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Cologne protocol efficacy: two unusual case studies

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Abstract

The efficacy of the Cologne Protocol as a diagnostic tool in the absence of IRMS is demonstrated from two unusual case studies. The data obtained from the Protocol study, when compared with the previously obtained longitudinal data, provided revelatory and essential information about the two athletes. In one case the existence of an inherent abnormal physiological pattern in the athlete, potentially due the consumption of alcohol, was uncovered. In the other case the likelihood that the athlete was using a doping agent during the Protocol itself was revealed. In both cases IRMS analysis does not indicate exogenous administration of steroids.

Introduction

A follow-up performed under partial controlled conditions (i.e., the "Cologne Protocol") is an important tool in assessing the abnormality of endogenous steroid profiles.[1] Even in those cases where IRMS analysis is available, this type of study can be very useful for a better understanding of the sources of unusual variability that affects the profile. During this Protocol every urine produced by an athlete during a period of three consecutive days is collected. In this way it is possible to have an in-depth picture of how the concentrations and the ratios of the most important endogenous steroids vary over time, and to establish a controlled steroid profile of the athlete. In addition, two blood samples are collected: one when the first collection of urine takes place, and the other with the last urine collection. The decision to perform such studies is made in agreement with the NADO.

Experimental

In both studies samples were prepared according to the confirmation procedure for T/E used in the laboratory. The quantification of T (Testosterone), E (Epitestosterone) and T/E is made by isotopic dilution, with the addition to all samples of a deuterated internal standard (ISTD) mixture. For the quantification of other steroids methyltestosterone is used as ISTD. The free

fraction is extracted with TBME at pH 7 to analyze for the possible deconjugation of T and E. Hydrolysis is done with β-glucuronidase. The conjugated fraction is extracted with n-pentane.
GC-MS analysis: Agilent 6890/5973 GC-MSD; Column: Agilent HP-ULTRA1, length 25 m, i.d. 0.2 mm, film thickness 0.11 µm; Carrier gas: Helium, flow 0.8 mL/min; Injector: 250 ºC, split 1:10, injection volume 2 µL; Temp. prog.: 196 ºC (0 min), 2 ºC/min to 237 ºC (0 min), 30 ºC/min to 300 ºC (3 min); Scan mode: SIM (m/z 272, 275, 290, 327, 417, 430, 431, 432, 433, 434, 435, 446).
LH and β-hCG analysis: Immulite 2000 DPC.

Results

***1st Study*:** During a follow-up study (FUS) performed by the Portuguese NADO (CNAD) the observed variability for the T/E ratio (37%) was slightly higher than acceptable for physiological excretion. This variability was caused by the suppression of the steroid excretion in the urine sample that initiated the study from an athlete already showing a low E excretion (Figure 1). In this particular case the ethanol was also measured, showing a value of 1.2 g/L. A Cologne Protocol was then suggested, and performed (Figure 2). All the values presented here were corrected for specific gravity.

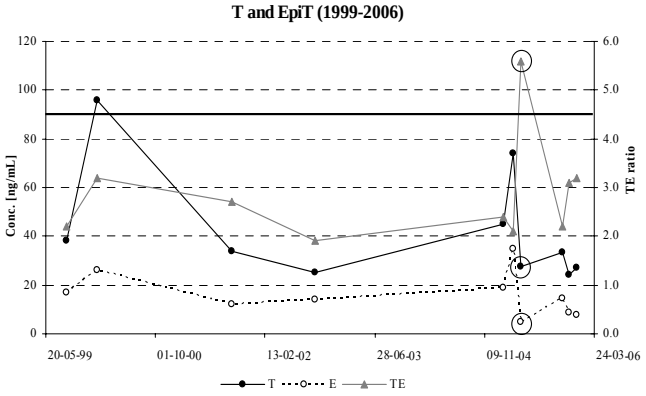

Figure 1: T/E ratio, T and E during the 6 years of the FUS

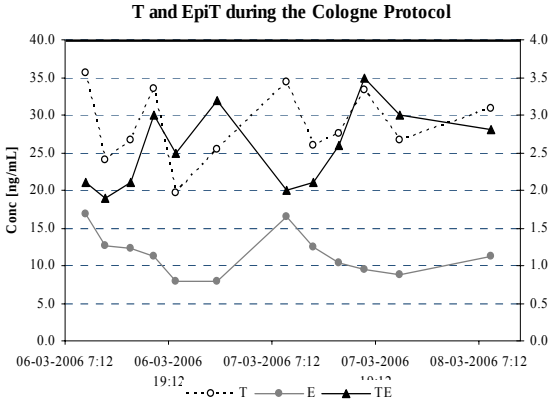

Figure 2: T/E ratio, T and E during the Cologne Protocol

***2nd Study*:** The athlete showed high variability of the endogenous steroids over the years, and a marked suppression of androsterone (A) and etiocholanolone (Et), probably due to the administration of glucocorticoids (Table 1). In some samples betamethasone was detected, and through the years the athlete was covered by TUEs. Some of the samples were sent (to the WADA accredited lab of Cologne) for IRMS analysis, but all were negative.
In 2008 a Cologne Protocol was performed on this athlete, and the values were compared with those obtained during the 5 previous years (Table 1).

Collection Date	SG *	Concentrations * [ng/mL] A	Et	T	E	Metabolic Ratios T/E	A/T	A/Et
13-03-2004	1.027	455	556	63.0	21.3	3.0	7.2	0.82
06-02-2005	1.026	1259	977	36.2	11.5	3.1	34.8	1.29
20-03-2005	**1.027**	**1289**	**761**	**76.4**	**17.3**	**4.3**	**16.9**	**1.69**
24-03-2005	**1.029**	**2374**	**1514**	**126.8**	**25.5**	**4.9**	**18.7**	**1.57**
26-03-2005	1.024	1338	750	57.8	15.1	3.7	23.2	1.78
01-07-2005	**1.032**	**611**	**654**	**96.2**	**17.1**	**5.3**	**6.3**	**0.93**
06-08-2005	**1.024**	**1110**	**1266**	**88.9**	**18.4**	**4.6**	**12.5**	**0.88**
11-08-2005	**1.031**	**923**	**1295**	**84.3**	**18.3**	**4.2**	**11.0**	**0.71**
12-08-2005	**1.027**	**776**	**944**	**89.9**	**20.8**	**4.0**	**8.6**	**0.82**
01-01-2006	1.030	869	603	33.7	10.3	3.3	25.8	1.44
28-06-2006	1.025	699	639	47.7	19.3	2.4	14.7	1.1
02-07-2007	1.028	911	634	47.1	17.1	3.3	19.3	1.4
13-07-2007	1.013	577	729	38.5	15.4	2.8	15.0	0.8
07-08-2007	**1.026**	**812**	**782**	**60.1**	**16.5**	**4.0**	**13.5**	**1.0**
09-08-2007	**1.027**	**811**	**887**	**82.9**	**20.9**	**4.2**	**9.8**	**0.9**
11-08-2007	1.023	974	1006	80.2	27.0	3.2	12.1	1.0
12-08-2007	1.027	865	960	86.7	28.3	3.3	10.0	0.9
13-08-2007	1.025	785	805	62.1	21.0	3.1	12.6	1.0
06-07-2008	**1.027**	**542**	**464**	**40.7**	**11.4**	**4.6**	**13.3**	**1.2**
29-01-2009	1.019	2105	1568	71.7	26.6	2.7	29.3	1.4
Mean		1004	890	68.5	19.0	3.7	15.7	1.1
s.d.		488	310	24.2	5.1	0.8	7.5	0.3
CV%		**48.6**	**34.8**	**35.3**	27.1	21.6	**47.8**	28.5
Mean + 3SD		**2469**	**1820**	**141.1**	**34.4**	**6.1**	**38.3**	**2.1**

Table 1: Data collected in Lisbon 2004 through 2009.

	Concentrations [ng/mL] LH	A	Et	T	E	Metabolic Ratios T/E	A/T
Mean	45	1248	1233	153.9	34.3	4.6	8.1
s.d.	28	293	386	19.0	5.5	0.8	1.6
CV%	**63.0**	23.5	**31.3**	12.4	15.9	17.7	20.3

Table 2: Statistics related to the Protocol data

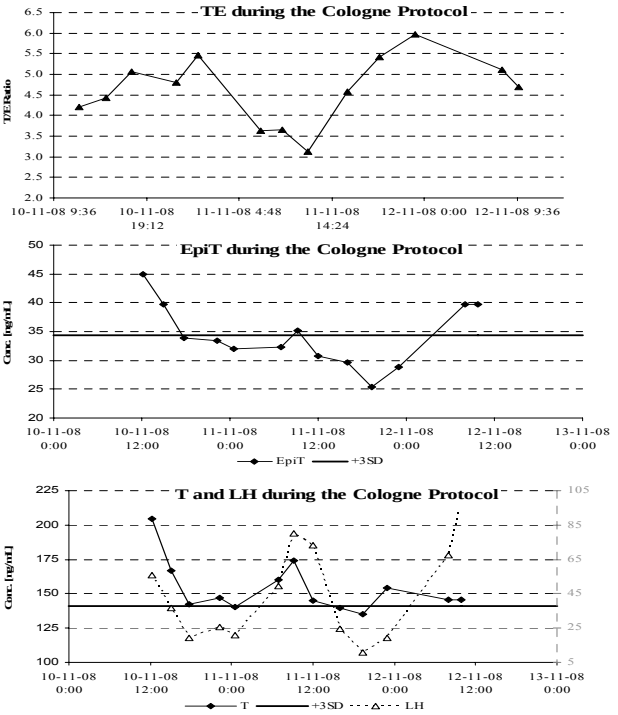

Figure 3: TE ratio, E, T and LH (Luteinizing Hormone) during the Cologne Protocol. LH excretion showed a diurnal cycle variation The lines represent the mean of the values presented in Table 1 plus three standard deviations.

hCG (Chorionic Gonadotrophin) was also measured and was normal in all the samples.

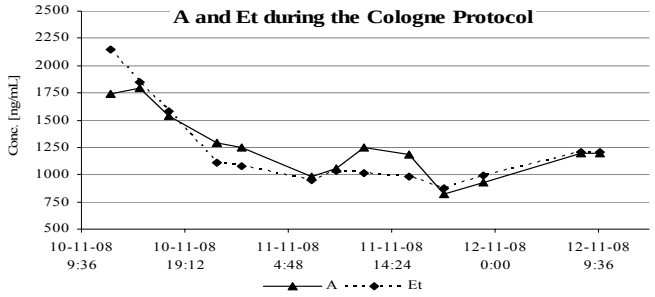

Figure 4: A and Et during the Cologne Protocol. A slight decline of the concentrations over time is observed.

	10-11-2008	12-11-2008	Reference Values
FSH	2.8	2.6	1.4 - 18.1
LH	5.6	4.5	1.5 - 9.3
17OH-Progesterone	**2.8**	**2.4**	0.50 - 2.40
DHEA	**12.5**	9.7	1.3 - 12.5
Total Testosterone	8.99	8.12	1.5 - 14.0
ACTH	**73.0**	8.9	<46
Cortisol	20.8	14.6	5.0 - 25.0

Table 3: Blood values at the time of the first and last urines of the Cologne Protocol.

Discussion and Conclusion

1st Study: Two factors have to be taken in consideration in this case. The sample that prompted the follow-up study was collected at an unusual time (2:30 am). The value of E in this sample was extremely low. During the Cologne protocol the athlete showed a marked diurnal variation in steroid concentrations and T/E ratio. The two urines collected during the night had low values. In addition the suspected sample showed a high value of ethanol and it is known that the intake of ethanol may increase the T/E ratio.[2] If the two factors coexisted, T

was increased by ethanol and E was suppressed, resulting in a T/E that was abnormally high. Based on these studies, the athlete was not sanctioned.

2nd Study: The values obtained from the longitudinal study showed high variability for almost all the parameters of the steroid profile (Table 1). The T/E values higher than 4 correspond in time to an international competition in Portugal. Although there is some evidence in the literature that effort and adrenal stress can modify steroid excretion, in this particular case it is highly likely that other factors may explain the observation made.

Samples collected during the Cologne protocol showed values clearly outside the ranges established for this athlete from the longitudinal study. A and Et were increased to values within the normal reference range (while lightly suppressed for the samples collected outside the controlled protocol) and T values were actually risen above the athlete's based range.

In this case blood values may provide key clues (Table 3). The only value outside the reference range was the initial ACTH (Adrenocorticotropic hormone) value (which then declined at the end of the study). This is in agreement with the decline of the urinary A and Et concentrations (Figure 4) and plasma cortisol values. The urinary T and E values followed a similar pattern except for the response to the LH pulse.[3]

Since the athlete previously showed suppression of A and Et (Table 1) due to the intake of glucocorticoids it is possible that ACTH could have been used to reestablish the adrenal function, explaining the higher values seen in the samples from the Cologne Protocol and 2009. Can ACTH also be responsible for the abnormally high values of T? Some studies have described the adrenal origin of endogenous T and E even though not conclusive.[4] It would of interest to explore in depth this area in the future.

References

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