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Longitudinal studies in steroid profiling – a multivariate approach.

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Introduction.

It is well known that the alteration of one or more variables of the urinary steroid profile in comparison to a population-based or individual range may be caused by the administration of testosterone or its precursors. One way to prove the exogenous administration of testosterone or its precursors is the use of isotope ratio mass spectrometry (IRMS). In case IRMS remains inconclusive longitudinal analyses can be performed. At present, just univariate methods have been used to evaluate a potential "adverse analytical finding" from the rest of the data:subject-based reference limits, outliers test, predictive model (Bayesian test), etc [1-3].

Here we propose a multivariate analysis (MVA) approach for the longitudinal studies, taking into account the steroid profile and some metabolites ratios, analyzed globally. The goal is to build up a predictive model to judge whether a sample showed an abnormal profile using principal components analysis (PCA) as MVA method. Some authors have already proposed this kind of approach in the human antidoping area, but using population studies [4-5].

In a first step we have analyzed samples collected from subjects of two different ethnic origins (Caucasians and Asians) treated with intramuscular testosterone enanthate to prove the effectiveness of the approach. In a second step, we applied the method to anonymous real longitudinal studies and the results were compared with the current WADA (World Antidoping Agency) approach.

Experimental.

Subjects.

- Intramuscular testosterone enanthate.

  Two groups (5 Caucasians and 6 Asians, age 25 ± 3 yrs and 20 ± 2 yrs, respectively) received 250 mg of testosterone enanthate (Testoviron Depot®, Schering, Japan) intramuscularly, equivalent to 180 mg testosterone,.

  Urine samples were collected every 8 hours for 3 days before, and for 15 days after drug administration. (Hospital del Mar (Barcelona, Spain) Ethical Committee approved CEIC: nº 94/467, 3/10/1994). The UGT2B17 polymorphism was not genotyped, nor the IRMS analysis.

- Real cases.

Subjects included in three longitudinal studies from Italian NADO (CONI) suspected for testosterone or its precursors abuse, all negative to IRMS analysis, after rigorous anonymization.

Urinary Steroid.

Urinary unconjugated steroids plus steroid glucuronides were determined by gas chromatography mass spectrometry (GC/MS) after hydrolysis of the conjugates with β-glucuronidase as previously described [7]. The steroids included in this study are: testosterone, epitestosterone, androsterone, etiocholanolone, 5α-androstandiol, 5β-androstandiol and some metabolic ratios (i.e. T/E).

Data Analysis.

The metabolic profile data were imported into SIMCA-P+ 12 (Umetrics, Umea, Sweden) for statistical data analysis. Principal component analysis (PCA) was performed with mean centering, logarithm transformation and Pareto scaling as data pre-treatment.

Results and Discussion.

Intramuscular testosterone enanthate.

Preliminary PCA analysis was performed on all samples (Figure 1): this unsupervised MVA allow us to know the possible structure of the data. The score plot of the first two PC's highlighted the clear cluster, between Caucasian and Asians individuals. This model also allowed to show that the inter-individual variation is greater than intra-individual one (data not shown). All subjects were analyzed separately, using the samples before administration as individual own controls. In the score plot of the first two PC's, it was verified that testosterone administration caused two well separated clusters, with some samples in a transition area (Figure 2).

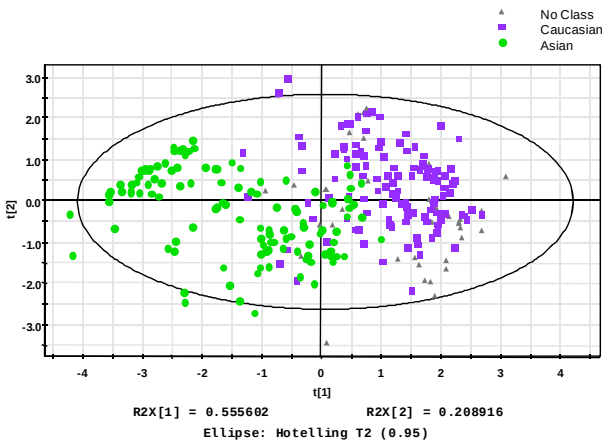

Figure 1. PCA score plot performed by considering all samples.

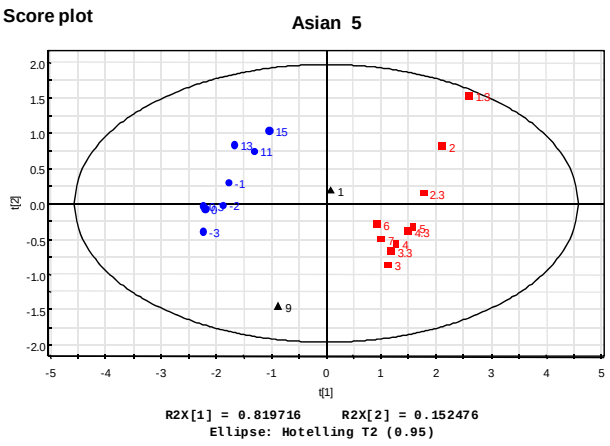

Figure 2. Characteristic example of PCA score plot of an individual subject.
● : blank samples; ■ : positive samples; ▲: no class

The Cooman's plot permits a good visualization of the two class separation. Here two independent PCA models are calculated defining two separate classes of samples, and the residual distances of the samples to each of the two models are plotted against each other (called Soft Independent Modeling of Class Analogy, SIMCA). Mapping unknown samples into the models will classify these samples as belonging to one of the two predefined classes, to both classes or to none of the two classes (Figure3).

In Table 1 we report the numbers of positive samples by the two different approaches showing how the proposed method has better results than the WADA method for Asian population and slightly similar for Caucasian.

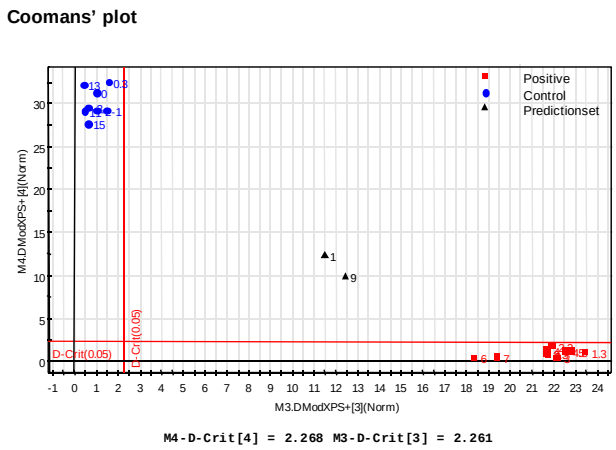

Figure 3. Example of Cooman's plot.Asian-5 subject.

Table 1. Numbers of positive samples detected after the administration by the application of the two approaches divided by subject, and ethnic group.

	N° Positive			
	Asian		Caucasian	
Subject	WADA (T/E)	PCA	WADA (T/E)	PCA
1	3	13	12	9
2	0	13	16	15
3	7	14	14	14
4	3	12	13	14
5	0	13	14	15
6	6	13		

Real cases.

One way to predict if one suspicious sample belongs to the group of samples of the same athlete is to create the PCA model excluding this particular sample, called training set (longitudinal study) and predicted set (suspected sample).Then we calculate the distance to the model in the X space (DModXPS+), used to detect moderate outliers and T2RangePS, which measure the subject distance from the center of the model, used to detect strong outliers (Figure 4). The same procedure was followed for the other two cases (one positive and one negative). In these examples the MVA approach and the WADA protocol gave the same results.

Conclusions

Taking into account the results of these studies we suggest that PCA can be a powerful, complementary technique for the overall longitudinal studies data evaluation and for its use in the endocrinological passport including steroid profiling, as proposed by the WADA itself. In

controlled studies, we obtained best predictive result for the Asian population where some difficulties with the traditional approach remain.

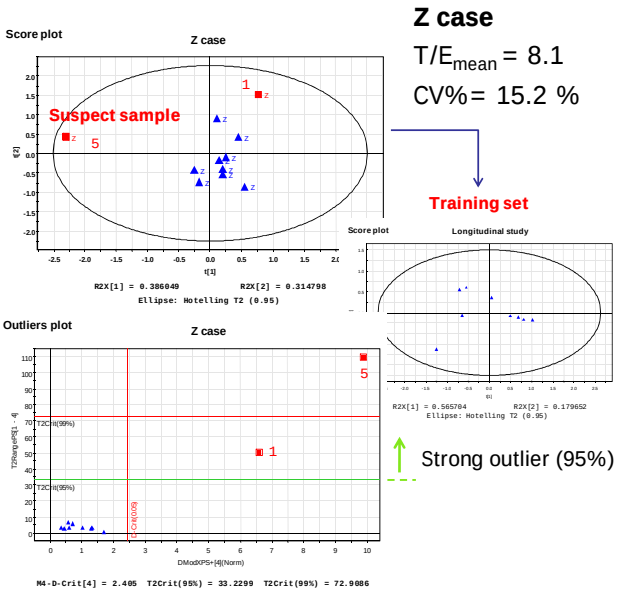

Figure 4. Score plots and Outliers plot for the Z case.

The two suspected samples (■) have a T2RangePS values greater than the model critical distance ($T2Crit_{0.05}$=33.3). Then, these samples have a different behaviour, compared with the normal model built with the variables of the steroid profile, they do not belong to normal model. The samples can be declared abnormal, contrary to WADA approach.

References

1.-Queraltó JM, Boyd JC, Harris EK. On the calculation of reference change values with examples from a long-term study.ClinChem, 1993; 39:1398-403.

2.- Sharpe K, Ashenden MJ, Schumacher YO. A third generation approach to detect erythropoietin abuse in athletes. Haematologica, 2006; 91:356-63.

3.-Jakobsson-Schulze JJ, Lundmark J, Garle M, Ekström L, Sottas PE and Rane A. Substantial advantage of a combined Bayesian and genotyping approach in testosterone doping tests. Steroid, 2009; 74(3):365-8.

4.-Norli HR, Esbensen K, Westad F, Birkeland KI, Hemmersbach P.Chemometric evaluation of urinary steroid profiles in doping control. J. Steroid Biochemistry Molecular Biology, 1995; 54(1/2):83-88.

5.-Cawley AT, Trout GJ, Kazlauskasa R, Howe CJ and George AV. Carbon isotope ratio (δ13C) values of urinary steroids for doping control in sport. Steroids, 2009; 74(3) 379-392.

6.- Erikson L, Johansson E, Kettaneh-Wold N, Trygg J, Wikström C,Wold S. Multi- and magavariate data analysis. Part I. Basic principles and applications. Second Edition. Umetrics Academy . Sweden. 2006.

7.-Mazzarino M, Abate MG, Alocci R, Rossi F, Stinchelli R, Molaioni F, de la Torre X, Botre F. Urine stability and steroid profile: Towards a screening index of urine sample degradation for anti-doping purpose. Analytica Chimica Acta 683 (2011) 221–226.