Reprint from

RECENT ADVANCES IN DOPING ANALYSIS (9)

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Sport und Buch Strauß, Köln, 2001

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In: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (eds.) Recent advances in doping analysis (9). Sport und Buch Strauß, Köln, (2001) 133-143

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GC/C/IRMS and GC/MS in "Natural" Steroids Testing

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Introduction

Testing for the administration of natural steroids is a complex task requiring the identification and quantification of a number of parameters of the steroid profiles, one of which being the T/E[1] value. Complementary diagnostic information is gained from the determination of the urinary concentration of testosterone, epitestosterone, androsterone and etiocholanolone. By 1997, three different groups had proposed the GC/C/IRMS as a promising tool for the detection of the administration of testosterone (Aguilera et al., 1996; Becchi et al., 1994; Horning et al., 1997; Shackleton et al., 1997) and since then, others have described the values of the $^{13}C/^{12}C$ ($\delta\ ^{13}C\ ^{0}/_{00}$) of urinary androgens within the athletic populations and following the administration of DHT, epitestosterone or testosterone precursors such as DHEA.

In this paper, we present the results afforded by the analysis of urine samples collected following the administration of testosterone, androstenedione and DHEA as well as examples of the combined GC/MS and GC/C/IRMS individual "profiles" which have been proven to be useful in the investigation of putative doping cases, to discriminate inter alias, between the administration of a source of testosterone and 19-nortestosterone from a natural and systematic excretion of elevate T/E values or an increased endogenous norandrosterone production.

Experimental

"Androstenedione complex", DHEA and Nordione were purchased with an authorization from Health Canada (8572.090.98) from Price's Power International (International Nutrition and

[1] The T/E value is the corrected area ratio of both peaks.

Export, Newport News, Virginia 23608, USA). One capsule was administered to volunteers after verification of its content by GC/MS analysis. Urine samples were collected 24 hours before and up to one week after the administration and were prepared and analysed according to anabolic agents' procedure for the GC/MS steroid profiling. The preparation of the specimens for the GC/C/IRMS was carried out according to a modification of the method proposed by Shackleton (1997) for the analysis of testosterone and precursors. The underivatized extracts of hydrolysed steroids (Ayotte et al., 1996) were analysed as such when the concentration of norandrosterone was greater than 30 ng/mL or following an HPLC fractionation for lower levels. The identity of the peaks was verified by GC/MS. Authentic standards were purchased from Steraloids Inc. (Wilton, NH 03086).

Results and discussion

Testosterone, androstenedione and DHEA:

The administration of testosterone and precursors, androstenedione and DHEA can be detected by the GC/MS determination of parameters of the urinary steroid profile that are abnormal when compared to the ranges of values normally found in human (Donike et al., 1983, 1993). The oral intake of androstenedione and DHEA was shown to transiently increase the excreted T/E value in females and, although not systematically, in some males (Bowers, 1999; Bosy et al., 1998; Van Eenoo et al., 1998; Garle and Palonek, 1998, Uralets and Gillette, 1999; Lévesque and Ayotte, 1999; Lévesque et al., 1999). Other alterations of the urinary steroid profile, such as an abnormally high concentration of androsterone and etiocholanolone, the presence of the characteristic hydroxylated metabolites glucuro- and sulfoconjugated, 6α-hydroxyandrostenedione, 6β-hydroxyepiandrosterone for androstenedione and for DHEA, the increased excretion of 7β-hydroxydehydroepiandrosterone with suppression of 16α-hydroxyandrosterone, served as basis for reporting positive findings (Lévesque and Ayotte, 1999; Lévesque et al., 1999). The disruption of the normal urinary profiles of androgens metabolites can be demonstrated by comparison with the described population reference ranges that are summarised in table 1 (Ayotte, 1997 and reference cited therein; Lévesque and Ayotte, 1999; Catlin et al., 1997; Geyer et al., 1997; Baenzinger and Bowers, 1994). It also requires the

investigation of the athlete's previous or subsequent tests results in order to exclude the few individuals who naturally produce urine samples in which elevated T/E values are systematically measured. A systematic excretion of elevate T/E values reflects a normal condition, while a sudden increase deviating from the athlete's norm, is consequent with the administration of a source of testosterone, including the precursors (Ayotte, 1997; Geyer et al., 1997). The evaluation of some cases that we have made in the past years, based upon the variation of the individual's T/E values, are illustrated in Table 2.

Table 1: Description of the steroid profiles in the athletes' reference populations

Parameter	Description (97.5%)		Reference
	Male athletes	Female athletes	
T/E values	4.2 (n = 11000)	3.2 (n = 4667)	Lévesque and Ayotte, 1999
	5.2 (n = 5000)	6.3 (n = 1700)	Geyer et al., 1997
	6.5-7.0 (99%; n = 22806)		Baenzinger and Bowers, 1994
	5.6 (99%; n = 3700)		Catlin et al., 1997
T (ng/mL)	106 ng/mL (n = 9500)	26.4 ng/mL (n = 3740)	Lévesque and Ayotte, 1999
	137 ng/mL (n = 5000)	57 ng/mL (n = 1700)	Geyer et al., 1997
A (ng/mL)	6703 ng/mL (n = 9500)	5170 ng/mL (n = 4200)	Lévesque and Ayotte, 1999
	6689 ng/mL (n = 5000)	6439 ng/mL (n = 1700)	Geyer et al., 1997
Etio (ng/mL)	5294 ng/mL (n = 9500)	4938 ng/mL (n = 4200)	Lévesque and Ayotte, 1999
	4716 ng/mL (n = 5000)	6107 ng/mL (n = 1700)	Geyer et al., 1997

Table 2: Evaluation of elevate T/E values in athletes' samples

	T/E value			Remarks
Normally elevated cases				
	Mean	Variation	Range	
Male 1	4,8	23%	2,9 to 6,9 (n = 20)	
Male 2	1,3	13%	1,0 to 1,5 (n = 11)	
Male 3	5,8	25%	3,1 to 8,7 (n = 14)	
Female 1	0,8	38%	0,3 to 1,2 (n = 16)	
Female 2	1,1	43%	0,5 to 2,1 (n = 13)	
Female 3	6,1	39%	3,0 to 9.2 (n = 11)	
Positive cases				
Male 4	3,9	68%	2,3 to 10,6 (n = 8)	Positive test result: 10,6
	2,9	24%	2,2 to 3,2 (n = 7)	Excluding positive test result
Male 5	1,8	70%	0,9 to 7,3 (n = 21)	Positive test result: 7,3
	1,5	22%	0,9 to 2,2 (n = 20)	Excluding positive test result
Female 4	2,0	132%	0,6 to 11,6 (n = 16)	Positive test result: 11,6
	1,2	38%	0,6 to 1,9 (n = 14)	Excluding two elevate test results

Several groups have reported significantly changed carbon isotopic $^{13}C/^{12}C$ values (expressed as $\delta\ ^{13}C\ ^{0}/_{00}$) in the androgens metabolites excreted following the administration of testosterone (Aguilera et al., 1999, 2000, 2001), DHT, epitestosterone, DHEA (Shackleton et al., 1997; Ueki and Okano, 1999), and corticosteroids (Bourgogne et al., 2000). The methods described are based upon the comparison of the $\delta\ ^{13}C\ ^{0}/_{00}$ values of the urinary androgens metabolites to reference endogenous steroids, end products from earlier processes of the endogenous biosynthetic pathways. The urinary androgens metabolites most frequently analysed are androsterone, etiocholanolone, 5α- and 5β-androstane-3α,17β-diol, whereas the endogenous unaltered reference steroids are pregnanediol, pregnanetriol and cholesterol. The analysis of $\delta\ ^{13}C\ ^{0}/_{00}$ natural values amongst athletes did not indicate differences related to the nationality but to the diet (Aquilera et al., 1999, 2000, 2001; Shackleton et al., 1997, Ueki and Okano, 1999). To compensate for the different analytical methodologies and to allow for inter-laboratory comparison, it was proposed to use the ratio of the values of the androgens metabolites to those of the endogenous reference steroids in each sample (Shackleton et al., 1997).

19-nortestosterone and precursors, norandrostenedione and norandrostenediol:

The administration of 19-nortestosterone and of its precursors, 19-norandrostenedione and 19-norandrostenediol, results mainly in the excretion of 19-norandrosterone (NA) and 19-noretiocholanolone (NE), mostly found in the glucuroconjugated form. The period during which the metabolites can be detected, is drastically reduced when the oral preparations are taken and the relative amounts of NA and NE can vary based upon the individual and the product taken (Engel et al., 1958; Massé et al., 1985; Schänzer, 1996; Kintz et al., 1999). The low excretion of endogenous norandrosterone is normally not detected in human urine samples during routine doping control testing, with limits of detection of around 0.2 to 0.5 ng/mL, excepting specimens collected during pregnancy (Reznik et al., 1987). A more sensitive analytical technique such as the GC/HRMS, a larger volume of urine extracted and an extensive sample clean-up are needed to detect, identify and quantify endogenous NA, which in some male specimens was quantified at levels varying around 0.01 to 0.32 or 0.05 to 0.6 ng/mL, well below the limit of the IOC (Jeanneau et al., 1999; Le Bizec et al., 1999; Dehennin, 1999). In females, the level of NA is normally also very low, reaching a mean maximum value of 0.6 ng/mL during the ovulation

(Hemmersbach et al., 2000). The statistics collated by the IOC indicate that each year, around 0.24% of the A-samples analysed were reported to contain norandrosterone, making nortestosterone one of the anabolic agents most frequently found in athletes' samples along with testosterone.

Natural Steroids $\delta^{13}C\ ^{0}/_{00}$ values

We have measured the $\delta\ ^{13}C\ ^{0}/_{00}$ values of urinary steroids excreted in mixed athletes' samples of different nationalities and found them to be similar to those reported by others (table 3).

Table 3: Reference $\delta\ ^{13}C\ ^{0}/_{00}$ values measured in athletes' samples

		$\delta\ ^{13}C\ ^{0}/_{00}$		
Steroid	n	Mean	Std dev.	Range
Androsterone	78	-23,4	1,6	-19,9 to –26,6
Etiocholanolone	78	-23,3	1,3	-20,0 to –26,2
Pregnanediol	56	-24,9	1,2	-22,1 to –26,8
Pregnanetriol	68	-23,8	1,5	-20,8 to –26,8
Cholesterol	68	-24,2	1,5	-21,1 to –26,8

Synthetic $\delta^{13}C\ ^{0}/_{00}$ values

The GC/C/IRMS analysis of samples collected before and following the administration of testosterone and of a single oral dose of androstenedione and DHEA have shown that the ^{13}C content of the androgens metabolites differed significantly from their original value (stable within the course of the study) and from those of the reference steroids, going to the depleted values measured in synthetic steroids. Examples of results are summarised in tables 4, 5 and in figures 1, 2.

We are now using the combination of GC/MS and GC/C/IRMS to evaluate "testosterone" cases. Examples of normally and abnormally elevate T/E excretion are presented in table 6.

Table 4: $\delta\ ^{13}C\ ^{0}/_{00}$ values of urinary steroids modified by the administration of androstenedione.

Androstenedione			α-adiol	β-adiol	Etio	A	Pd	Pt	Chol.
Vol. 1	Before	Mean	-24,4	-24,3	-24,6	-24,3	-25,5	-23,8	-23,6
		Std dev	1,7	0,5	0,3	0,1	0,3	0,5	0,5
	After	Mean	-31,4	-32,9	-33,7	-31,7	-25,3	-24,1	-23,2
		Std dev	2,6	-3,2	1,9	2,4	0,5	0,8	0,8
	Difference		-7	-8,5	-9,1	-7,3	0,3	0,3	0,3
Vol. 2	Before	Mean	-[2]	-	-24,4	-23,8	-22,3	-23,0	-22,4
		Std dev	-	-	0,3	0,2	0,2	0,3	0,6
	After	Mean	-31,4	-32,4	-33,8	-33,9	-22,5	-23,7	-23,2
		Std dev	3,8	2,4	1,7	2,2	0,6	0,8	0,8
	Difference		-	-	-9,4	-10,1	-0,2	-0,6	-0,8

Table 5: $\delta\ ^{13}C\ ^{0}/_{00}$ values of urinary steroids modified by the administration of DHEA

DHEA			α-adiol	β-adiol	Etio	A	Pd	Pt	Chol.
Vol. 1	Before	Mean	-23,5	-25,0	-25,7	-25,8	-24,7	-24,4	-23,0
		Std dev	0,8	1	0,7	0,9	0,5	0,3	0,7
	After	Mean	-33,8	-31,8	-33,6	-34,0	-25,2	-24,5	-23,0
		Std dev	2,4	4,7	2,3	2,6	0,6	0,4	0,7
	Difference		-10,3	-6,8	-7,9	-8,3	0,5	0,1	0,1
Vol. 2	Before	Mean	-[2]	-23,3	-25,8	-24,8	-24,9	-23,9	-23,8
		Std dev	-	1,1	0,9	0,8	0,5	0,6	0,7
	After	Mean	-32,6	-33,7	-35,7	-34,1	-24,8	-24,5	-24,4
		Std dev	2,6	3,1	1,2	2,1	0,8	1,1	0,8
	Difference		-	-10,4	-9,9	-9,3	0,1	-0,6	-0,6

[2] The signal was too low to permit an accurate determination.

Table 6: Evaluation of testosterone cases using the combination of GC/MS and GC/C/IRMS

				$\delta\ ^{13}C\ ^{0}/_{00}$		
	T/E	Etio	A	Pd	Pt	A/Pd
Testosterone [3]	15,7	-28,5	-28,1	-23,8	-24,0	1,2
(Variation %)	(32%)	(2%)	(2%)	(3%)	(3%)	(3%)
Athlete 1 (negative)	4,2[4] (40%)	-22,6	-23,7	-23,7	-23,8	1,0
Athlete 2 (negative)	6,7[5]	-26,0	-26,3	-26,7	-26,2	1,0
	(23%)	(4%)	(3%)	(4%)	(0,3%)	
Athlete 3 (negative)	6,1[6]	-21,7	-22,2	-23,0	-23,5	1,0
	(25%)	(6,5%)	(3,8%)	(0,3%)	(6,1%)	
Athlete 4 (positive)	38	-31,3	-31,9	Cholesterol	-25,3	A/Cholesterol
				-25,7		1,2

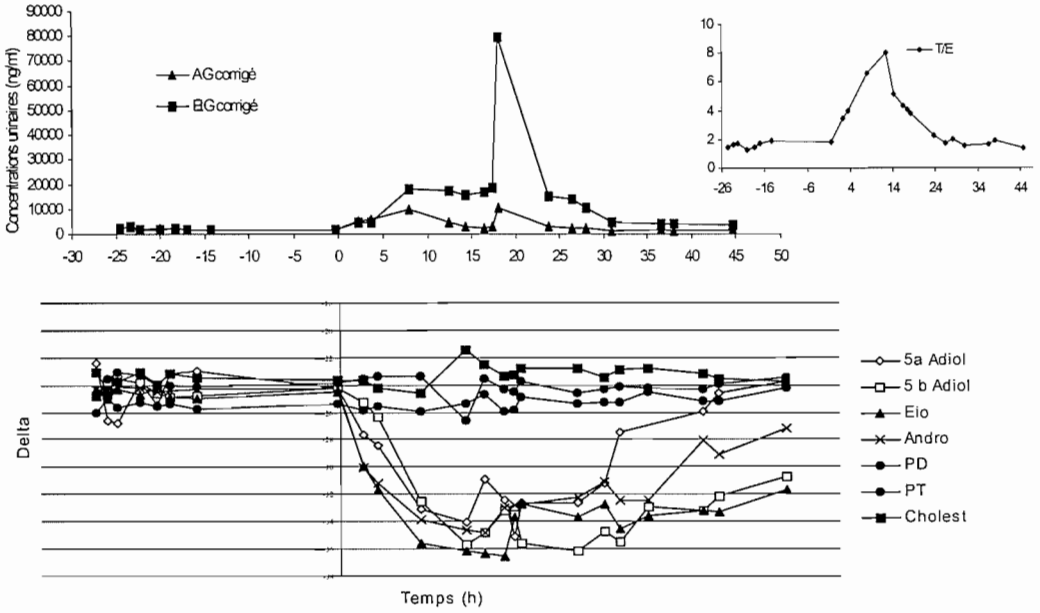

Figure 1: Variation of T/E values, corrected concentration of A and Etio and of $\delta\ ^{13}C\ ^{0}/_{00}$ values following the administration of a single dose of androstenedione in male volunteer 1.

[3] n = 15 urine samples (Medical treatment: testosterone enanthate once per week, two weeks collection)
[4] T/E : n = 8 $\delta\ ^{13}C\ ^{0}/_{00}$: single determination
[5] T/E : n = 4 $\delta\ ^{13}C\ ^{0}/_{00}$: n = 3
[6] T/E : n = 2 $\delta\ ^{13}C\ ^{0}/_{00}$: n = 2

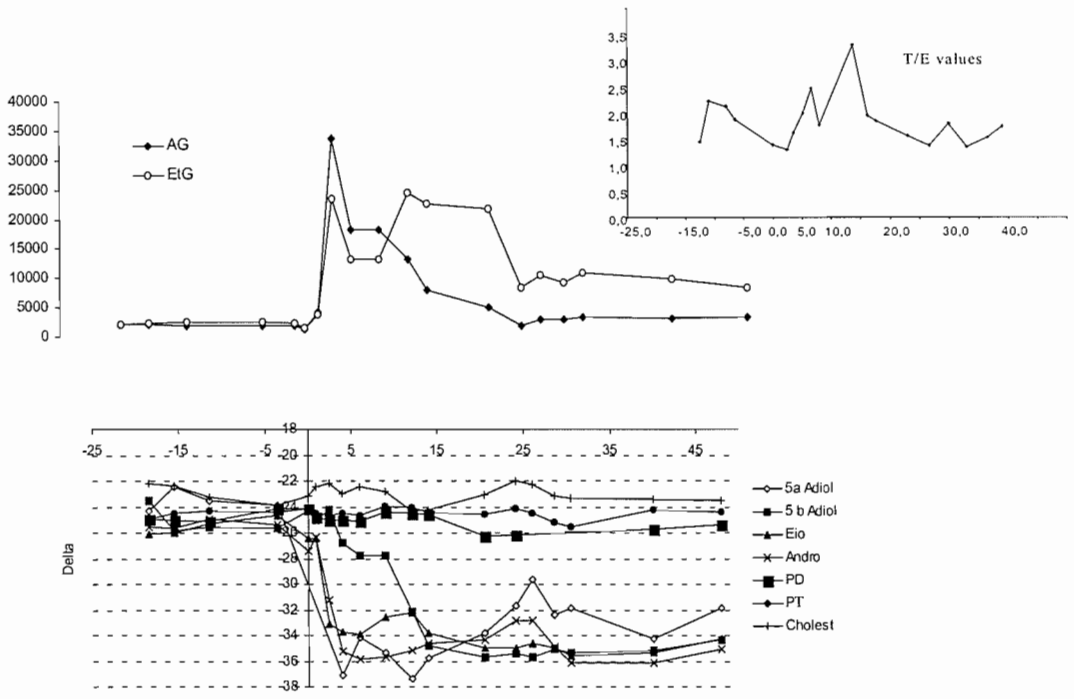

Figure 2: Variation of T/E values, corrected concentration of A and Etio and of δ ^{13}C $^{0}/_{00}$ values following the administration of a single dose of DHEA (200 mg) in male volunteer 3.

19-norsteroids

Twenty-five urine samples, analysed between 1992 to 1999, containing norandrosterone in amounts ranging from 35 to 11000 ng/mL, were re-analysed by GC/C/IRMS and so were the urine samples collected after the administration of norandrostenedione (commercial Nordione). The δ ^{13}C $^{0}/_{00}$ values measured in the excreted norandrosterone and noretiocholanolone were both significantly different than those of the reference steroids and of androsterone and etiocholanolone, excepting of course, athletes' samples in which elevate T/E values were also measured.

Endogenous norandrosterone (around 2 ng/mL), excreted during the 12th and 14th weeks of pregnancy of two different persons, was analysed by GC/C/IRMS following its isolation from a large volume of urine. The δ ^{13}C $^{0}/_{00}$ values then measured were found to be similar to the other endogenous steroids, androsterone, etiocholanolone, pregnanediol and pregnanetriol. The results are summarised in table 7.

Table 7: $\delta\ ^{13}C\ ^{0}/_{00}$ values of norandrosterone in 1) nortestosterone positive samples 2) norandrostenedione samples 3) pregnancy samples

	$\delta\ ^{13}C\ ^{0}/_{00}$ (mean values)					
	NA	NE	A	Pd	Pt	NA/Pd
Nortestosterone[7]	-31,1	-31,5		-23,0	-23,0	1,35
Norandrostenedione[8]	-31,6	-30,4	-22,7	-23,4	-22,4	1,36
Pregnancy 12th week[9]	-23,6	-	-24,9	-23,1		1,02
Pregnancy 14th week	-23,0	-	-26,1	-23,0		1,00

Conclusion

The combination of GC/MS and GC/C/IRMS has been proven useful to the evaluation of cases where the administration of "natural" steroids was suspected. The population reference ranges of the diagnostic parameters of both techniques are now well described. The ratio of the $\delta\ ^{13}C\ ^{0}/_{00}$ values measured in the steroids metabolites to the unaltered reference steroids is the only way to compensate for the many diets and for the different methods that are currently used in the laboratories. Alone, absolute values associated with either synthetic or natural steroids cannot form basis of the decision, significant differences having reported for the reference populations. The ratio of 1,1: 1,0 proposed by Shackleton (1997) for testosterone seems also adequate for the other steroids. The choice of the reference steroids must be made taking into account the alterations of the values that could be produced following the administration of precursors such as pregnenolone. When the concentration and the volume of specimen available permitted the injection of more than 20 ng, the ratios of the urinary synthetic norandrosterone to the unaltered reference steroids were always high since the contribution of natural norandrosterone can be virtually excluded. Natural values, similar to those of the reference steroids, were obtained during pregnancy.

[7] n = 25 urine samples
[8] Results of one excretion study. The mean value of commercial capsules: -32,7
[9] One determination: extracted from 560 mL and 380 mL respectively

Acknowledgements

The financial support of the Canadian Centre for Ethics in Sports (CCES), of the International Association of Athletic Federations (IAAF), the International Drug Testing and Management (IDTM) through their testing programmes is gratefully acknowledged. The skilled contribution of Alain Charlebois and Jean-Pierre Couture is invaluable. Jean-François Lévesque and André Lajeunesse received grants from the FCAR and from Sport Canada for which we are grateful.

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