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The Cologne protocol to follow up high testosterone/ epitestosterone ratios

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Introduction

The most important parameter for the detection of doping with exogenous testosterone is an elevated ratio testosterone/epitestosterone (1, 2). Before a dope control sample is declared positive for testosterone, it has to be proven, according to the rules of the IOC (3), that the elevated ratio testosterone/epitestosterone is not natural. Therefore we have developped in cooperation with the Antidoping Commission of the International Cycling Federation (UCI) a complex protocol to follow up high testosterone/epitestosterone values. The main intention of this protocol is the prevention of false positive results.

The knowledge of the „normal" steroidprofile as basis for the detection of doping with testosterone

The basis for the detection of doping with testosterone and other endogenous steroids is the knowledge of the „normal" steroid profile, i.e. population based reference ranges of endogenous steroids. Therefore we have analysed within our screening procedure for anabolic steroids steroidprofiles of dope control urines for several years. All samples were analysed and quantified with the same method, which was described in detail several times (4, 5). In table 1 are presented the endogenous steroids, which are monitored for steroidprofiling in the Cologne laboratory.

Tab. 1: Retention times, retention indices and ion traces of the endogenous steroids which are monitored for steroidprofiling in the Cologne laboratory. Also added are the internal standards (all steroids as per-trimethylsilyl derivatives).

Substanz	Abbreviations	RT	Index	m/e
5ß-androstane-3α,17α-diol		8.64	2419	241
5ß-androstane-3ß,17α-diol		8.99	2437	241
5α-androstane-3α,17α-diol		9.12	2433	241
5ß-androstandion		9.19	2437	432
d_5-androsterone	d5-AND	10.28	2501	439
androsterone	AND	10.36	2506	434
d_4-etiocholanolone	d4-ETIO	10.46	2511	438
etiocholanolone	ETIO	10.54	2515	434
5α -androstane-3α,17ß-diol	5αA3αD	10.68	2522	241
5ß-androstane-3α,17ß-diol	5ßA3αD	10.82	2529	241
dehydroepiandrosterone	DHEA	11.66	2572	432
epiandrosterone	EPIAND	11.78	2578	434
5α-androstandion		12.11	2595	432
5-androsten-3ß,17ß-diol		12.13	2596	434
5α -androstane-3ß,17ß-diol	5αA3ßD	12.15	2597	421
d_3-epitestosterone	d3-EPIT	12.15	2597	435
epitestosterone	EPIT	12.19	2599	432
dihydrotestosterone	DHT	12.41	2610	434
4-androstendion		12.85	2633	430
d_3-testosterone	d3-TEST	12.99	2640	435
testosterone	TEST	13.03	2642	432
11ß-hydroxy-androsterone	11OHAN	13.39	2660	522
11ß-hydroxy-etiocholanolone	11OHET	13.65	2673	522
methyltestosterone	MTEST	14.82	2733	446
pregnanediol*		15.37	2761	117
pregnanetriol**		15.98	2792	255

* 5ß-pregnane-3α,20α-diol

** 5ß-pregnane-3α,17α,20α-triol

The descriptive statistics and the calculated population based reference ranges of the steroidprofile parameters are presented in table 2-5. All values and details of the statistical calculation (reference population, reference sample group etc.) are published in the thesis of S. RAUTH (6). The calculation of the population based reference ranges were performed according to a concept and to recommendations of the International Federation of Clinical Chemistry (IFCC). All concentrations are corrected to a urine density of 1.020 g/cm^3.

Tab. 2: Descriptive statistics of the concentration of endogenous steroids and the concentration of the reference sample group men. (6)

concentrations	N	$\tilde{x}$ [ng/ml]	10.Perc. [ng/ml]	90.Perc. [ng/ml]	$\bar{x}$ [ng/ml]	s [ng/ml]	skewness	excess
AND	5101	2671	1322	4986	2975	1545.23	1.31	2.94
ETIO	5101	2032	1020	3797	2279	1209.92	1.58	4.88
EPIT	5095	27.7	9.2	70.6	35.7	30.03	2.77	15.49
TEST	5100	36.8	6.3	88.3	44.6	37.94	2.78	20.06
Adiol	5088	49.6	22.1	108.3	60.4	44.87	3.84	33.30
Bdiol	5088	128.1	38.0	342.8	166.5	141.79	2.28	9.26
Pregnd	5088	279.9	118.0	616.7	335.0	231.51	2.39	11.82
ratios	N	$\tilde{x}$	10.Perc.	90.Perc.	$\bar{x}$	s	skewness	excess
AND/ETIO	5283	1.32	0.77	2.22	1.43	0.60	1.21	2.78
TEST/EPIT	5277	1.35	0.25	3.29	1.65	1.41	3.54	41.49
AND/TEST	5282	72.5	32.7	400.5	191.0	620.82	23.46	904.87
AND/EPIT	5283	94.0	43.0	244.6	126.7	110.18	4.03	34.93
ETIO/TEST	5283	54.9	24.8	294.8	143.2	480.82	26.94	1120.79
ETIO/EPIT	5277	72.9	30.1	195.3	99.9	99.06	5.54	67.61
Adiol/Bdiol	5283	0.41	0.17	1.03	0.52	0.41	3.60	41.22

Tab. 3: Descriptive statistics of the concentrations of the endogenous steroids and the concentration ratios of the reference sample group women. (6)

concentrations	N	$\tilde{x}$ [ng/ml]	10.Perc. [ng/ml]	90.Perc. [ng/ml]	$\bar{x}$ [ng/ml]	s [ng/ml]	skewness	excess
AND	1696	1859	802	4199	2286	1629.40	2.22	7.99
ETIO	1696	1990	891	4050	2326	1548.73	3.15	27.53
EPIT	1693	6.0	2.1	22.1	10.0	12.72	4.97	41.95
TEST	1693	8.2	2.1	25.5	12.6	15.21	4.09	24.86
Adiol	1694	18.5	8.2	48.1	25.1	24.30	3.76	20.77
Bdiol	1694	64.1	18.1	190.3	92.5	92.88	2.70	10.90
Pregnd	1695	390.5	146.2	1320.6	642.5	884.49	4.98	36.29
ratios	N	$\tilde{x}$	10.Perc.	90.Perc.	$\bar{x}$	s	skewness	excess
AND/ETIO	1742	0.96	0.58	1.59	1.04	0.44	1.47	4.53
TEST/EPIT	1736	1.34	0.34	3.87	1.83	1.82	3.90	31.34
AND/TEST	1739	220.6	82.2	777.0	449.9	1384.28	21.15	621.99
AND/EPIT	1742	304.3	119.0	657.7	355.4	230.63	1.45	3.31
ETIO/TEST	1742	232.1	90.0	746.3	462.7	1181.14	12.35	207.04
ETIO/EPIT	1739	321.5	109.0	812.9	399.5	301.30	1.56	3.06
Adiol/Bdiol	1742	0.31	0.11	0.84	0.41	0.36	4.80	61.35

Tab. 4: Reference limits of the non-parametric 95% reference range and the respective 90% confidence intervals for the reference sample group of men. ($N_{(concentrations)}$=5101, $N_{(ratios)}$=5283 (6)

	Reference limits		90% confidence interval			
	2.5%	97.5%	lower limit		upper limit	
concentrations	[ng/ml]	[ng/ml]	[ng/ml]		[ng/ml]	
AND	867.2	6703	834.5	894.3	6533.	6881
ETIO	673.7	5294	654.3	697.1	5107	5497
EPIT	4.9	112	4.56	5.39	106.7	118.4
TEST	2.06	137.4	1.87	2.18	131.4	143.8
5αA3αD	13.85	166.5	13.15	14.72	161.4	173.3
5ßA3αD	19.63	550.1	18.44	21.29	531.3	572.8
Pregnd	73.2	951.2	70.6	77.2	910.7	984.1
	Reference limits		90% confidence interval			
ratios	2.5%	97.5%	lower limit		upper limit	
AND/ETIO	0.55	2.869	0.53	0.57	2.81	2.93
TEST/EPIT	0.08	5.19	0.07	0.08	5.01	5.4
AND/TEST	22.5	1164	21.7	23.2	1052	1269
AND/EPIT	27.7	406.3	26.6	28.7	386.8.	426.7
ETIO/TEST	16.7	819.2	16.3	17.2	768.6	905.9
ETIO/EPIT	20.5	345.8	19.92	21.42	326.8	362.3
Adiol/Bdiol	0.11	1.56	0.11	0.12	1.51	1.61

Tab. 5: Reference limits of the non-parametric 95% reference range and the respective 90% confidence intervals for the reference sample group of women. ($N_{(concentrations)}$=1694, $N_{(ratios)}$=1742) (6)

	Reference limits		90% confidence interval			
	2.5%	97.5%	lower limit		upper limit	
concentrations	[ng/ml]	[ng/ml]	[ng/ml]		[ng/ml]	
AND	404.1	6439	368.8	458.4	6135	6964
ETIO	473.2	6107	395.2	503.1	5698	6564
EPIT	1.11	42.2	1.04	1.21	37.2	47.9
TEST	0.65	57.3	0.55	0.83	48.1	62.1
5αA3αD	4.74	91	4.12	5.14	83.1	98.3
5ßA3αD	9.14	366.4	7.97	10.1	312.4	387.8
Pregnd	83.1	3089	76.9	89.3	2659	3382
	Reference limits		90% confidence interval			
ratios	2.5%	97.5%	lower limit		upper limit	
AND/ETIO	0.42	2.15	0.39	0.44	2.03	2.22
TEST/EPIT	0.11	6.31	0.1	0.13	5.91	6.7
AND/TEST	47.9	2184	40.0	52.1	1704	2591
AND/EPIT	65.3	939.4	60	74.3	893.9	986.8
ETIO/TEST	37.0	2397	31.8	42.0	1998	2905
ETIO/EPIT	60.5	1205	53.1	65.0	1152	1289
Adiol/Bdiol	0.07	1.24	0.07	0.08	1.16	1.28

Parameters for the detection of doping with testosterone

The application of exogenous testosterone leads to characteristic changes of the normal steroidprofile (1). The most obvious change is the **increase of the ratio TEST/EPIT**. A value of this parameter above the upper limit of the reference range, i.e. higher than 6, indicates a suspicion for testosterone doping. Beside the TEST/EPIT ratio, two other parameters are important for the detection of testosterone doping. These parameters are a **decreased ratio of AND/TEST** and an **increased concentration of TEST (cTEST).**

If all three parameters are outside of the limits of the reference values (see table 6) and other influences, e.g. bacterial activities, can be excluded, there is a high probability, that the unnormal steroidprofile is caused by a testosterone application. But because the T/E ratio is the most sensitive parameter for the detection of doping with testosterone, a suspicion is already given, if the T/E ratio is higher than the upper reference limit.

Tab. 6: Parameters of the steroidprofile, which make a urine suspicious for an application of exogenous TEST.

Parameter	**Men**	**Women**
TEST/EPIT	> 6	> 6
AND/TEST	< 20	< 40
c TEST [ng/ml] *	> 130	> 60

* concentration corrected to a urine density of 1.020

To study, if the high T/E ratio is natural, caused by a testosterone application or caused by other influences, e.g bacterial, pathological or analytical influences, we start with the following protocol.

Screening results -Search for other factors which may increase the ratio TEST/EPIT

If there was found a ratio TEST/EPIT > 6 we look first in our screening results for other factors, which may lead, beside the TEST application, to increased TEST/EPIT ratios or change the other testosterone related parameters AND/TEST and cTEST. In our screening method we have included some controls to recognize these factors:

Mistakes in the sample preparation and changes of the GC/MS conditions may change the TEST/EPIT ratio and the other testosterone related parameters. This is controled by the use of the internal standards d3-TEST, d3-EPIT and d4-ETIO. The d3-TEST and d3-EPIT are

added to each sample in a ratio of 6:1 (90 ng/ml / 15 ng/ml) so that the ratios TEST/EPIT can be corrected (7).

Bacterial activities in the urine can be recognized by the presence of 5α- and 5ß-androstandion (4), which are monitored in our screening procedure. Also high amounts of DHT are found. The AND/TEST ratios are often low. These changes of the steroidprofile are mostly connected with high pH values. Further studies of such samples have shown, that in all of these samples high amounts of the steroid glucuronides are hydrolysed, and that the urine contains different enzyme activities as 3-hydroxy-steroid-dehydrogenase activity or steroid-Δ-isomerase etc. which can transform unconjugated androsterone to 5α-androstandion or unconjugated 5-androstendiol to testosterone (5). In these cases, the steroidprofile parameters are worthless and we stop the follow up protocol. We inform the federation about the presence of bacterias, which are mostly caused by insuitable transport or storage conditions and recommend to repeat the dope controls and to improve the transport and storage conditions.

Bacterial activities in the phosphate buffer used for the hydrolysis can not be recognized from our screening chromatograms. We control such side activities in the hydrolysis step by spiking a buffer solution with 5 µg of 5-androstene-3ß,17ß-diol and DHEA (8). If TEST or 5-androstendion is found after incubation of 1 hour at 50° C, the sample preparation has to be repeated.
We prevent such side activities by heating the buffer for several minutes before use (8).

Incomplete hydrolysis leads to a low and therefore suspicious ratio AND/TEST because AND-glucuronide is hydrolysed slower than TEST-glucuronide (5). We control the hydrolysis with the internal standard [2,2,3,4,4-$^{2}H_5$]-androsterone-D-17-glucuronide, which is added in an amount of 500 ng/ml to each sample. In case of an incomplete hydrolysis, we repeat the sample preparation.

Incomplete derivatisation may lead to wrong steroidprofile values, e.g. to low AND/TEST values. The derivatisation is controled by the monitoring of the ion 272 (molecular ion -90) for AND and ETIO, mono-TMS. If these two substances are detected, the derivatisation is

incomplete and we add to the derivatised sample (ca. 80 µl) once more 20 µl of derivatisation reagent with a higher concentration of the catalast NH_4I (2%).

If we have excluded all above mentioned factors for the high TEST/EPIT ratio we start with the confirmation of the sample.

Confirmation of suspicous TEST/EPIT samples

Our procedure for the confirmation of samples with high TEST/EPIT ratios was exactly described by NOLTEERNSTING et al. (7).

The most important points are:

Reproducibility

To examine the reproducibility of the method, we prepare three aliquots of the sample which are injected twice. The variation coefficient of the TEST/EPIT ratios should be better than 5%. The mean of the TEST/EPIT values of the replicates minus tthree times the standard deviation should result in a value higher than 6.

Correction with deuterated standards

For the correction of the ratios TEST/EPIT the internal standards d3-TEST/d3-EPIT 6:1 are added to each sample.

Full scan spectrum of TEST and EPIT

Additional to the 3 aliquots we prepare one aliquot without internal standard to achieve full scan spectras of TEST and EPIT. This is done to show, that no other substance is coeluted with TEST or EPIT.

Increased urine volume

The used urine volume is increased to achieve a good GC signal especially for EPIT.

Separation of the free fraction

Before the hydrolysis, the free steroids are separated by extraction with ether. By this step, we prevent false positive results by unconjugated, bacterial produced, TEST (5).

Heating of the buffer

To avoid testosterone producing side activities from the phosphate buffer (8) during the hydrolysis, we boil the phosphate buffer for several minutes before the use.

Hydrolysis with ß-glucuronidase from E.coli

For the hydrolysis of the glucuronides we only use ß-glucuronidase from E. coli, because we have never observed testosterone producing side activities in this enzyme preparation. Such activities can be found in the ß-glucuronidase/ aylsulfatase of Helix Pomatia preparations (9).

n-pentane extraction

After hydrolysis the extraction of the steroids is performed with n-pentane instead of tert. butylmethyl ether. This is an additional clean up step to reduce the biological background for the detection of TEST and EPIT (10).

Changed MS parameters

For the MS detection is used a SIM method with only 5 ions. The molecular ions of the bis-TMS derivatives of TEST, EPIT, d3TEST and d3EPIT are registered with dwell times of 200 msec (7).

Measurement of LH, FSH and HCG

As proposed by KICMAN et al.(11) and COWAN et al.(12) we analyse also the gonadotropines LH, FSH and HCG. Of most importance is the ratio LH/TEST.

Measurement of ethanol

Several studies have shown that the application of ethanol may increase the TEST/EPIT ratio and decrease the AND/TEST ratio (13). Additionally we have found, that these changes of the steroidprofile are always connected with the presence of ethanol in urine (13). Therefore we analyse samples with elevated TEST/EPIT ratios also for ethanol by Headspace/GC.

If we find ethanol, we stop the follow up protocol and don´t give the sample positive. Until now we observed such cases only in Out of Competition Controls.

If the TEST/EPIT ratio > 6 is confirmed and the additional analyses are performed, we continue with the following steps.

Information of the Federation and request for further data

We inform the federation about the high TEST/EPIT ratio and ask for retrospective (code numbers of former dope controls) or prospective data (further dope controls) . For retrospective data, which are archived in other laboratories, we recommend the federation to provide the information listed in the formsheet for high TEST/EPIT values (Fig. 1).
For the decision making process, we need at least data from 4 samples of the same athlete.
The statistical evaluation of the datas is performed as presented in the following chapter

If the retrospective or prospective data are not sufficient for the decision (e.g. not enough data, strong variation of the data), an endocrinological study is performed.

Elevated TEST/EPIT value hg/doku/s41/inafte

Laboratory	
Sample Code	
Laboratory Code	
Event	
Date of dope control	
Declaration of medication	

Screening Results

pH	
specific gravity [g/cm^3]	
androsterone [ng/ml]	
etiocholanolone [ng/ml]	
testosterone [ng/ml]	
epitestosterone [ng/ml]	
5α-androstane-3α,17ß-diol [ng/ml]	
5ß-androstane-3α,17ß-diol [ng/ml]	
LH [miU/ml]	
ratio of testosterone / epitestosterone (corrected)	
ratio androsterone / testosterone	
ratio androsterone / etiocholanolone	
ratio testosterone / LH [nmol/IU]	

Screening method

fraction (free/conjugated/combined)	
enzyme preparation for hydrolysis	
correction of ratio testosterone/epitestosterone (deuterated standards, calibration curve)	

Confirmation results

ratio testosterone/epitestosterone	

Confirmation method

fraction (free/conjugated/combined)	
enzyme preparation for hydrolysis	
extraction (n-pentane/ether)	
correction of ratio testosterone/epitestosterone (deuterated standards, calibration curve)	
mass spectrum of testosterone, epitestosterone	

Remarks

Fig. 1: Formsheet for collection of datas from other laboratories.

Endocrinological study (ES)

The aim of an ES is to calculate the subject based refernce range for the TEST/EPIT ratio and the normal variation of the other TEST related steroid ratios. The basis for the judgement of the results is our knowledge about the stability of steroidprofile parameters in rest and exercice (14, 15, 16). The decision, positive or negative for the suspicious sample, is based upon a comparison of the steroidprofile values of the suspicious sample and the values of the ES.

The ES takes 2 days .During this time the athlete lives near by the laboratory. He can keep normal nutrition and training. The application of ethanol is not permitted.

Procedure

- Information of athlete about the aim and the procedure of the ES.
- Answering of a questionnaire (see Fig. 2)
- Collection of all urine fractions in a time period of at least 48 hours (ca. 13 - 15 samples according to a collection schedule (see Fig. 3).
  At least 5 urine collections have to take place under observation of a member of the laboratory staff.
- Single blood sample collection (10 ml) performed by medical staff.

Analysis

Parameter	*Single parameter*	*Analysentechnik*
Steroidprofile parameters in urine (concentrations, excretion rates, ratios)	Glucuronides of androsterone, etiocholanolone, testosterone, epitestosterone, 5α-androstan-3α,17ß-diol, 5ß-androstan-3α,17ß-diol, dihydrotestosterone, tetrahydrocortisol, 11ß-hydroxyandrosterone, 11ß-hydroxyetiocholanolone, pregnandiol, pregnantriol	GC/MSD
Peptide hormones in urine	LH, FSH, HCG	Enzymimmunoassay
Blood parameters	Testosterone, 17α-hydroxyprogesterone (eventually esters of testosterone)	GC/HRMS

Evaluation

- Statistical evaluation of the steroidprofile parameters and of the peptide hormone values (mean, standard deviation, coefficient of variation) for the determination of the individual reference ranges.
- Comparison of the values of the ES with the values of the suspicious sample.
- Comparison of the values of the ES with population based reference ranges.

Endocrinological study Cologne,

Last name:	Date of birth:
First name:	Sex: male. O female O
Street:	Size [cm]:
City:	Weight: [kg]:
Country:	

Federation:	Professional: O Amateur: O*
Major Discipline:	Years of training:
Coach:	Training sessions per week:
Federation doctor:	Training hours per week:

Current training:			
Current health status:			
Current medication:			
Former dope controls: *Date*	*City/Event*	*Code-Nr.*	*Laboratory*
Remarks:			

Fig. 2: Questionnaire for the endocrinological study

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Urine Collection Schedule

Name:

Day/Date	Collection time	Actual collection time	Volume [ml]	Remarks
1. day	7.00			
	10.00			observation
	13.00			
	16.00			observation
	19.00			
	22.00			
2. day	7.00			
	10.00			observation blood sample collectionn
	13.00			
	16.00			observation
	19.00			
	22.00			
3. day	7.00			
	10.00			observation
	13.00			

Fig. 3: Urine collection schedule for the endocrinological study. Several collections take place under observation.

Data evaluation of endocrinological studies or longitudinal studies

In table 7 and 8 are presented the results of an endocrinological study of a male athlete with 12 samples. The coefficients of variation (cv in %) for the presented steroid ratios should be

within 10 % and 25 % (14,15,16). If we have higher variations, e.g. in longitudinal studies with only 3 or 4 samples, we cannot calculate the subject based refernce range for the TEST/EPIT ratio. For the ratio TEST/LH we observe always variations higher than 20%.

Tab. 7: Results of an ES. Concentrations of endogenous steroids.

#	time	AND [ng/ml]	ETIO [ng/ml]	EPIT [ng/ml]	TEST [ng/ml]
1	10:15	6133	7138	18,6	83,4
2	13:45	2334	2580	6,1	34,5
3	16:05	2917	3252	5,9	35,2
4	19:00	3691	3930	9,6	51,2
5	00:05	2905	3657	9,0	40,1
6	10:45	2286	3103	7,0	45,9
7	12:15	3130	3926	9,0	67,2
8	16:25	4304	4582	13,5	96,3
9	19:15	2164	2389	5,5	36,8
10	22:00	2412	3201	7,3	46,4
11	07:35	2047	2437	5,2	36,1
12	10:45	3400	4525	10,0	75,2

Tab. 8: Results of an ES. Ratios of endogenous steroids and LH values. The ratios TEST/EPIT are corrected with the internal standards d3-TEST/d3-EPIT 6:1 (7). Presented are also the mean, standard deviation (stdev) and coefficient of variation in percent (cv %).

#	time	TEST/ EPIT corr.	AND/ TEST conc	AND/ EPIT conc	AND/ ETIO conc	LH [mIU/ml	TEST/LH [nmol/IU]
1	10:15	4,6	73,5	330,1	0,9	9,4	30,9
2	13:45	4,7	67,7	382,6	0,9	2,1	57,6
3	16:05	5,6	82,9	494,4	0,9	3,1	39,4
4	19:00	4,5	72,1	386,5	0,9	1,9	91,6
5	00:05	4,7	72,4	322,7	0,8	2,7	51,6
6	10:45	5,9	49,8	326,1	0,7	6,9	23,2
7	12:15	6,9	46,6	347,7	0,8	8,0	29,1
8	16:25	6,8	44,7	318,8	0,9	7,8	42,7
9	19:15	5,8	58,8	392,1	0,9	4,6	27,9
10	22:00	5,7	52,0	330,4	0,8	6,7	24,0
11	07:35	6,1	56,7	392,8	0,8	5,8	21,4
12	10:45	6,9	45,2	340,7	0,8	6,4	40,8
mean		**5,7**	**60,2**	**363,7**	**0,8**	**5,5**	**40,0**
stdev		**0,9**	**13,1**	**50,2**	**0,1**	**2,5**	**19,9**
cv %		**15,9**	**21,7**	**13,8**	**8,9**	**46,2**	**49,7**

From mean and standard deviation (stdev) of the corrected TEST/EPIT ratios we calculate the limits of the subjected based refernce range according to the formula (17):

- upper limit of subject based refernce range = mean + 3 x stdev
- lower limit of subject based refernce range = mean - 3 x stdev

In figure 4 are presented the single TEST/EPIT values of an ES and the corresponding reference limits

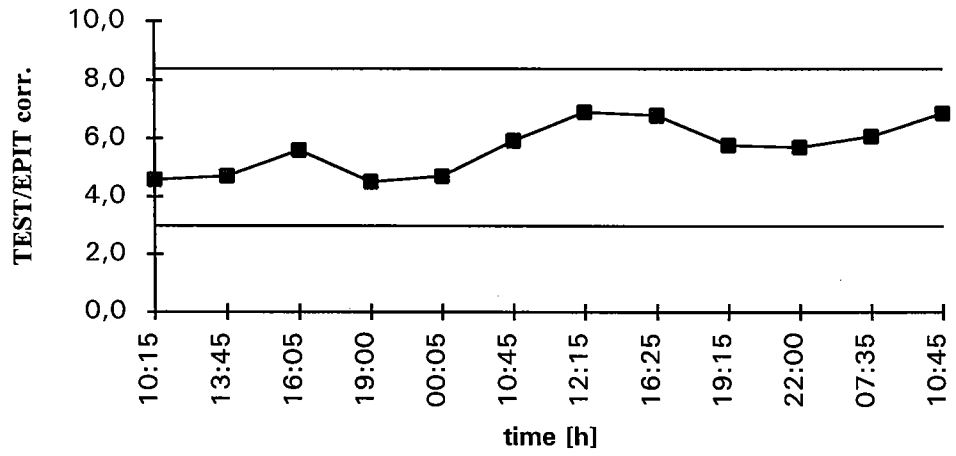

Fig. 4: TEST/EPIT values of an ES and corresponding limits of the subject based reference range (see table 8).

Blood samples in the ES

During the ES we take 10 ml of blood. We take this sample to receive additional TEST related information, e.g. about the ratio TEST/17α - hydroxyprogesteron. The analysis is performed according to the GC/HRMS method described by HORNING et al. (18).
With this blood sample we have also the possibility, to prove an application of TEST during the ES by the detection of TEST-esters in the plasma.

Ketococonazole Test during the ES

A Ketoconazole test is an effective method for the differentiation between a natural elevated TEST/EPIT and a TEST abuse by an athlete (19). Such a test may be useful to detect a TEST application during the ES. But because of possible health risks by side effects of this drug, we hesitate to perform this test.

Interpretation of the results of an ES or a longitudinal study and recommendation to the federation

If the TEST/EPIT values and the other TEST related ratios of the suspicious samples lie within the subject based reference range and the variations of the ES values, we recommend to give the sample negative. In this case, we have a natural elevated TEST/EPIT ratio. The comparison of the ES values with the population based reference ranges allows to recognize the reason for the increased TEST/EPIT ratio. Most often a low EPIT concentration is responsible for the natural elevated TEST/EPIT ratio. Such a case is presented in tables 7 and 8. A low EPIT case is characterised by the following parameters:

- Concentration of EPIT near or outside the lower limit of the refernce range
- Ratio AND/EPIT near or outside the upper limit of the refernce range
- Ratio AND/TEST near the mean and median of this parameter
- Concentration of TEST near the mean and median of this parameter.

If we have a low EPIT case, we recommend to the federation to give a certification (naturally elevated T/E ratio) to the athlete. The certification should have the following consequences: In case a laboratory shows a "positive " result for TEST related parameters, the Antidoping Commission of the federation should be informed at once and before disciplinary measures be taken against the athlete. The decision positive or negative should be made only after the comparison of the suspicious values with the archived values of the athlete.

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