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T/E Values: Measurements and Observations
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T/E values : measurements and observations

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This paper deals with the measurement of T/E values as it is now performed in IOC accredited laboratories. Using the laboratory reports of 1995 reaccreditation proficiency tests, we present a survey of the different protocols used and a comparison of the results in terms of accuracy and reproducibility. Some problems observed are presented as well as recommendations. On the other hand, since longitudinal studies built from results collected from several laboratories were available, we present some examples of intra- and inter-laboratory results comparison.

When dealing with the administration of natural steroids, such as testosterone and DHT, the laboratory must produce quantitative measurements. The cut-of-levels are determined from statistical evaluation of results collected from a population of individuals : Donike et al. defined several population-based reference ranges and other groups verified these within their own population of athletes[1]. These longitudinal studies may be complex and composed of several values ranging from area ratios to precise concentrations. As a minimal requirement, the comparison of previous and subsequent T/E values is a mandatory step in the determination of a positive finding (IOC Medical Code) and it was proven useful in several occasions[1b]. Investigating the nature of a positive finding requires the comparison of ratios obtained from stringent confirmation protocols and routine day-to-day operations. Baenzinger et al.[2] have shown that the inter-individual variation of the T/E values is a direct result of the accuracy of the laboratory determinations. Finally, since the athletes performing in Olympic sports often compete internationally and train in different countries, the comparison of results gathered from different laboratories using different protocols must be possible.

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[1] Donike et al., in *Proceedings of the 10th Workshop on Dope Analysis, (1992), p. 47,* ibidem, p.69 , b) C. Ayotte, A. Charlebois and D. Goudreault, *J. Chromatogr Biomed. Appl., 687 (1996) p. 3.*

[2] J. Baenziger and L. Bowers, *in Proceedings of the 11th Workshop on Dope Analysis, (1992) p. 41*

Screening and confirmation protocols

A review of the different protocols of the routine T/E determination showed that two are most frequent. In the first one, values are determined by comparing with one standard T/E value of 6 (more frequent) or 10. For this purpose, several laboratories use the $D_3T/D_3E = 6$ prepared and distributed by the Köln laboratory[3], others make use of their own undeuterated standards. The second approach is based on the establishment of calibration curves made of several different T/E values and in some protocols, a quality control sample of a known value is analysed within each batch of samples. When confirming a positive sample, comparison is again made with either one T/E standard value of 6 or 10 (deuterated or not) or with a calibration curve composed of different T/E standards, or deuterated standards. Some protocols require the analysis of a T/E standard in the same concentrations than the suspicious sample and of quality control standards. The results reported by the 23 laboratories for the screening analysis of 10 different samples and for the confirmation a sample[4] of a mean 12.5 value are presented in table 1 to 3.

Table 1 : results obtained from screening T/E determination in different samples

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9
mean	1.8	0.6	0.1	1.1	3.2	2.6	0.8	0.9	0.9
std deviation	0.7	0.1	0.1	0.4	0.6	0.5	0.2	0.1	0.1
c.v. (%)	37.9	15.7	41.0	36.3	20.0	19.2	20.0	11.6	15.7
95% upper	3.0	0.7	0.2	1.8	4.0	3.4	1.0	1.0	1.0
5% lower	1.0	0.5	0.1	0.7	2.4	1.9	0.6	0.8	0.7

[3] W. Schanzer and M. Donike, *Proceedings of the 12th Cologne Workshop on Dope Analysis, Köln (1995) p. 93* ; E. Nolteernsting, H. Geyer, U. Mareck-Engelke, W. Shcnzer and M. Donike, idem, p. 113, E. Nolteernsting et al., *Proceedings on the 13th Workshop (1996) p. 191*
[4] This sample showed signs of bacterial degradation : some testosterone was found in the free form.

Table 2 : results obtained from the confirmation analysis of reaccreditation elevated T/E sample

Lab.	Spec. Gr.	pH	T/E	T/E raw	T/E std	D_3T/D_3E	Response factor deuterated stds	Response factor stds	Curve slope
1	1.030	6.0	12.3	-		6	1.23		
2	1.016	5.6	12.0	10.6	6			1.02	
3	1.016	6.0	9.2	8.8		6	1.08		
4	1.013	5.4	12.4	14.5		6	1.16		
5	1.017	5.5	13.4	13.7					
6	1.015	6.0	10.5	12.6		6	1.2		
7	1.017	6.5	12.1	13.5					1.12
8	1.019	5.7	12.2	-	6			0.79	
9	1.025	5.5	*19.1	-	6			1.2	
10	1.018	5.6	16.0	-					
11	1.020	5.5	14.7	15.6	6			1.15	
12	1.017	5.5	11.8	-	?10				
13	1.019	6.0	11.7	15	6 and 10			1.2	
14	1.019	5.5	12.7	-					1.09
15	1.018	5.5	15.5	15.1					0.97
16	1.014	5.5	*8.0	*17.8					[5]2.33
17	1.020	6.0	11.8	12.7		6	1.12		1.00
18	1.018	6.0	12.3	14.7		6	1.23		
19	1.019	6.1	13.0	-	?10				
20	1.020	6.5	13.0	14.9		6	1.37	1.15	1.15
21	1.020	6.0	13.4	14.8		6	1.15		
22	1.017	6.0	10.8	-					
23	1.020	5.3	*8.6	*6.6	ISTD-$_d$				
	mean (all)		**12.5**	**13.4**			**1.19**	**1.08**	**1.07**
	std. deviation		**2.4**	**2.7**			**0.08**	**0.15**	**0.07**
	c.v. (%)		**18.9%**	**20.5%**			**7%**	**14%**	**7%**

***** exclusion of these values shifted the mean to 12.54 (σ = 1.6, c.v. 12%)

[5] This value is excluded from the calculation of the mean

Table 3 : Comparison of protocols of T/E confirmation

T/E values			
	Method used		
	Calibration curves	**Deuterated standards**	**Standards**
	12.1	12.3	12.0
	12.7	9.2	12.2
	15.5	12.4	19.1
	11.8	10.5	14.7
	13.0	11.8	11.8
	8.0	12.3	11.7
		13.0	13.0
		13.4	
		8.6	
mean	**12.2**	**11.5**	**13.5**
std deviation	**1.3**	**1.3**	**2.5**
c.v. (%)	**10.8**	**11.2**	**18.4**

Table 4 : determination of testosterone and epitestosterone concentration in the elevated T/E sample

	testosterone glucuronide	**testosterone free and glucuronide**	**epitestosterone glucuronide**	**epitestosterone free and glucuronide**
n	6	11	6	10
mean	163.8	219.0	11.2	16.9
std deviation	38.7	42.4	3.3	4.5
c.v.(%)	24%	19%	29%	27%

As shown in tables 1 and 2, inter-laboratory comparison of screening or confirmed T/E values is generally acceptable : the c.v. of 11 different T/E determinations varies from 11% to 41%, 8 c.v. being equal or lower than 20%. The highest c.v. is not surprisingly obtained from a T/E value of 0.1, although all values are similar e.g. 0.1 or 0.2, considering the precision of the determination.

Comparing the precision and inter-laboratory reproducibility of the confirmation protocols presented in tables 2 and 3, we can observe the following trends. The protocols in which only one or two T/E standards are used are the ones that produce the more elevated inter-laboratory variation of T/E values : 18.4% and the more elevated inter-laboratory variation of the response factor : 14%. When the laboratories are sharing a common deuterated standard, a narrow 7%

inter-laboratory variation of the response factor is observed. Inter-laboratory measurements are in closer agreement e.g. 11.2%. However, the response factor of the D_3T/D_3E is slightly elevated, 1.19 (mean). Due to its isotopic purity, there may be a contribution of residual T-d_0. We have observed the underestimation of a 6.44 theoretical T/E value corrected using the deuterated standard (-13,7%) instead of the calibration curve (-1.0%). The contribution to the sample m/z 432.4 ion was also noticed from the comparison of the direct ratios of area : 6.43 (-0.16%) without the deuterated standards and 6.59 (+2.33%) when added. The measured direct D_3T/D_3E 6/1 area ratio is 7.11 and this explains the response factor. Finally, the analysis done with a several points calibration curve afforded also a close agreement with inter-laboratory T/E variation of 10.8% and response factor variation of 7%. A slightly lower mean response factor of 1.07 is also obtained.

Problems observed

Considering now the « outsider » results of 8.0, 8.6 and 19.1 reported by some laboratories, we attribute the major problems to one of the following : wrong baseline integration, poor chromatography or sensitivity. Frequent problems may also occur when comparing inter-laboratory T/E results depending on the fraction analysed either glucuronide or free and glucuronide. It is known that one effect of the microbial degradation of a specimen results in the deconjugation of the glucuronides[6]. Only few laboratories reported the unusual presence of testosterone in the free fraction and one described the sample as degraded.

Longitudinal studies

Some laboratories do not produce routinely accurate measurements and this adds to the problems depicted above. However, in most occasions, the evaluation of previous and subsequent test results collected from the same laboratory or from different laboratories permitted to draw conclusion in suspected analytical positive findings. A key-element in this process is the individual variation of the parameters of the steroid profile[7]. For example, it was shown that in

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[6] C. Ayotte et al, *Proceedings on the 14th Workshop,* in press and *J. Chromatogr. Biomed. Appl., 687 (1996) p.3,* H. Geyer et al., *Proceedings on the 14th Workshop,* in press

[7] U. Mareck-Engelke, H. Geyer and M. Donike, *Proceedings of the 10th Cologne Workshop on Dope Analysis, (1992), p. 87,* M. donike, S. Rauth, U. Mareck-Engelke, H. Geyer and R. Nitschke, *Proceedings of the 11th Cologne Workshop on Dope Analysis, (1993), p.33 ;* U. Mareck-Engelke, H. Geyer and M. Donike, *Proceedings of the 11th*

males, the T/E values generally vary within 30% from the mean. In females, due to the very low concentration of testosterone and epitestosterone often in levels near the lower level of quantification of the method, a higher variation may be expected[8]. It was proposed that the comparison of the suspected positive value be made with the rest of the negative values collected and that in males all values must lie within 3σ from the mean. In females, the same intra-individual variation was observed.

We verified the usefulness of the longitudinal studies in several cases of male and female athletes. When the laboratories are using similar protocols, the comparison of results permitted to draw a conclusion. Intra-laboratory and inter-laboratory values collected during many years from a given athlete show agreement within the described limit (2σ, 30%) and «outsider» values could be assessed. In few occasions, some results had to be excluded due to the uncertainty of the determination, principally concerning the potential degradation of the sample leading to the presence of testosterone in the free form when only glucuronides are analysed.

The concentration of testosterone and epitestosterone in the specimens may also give complementary information. We have described statistically the relationship between the concentration and the specific gravity within an athletic population[1]. We also observed that there is, for a given individual samples, a linear relationship between the concentration of testosterone and the specific gravity and this could be verified in several cases. As described in table 4, the inter-laboratory quantification of testosterone and epitestosterone in the elevated T/E accreditation sample was generally good.

Conclusion

The inter-laboratory comparison of T/E values is generally possible when a common sample is shared. When results are obtained from routine procedures, it may be more difficult. Some laboratories are not routinely measuring concentrations and some approximate T/E values. Since

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Cologne Workshop on Dope Analysis, (1993), p.85 ; U. Mareck-Engelke, H. Geyer and M. Donike, *Proceedings of the 12th Cologne Workshop on Dope Analysis, (1994), p.121 ;* ibidem, p. 135 ; M. Donike, U. Marck-Engelke and S. Rauth, idem, p. 157 ; U. Mareck-Engelke, U. Flenker and M. Donike, *Proceedings of the 12th Cologne Workshop on Dope Analysis, (1994), p.177*

[8] ibidem

some laboratories are analysing the glucuronide fraction only and some the combined free and glucuronide, there may be a underestimation of the testosterone concentration and T/E, since the microbial degradation may not cause other perturbation than deconjugation.

From the evaluation of the reaccreditation results, we can conclude the following : sharing common standards improve the inter-laboratory variation. Isotopically pure testosterone and epitestosterone deuterated standards could be synthesised and distributed. This paper only dealt the T/E value and testosterone quantification. However, we know that more precise measurements are needed and of other analytes such as LH, androsterone, etiocholanolone to name few. For example, to prove the administration of DHT[9], the concentration of androsterone, etiocholanolone, DHT and 5α- and 5β-androstandiol must also be obtained. The laboratories should determine precisely these concentrations.

[9] H. Geyer et al., *Proceedings of the 13th Cologne Workshop on Dope Analysis, (1996), p. 95 ;* W. Schanzer, S. Horning and M. Donike, *Proceedings of the 13th Cologne Workshop on Dope Analysis, (1996), p. 201 ;* H. Geyer, W. Schanzer, U. Schindler and M. Donike, idem p. 215 ; M. Ueki, M. Fujisaki, A. Idedita, T. Hiruma and M. Okano, idem p. 231