Martínez-Brito, D.[(1)], Correa Vidal, T. [(1)], García Mir, V. [(2)], González Hdez, S. [(1)], Montes de Oca, R. [(1)], Ledea Lozano, O. [(3)], Granda Fraga, M. [(1)]

Reference ranges of urinary steroid profile parameters in a Latin American population.

(1) Antidoping Laboratory, Sport Medicine Institute, Havana, Cuba, (2) Pharmacy and Food Institute, Havana University, Cuba, (3) Scientific Research National Center, Havana, Cuba

Introduction

Metabolism of endogenous anabolic androgenic steroids such as dehydroepiandrosterone, 4-androstenedione, dihydrotestosterone and 5-androstendiol induces alterations in urine concentrations of T itself as well as its metabolites. Determination of endogenous (physiological) or exogenous (pharmaceutical form) origin of these substances is highly complex which has led to the popularity of the consumption of endogenous steroids as a way to enhance athletic performance in recent years. [1-4]. Urinary steroid profile in the field of doping has been a widely described method in order to detect a substance prohibited. It is known that this profile is influenced by sex, age, exercise, diet, ethnicity, among others. [5-10] Samples received in the laboratory for doping control are anonymous, so the primary evaluation of steroid profile of an individual should be based on comparison with reference ranges obtained from a specific population. The data available in the field of doping belong to Caucasian populations mainly and is not currently described in the literature data from populations where the miscegenation predominates as in the case of Latin America. The aim of this paper is to determine the reference ranges of urinary endogenous steroid and the main ratios for a population of a geographic area and to describe their behavior in both sexes.

Materials and Methods

Samples used in the study were analyzed at the Antidoping Laboratory of Havana which were applied the following exclusion criteria: (i) positive samples for the presence of any compound included in the Prohibited List banned by WADA, (ii) samples containing a compound that alters the endogenous steroids profile, (iii) samples with a T/epiT higher than 4 and no physiological study concluded or IRMS analysis, (iv) samples with a lower density of 1.004 and (v) samples with signs of bacterial contamination. Samples were collected in and out of competition, top athletes from Cuba, Venezuela, Mexico, Dominican Republic, Guatemala and Chile, mainly. Given the endocrine differences, the estimated reference ranges were evaluated for females (n = 1181) and males (n = 2454) separately.

Sample analysis: 2.5 mL of urine were subjected to a liquid-liquid extraction with *tert*-butylmethylether at basic pH, after enzymatic hydrolysis with β-glucuronidase (*E. coli*) at pH7. Trimethylsilyl derivatives were analyzed using gas chromatography - quadrupole mass spectrometry. Quantification of steroids was carried out using a response factor with a calibrator sample containing analytes to be quantified at known concentrations. This validated assay is the same used in several External Quality Assessment Scheme rounds by WADA with good results. Following androgens were evaluated: testosterone (T), epitestosterone (epiT), androsterone (A), etiocholanolone (Et), 3α,5α-androstanediol (3α,5α-diol), 3α,5β-androstanediol (3α,5β-diol), dehydroepiandrosterone (DHEA) and ratios: T/epiT, A/Et, 3α,5α/3α,5β-diol and 3α,5α/epiT.

Data Manipulation: Correct integration of all chromatographic peaks was checked manually. In order to compare the measurements, the concentrations of the compounds in each sample were corrected for specific gravity. [11]. Evaluation of the data was performed using the statistical package SPSS (16.0) and STATITICA (6.0). Data distribution was determined by Kolmogorov-Smirnov test. Descriptive statistics for nonparametric data included the mean, median, percentiles at 2.5% and 97.5%. To compare male and female populations a Mann Whitney U test was applied. Correlations were assessed by Spearman's ρ test.

Results and Discussion

The application of the Kolmogorov-Smirnov test to assess the distribution of the data showed that neither compound nor ratio is normally distributed in both sexes. The distributions are characterized by wide differences between the mean and median and high values of standard deviation. Table I shows the results for male and female populations.

The evaluation of the androgens and ratios showed statistically significant differences between males and females when applying the Mann Whitney U test ($\alpha = 0.05$), except for T/epiT ratio. At 97.5% percentile, Et and DHEA, showed higher concentration values in females compared to males being more remarkable because DHEA is the main precursor of androgens. Statistical analysis used to determine a possible correlation between DHEA and other profile parameters showed no definite correlation between them in either sex (ρ Spearman, $\alpha = 0.05$). Uralets *et al*. [12] reported that the consumption of this substance leads an increase concentrations of metabolites with structure 5β (Et; 3α,5β-diol) and decreasing in urinary cortisol levels. The absence of positive statistical results between these parameters

and DHEA suggest that under normal physiological conditions this event do not occur.

Van Renterghem [3] published a similar study in a Caucasian population and reference limits for percentile 97.5% are lower than those described in this paper. Concentrations of T, epiT, A, Et, DHEA, 3α5β androstandiol and 3α5α androstandiol of the latin-american population are between 1.6 and 1.9 folds higher than the Caucasian population (Table II). These data confirm the genetic polymorphism of the UGT2B17 enzyme which showed differences between ethnic groups, being more common the suppression in Asians and Caucasians than in Afro-Americans. [13-17]. Ratios showed greater similarity in both populations. Exhaustive assessments of samples from a population of a high miscegenation should carry out when limits set by WADA is used. This situation can motive an increase in the samples test cost for the use of additional techniques like GC/C/IRMS. On the other hand, generates false negatives in samples from Asian athletes. One possible solution is provided by Starcevic [14] to propose the use of the urinary ratio T/epiT < 0.2 as the threshold limit for individuals with deletion polymorphism in the gene coding the UGT2B17 enzyme by ethnicity.

Conclusions:

This paper describes the reference limits of urinary steroids for a population representative of an area of Latin America. These limits allow a more accurate assessment of samples from athletes belong to this geographical area (high degree of miscegenation) as the limits taken so far, in doping control activity, correspond to Caucasian and Asian populations. These limits are close to the 97.5 % of the population in here presented corroborating genetic differences describe already. The statistical comparison between sexes showed significant differences in all parameters evaluated except for T/epiT ratio.

References:

1. Kennet D. Fitch. (2008) *Asian J Androl*, May, **10**: 384-390.
2. Kicman A.T and Gower DB. (2003) *Ann Clin Biochem*, **40**: 321-356.
3. Van Renterghem P., Van Eenoo P., Geyer H., Schänzer W., Delbeke F. T. (2010) Steroids **75**, 154-163.
4. Saudan C., Baume N., Robinson N., Avois L., Mangin P., Saugy M. (2006) *Br J Sport Med*, **40 (Suppl II)**, i21-i24
5. Donike M., Mareck-Engelke U., and Rauth S. (1995) In: Schänzer W., Geyer H., Gotzmann A., Mareck U. (eds.) Recent Advances in doping analysis (2), Köln, pp 157.
6. De la Torre X., Segura J., Yang Z., Li Y., Wu M. (1997) In: Schänzer W., Geyer H., Gotzmann A., Mareck U. (eds.) Recent Advances in doping analysis (2), Köln, pp 71.
7. Kicman A. and Cowan D. A. (2009) *Drug Test. Analysis*, **1,** 22-24.
8. De la Torre X.; Pascual JA.; Ortuño J. and Segura J. (1997) In: Schänzer W., Geyer H., Gotzmann A., Mareck U. (eds.) Recent Advances in doping analysis (4), Köln, pp 59.
9. Mareck U., Geyer H., Opfermann G., Thevis M. and Schänzer, W. (2008) *Journal of mass spectrometry*, Vol 43, Issue 7, pp 877-891
10. Ayotte C., Goudreault D., Charlebois A. (1996) *J. Chrom B,* 687, pp 3-25

11. World Anti-Doping Agency. Reporting and evaluation guidance for testosterone, epitestosterone, T/E ratio, and other endogenous steroids. Technical Document TD2004 EAAS.http://wada-ama.org/Documents/World_Anti-Doping_Program/WADP-IS-laboratories /WADA_TD2004EAAS_EN.pdf (access date 30.08.2011)
12. Uralets, V.P. and Gillette, P.A. (2002) In: Schänzer W., Geyer H., Gotzmann A., Mareck U. (eds.) Recent Advances in doping analysis (9), Köln, pp 85.
13. Sten T., Bichlmaier I., Kuuranne T., Leinonnen A., Yli-Kauhaluoma J., Finel M. (2009) The American Society for Pharmacology and experimental Therapeutics, DMD, **37**:417-423
14. Makoto U., Fujisaki M.M., Ikekita T., Okano M. (1996) In: Schänzer W., Geyer H., Gotzmann A., Mareck U. (eds.) Recent Advances in doping analysis (3), Köln, pp 231.
15. Coutts B.S., Kicman A., Cowan D. (1997) In: Schänzer W., Geyer H., Gotzmann A., Mareck U. (eds.) Recent Advances in doping analysis (4), Köln, pp 173.
16. Starcevic B. and Butch A.W. (2008) Clinical Chemistry **54:12**, 1945-1947.
17. Jakobsson J., Ekstrom L., Inotsume N., Garle M., Lorentzon M., Ohlsson C. (2006) *J Clin Endocrinol Metab*, **91**: 687-693

Table I. Results obtained for the mean, median and 2.5% and 97.5% percentile (maximum reference limit) and quartiles 1 and 3 for males.

Parameter (ng/mL)	Males (n = 2454)				Females (n = 1181)			
	Percentil 2.5%	Mean	Median	Percentil 97.5%	Percentil 2.5%	Mean	Median	Percentil 97.5%
Testosterone	4	60	51	181	2	13	9.3	54
Epitestosterone	8	56	45	167	3	21	16	81
Androsterone	334	3128	2300	10676	212	2318	1538	9466
Etiocholanolone	311	2586	1974	8578	283	2408	1747	9115
3α,5α-androstanediol	16	96	79	286	6.9	45	30	169
3α,5β-androstanediol	22	237	166	903	15.2	149	101	646
DHEA	14	66	55	193	14.7	79	62.5	240
T/epiT ratio	0.1	1.4	1.3	5.6	0.12	0.96	0.89	3.8
A/Et ratio	0.4	1.3	1.2	3.1	0.29	1.0	0.9	2.3
3α,5α/3α,5β ratio	0.14	0.6	0.47	1.8	0.1	0.5	0.3	1.7
3α,5α/epiT ratio	0.44	2.4	1.7	7.7	0.4	2.7	2.2	8.7

Table II. Median and percentile values of 97.5% described by Van Renterghem [3] for a Caucasian population and those obtained for the Latin American population (male sex).

Parameter (ng/mL)	**Caucasian population**		**Latin American population**	
	median	**Percentil 97.5 %**	**median**	**Percentil 97.5 %**
Testosterone	30.9	103	51.1	181
Epitestosterone	22.6	88.9	44.8	167
Dehydroepiandrosterone	34.5	117	55	193
Androsterone	2260	6700	2300	10676
Etiocholanolone	1580	4950	1974	8578
3α5α androstanediol	40.0	155	79	286
3α5β androstanediol	98.9	416	167	904
T/E ratio	1.39	4.3	1.29	5.62
And/Et ratio	1.46	3.64	1.17	3.09
3α5α /3α5β ratio	0.42	1.69	0.47	1.82