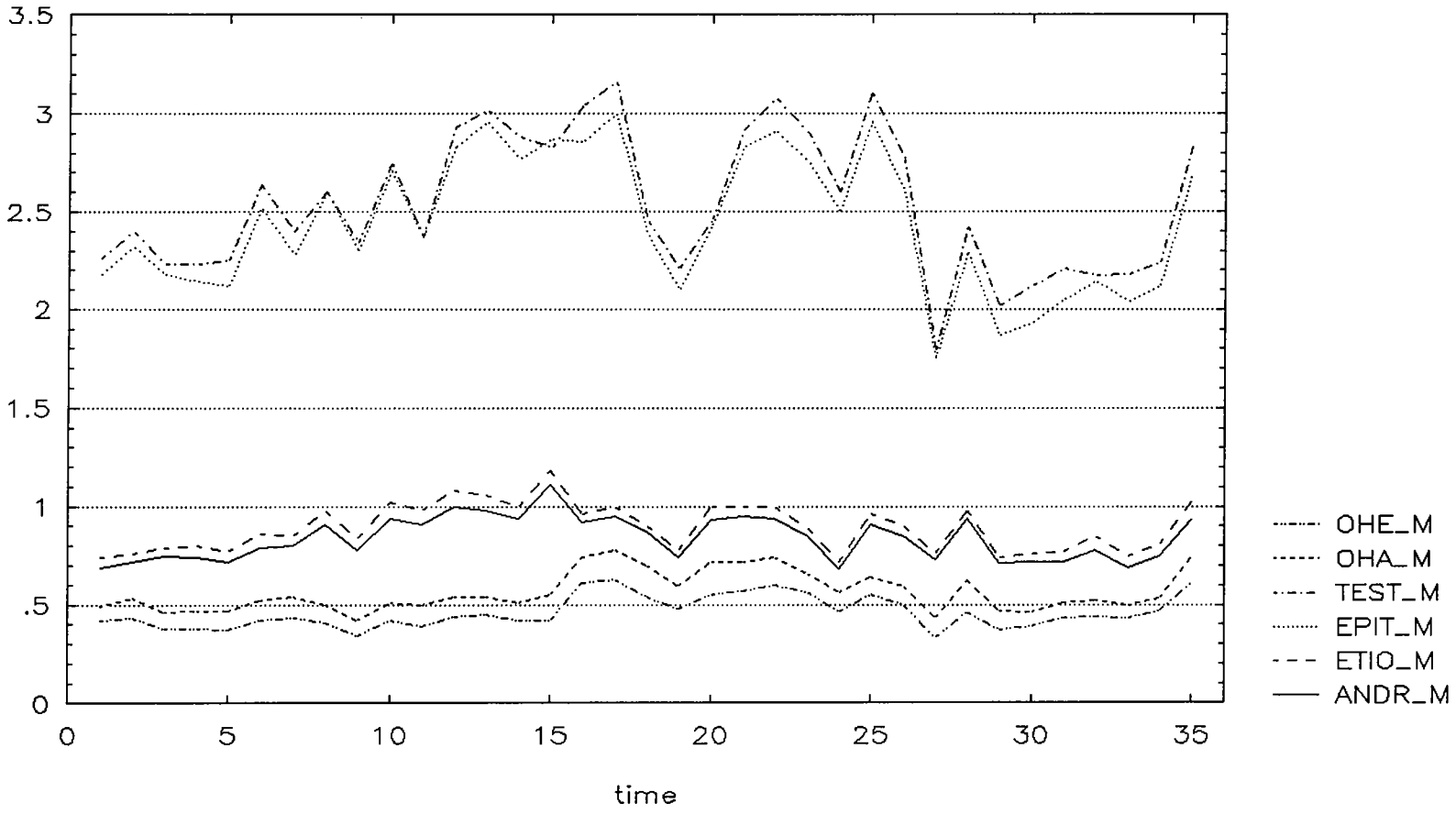

Figure 1: Shift in the calibration factors calculated with methyltestosterone as internal standard in pairs epitestosterone and testosterone (EPIT_M, TEST_M), androsterone and etiocholanolone (AND_M, ETIO_M), 11ß-hydroxy-androsterone and 11ß-hydroxy-etiocholanolone (OHA_M, OHE_M)

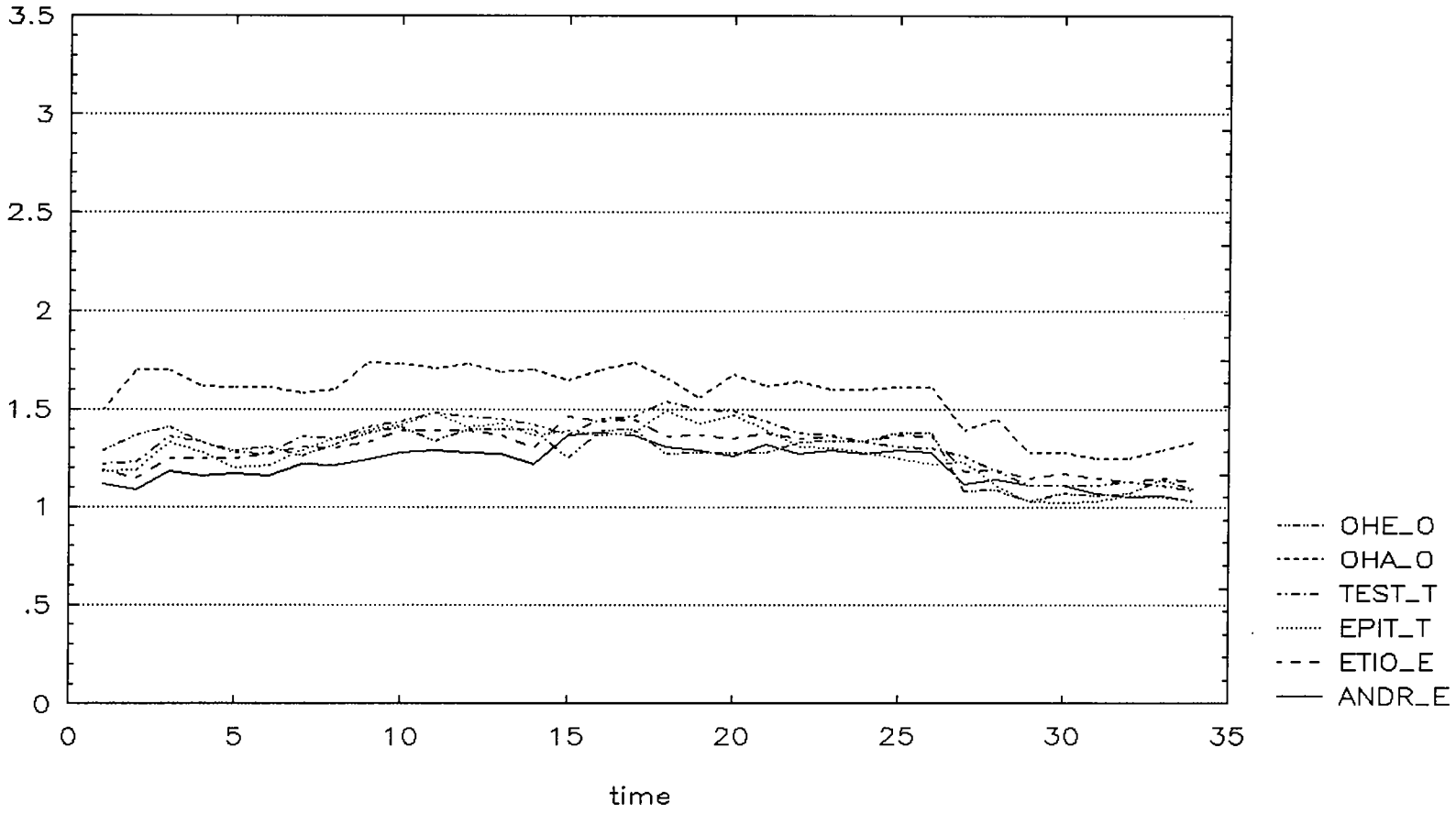

Figure 2: Calibration factors calculated with three deuterated internal standards. Androsterone and etiocholanolone with d4-etiocholanolone; epitestosterone and testosterone with d3-testosterone; and 11ß-hydroxy-androsterone and 11ß-hydroxy-etiocholanolone with d4-11ß-hydroxy-androsterone.

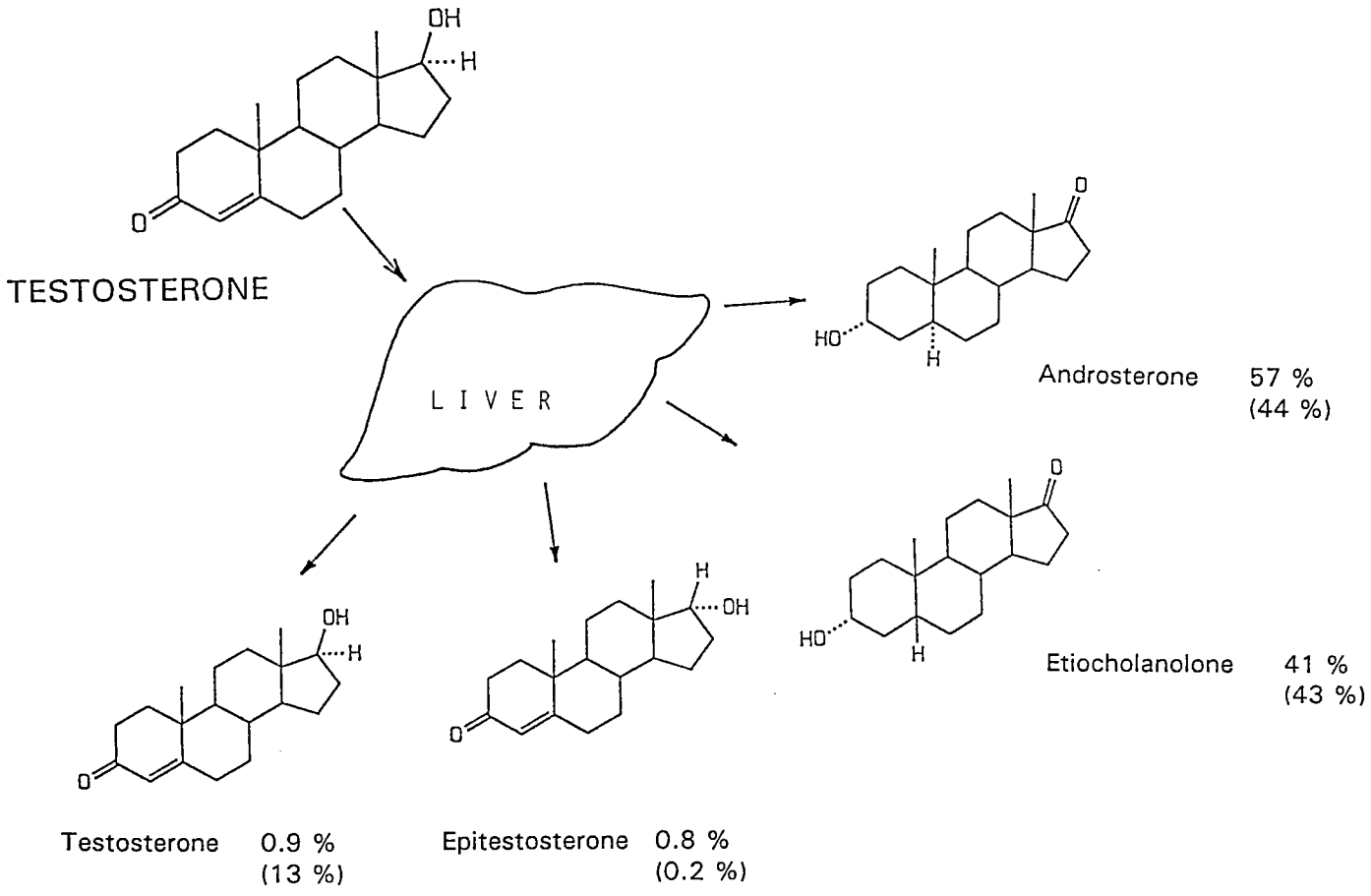

Figure 3: Relative excretion of testosterone metabolites, normally and after application of dideutero-testosterone (in brackets).

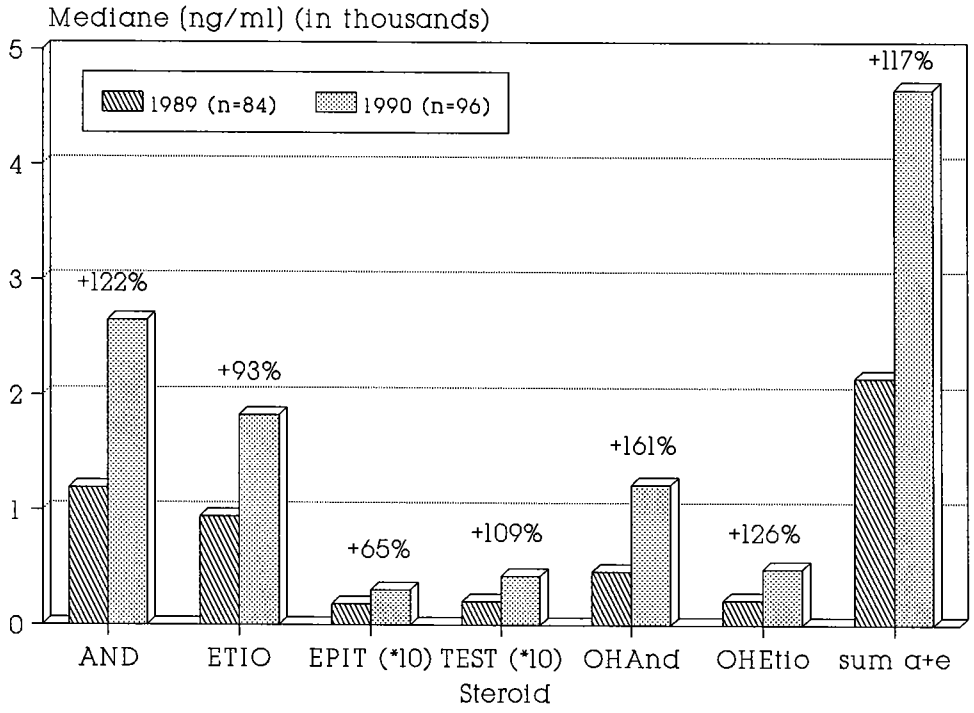

Figure 4: Mediane of the steroid concentrations of androsterone (AND), etiocholanolone (ETIO), epitestosterone (EPIT) times 10, testosterone (TEST) times 10, 11ß-hydroxy-androsterone (OHAnd), 11ß-hydroxy-etiocholanolone (OHEtio), and the sum of androsterone and etiocholanolone (sum a+e) of the participants of the Weightlifting Worldchampionships 1989 and 1990. All differences are highly significant.

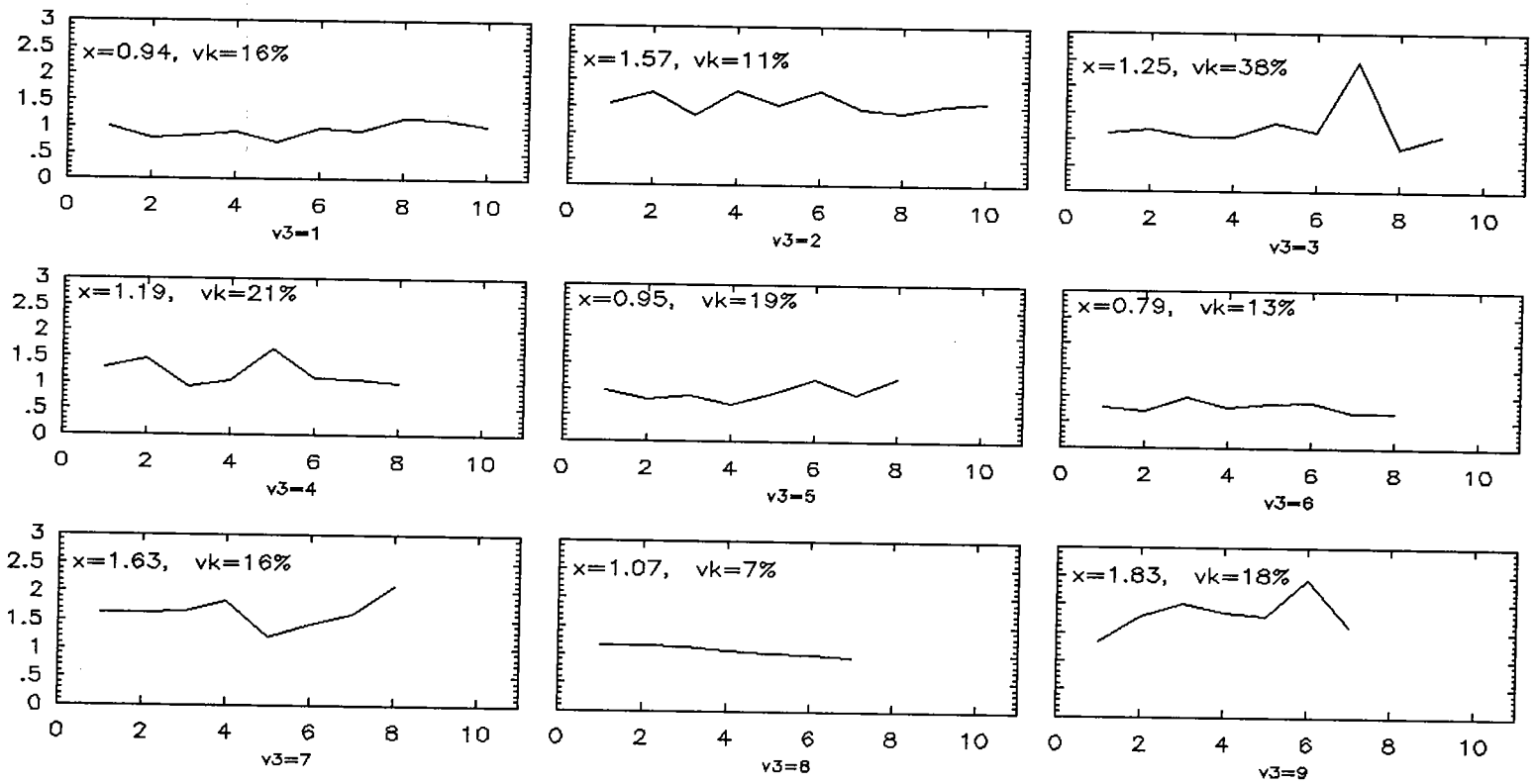

Figure 5: Androsterone/etiocholanolone ratio of 9 athletes of the German Decathlon Team, up to 10 controls within 6 months. x = mean, vk = coefficient of variation.

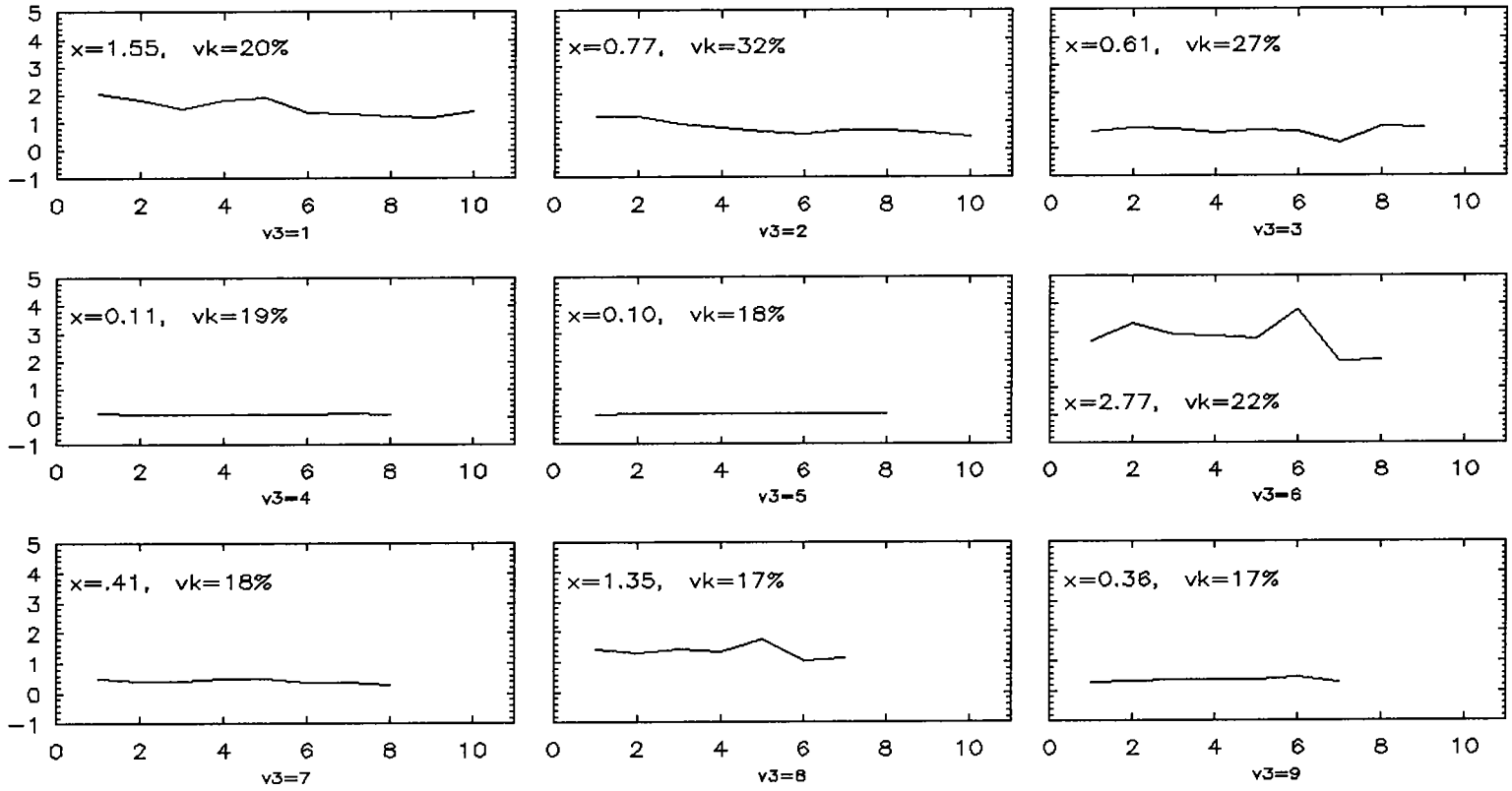

Figure 6: Testosterone/epitestosterone ratio of 9 athletes of the German Decathlon Team, up to 10 controls within 6 months. x = mean, vk = coefficient of variation.