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Longitudinal studies in steroid profiling - a multivariate approach: IRMS extension

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Abstract

In a previous work we have suggested that principal components analysis (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 WADA.

The success of multivariate analysis (MVA) began with the appropriate identification of the most diagnostic variables. The aim is to expand the detection window of steroid abuse, as to reduce the number of false negatives. In this sense the concentration of hydroxy-steroids [1] and the $\delta^{13}\text{C}$ values of some biomarkers (T, And, Etio, 5aDiol, 5bDiol, DHT) could represent very remarkable variables.

Three individuals with different baseline steroid pattern were selected for the study and they were administered with a single dose of Testosterone undecanoate. We have tested three variables groups: the "classic" steroid profile evaluation, the hydroxy-steroids and the IRMS data. The experimental data were processed by a MVA approach. As in previous work, the MVA improved WADA approach alone, in respect to subject detection time (window detection). On the other hand, comparing different groups of variables: the "classic" and the hydroxy-steroid variables are quite similar in their prediction capacity, while with the IRMS values the predictions improved with a lower occurrence of false negative results. An approach based on PCA becomes then more powerful, and we propose the IRMS values to be part of the endocrinological passport.

Introduction

PCA can be a powerful, complementary technique for the longitudinal studies data evaluation and for its use in the endocrinological passport including steroid profiling, as proposed by WADA. In controlled studies, good predictive results for the Asian population were obtained by PCA, where some difficulties with the traditional approach remained. Caucasian population analysis is more complex due to the greater intra-subject variability (ex. T/E). The success of MVA begins with the right selection of the experiment design: the appropriate identification of the most useful variables (numbers and types) is fundamental.

The aim is to expand the detection window of steroid abuse using, studying three variable blocks and to create a predictive model for every block using PCA as MVA method.

Experimental

Oral Testosterone Undecanoate

Three individuals (age 23, 30, 40 yrs) with different baseline steroid profiles were selected for the study and were administered with a single dose of Testosterone undecanoate. Every volunteer received orally 40 mg of testosterone undecanoate (equivalent to 25.3 mg of testosterone, Andriol[®], Organon Italia S.p.A).

Urine samples were collected according to schedule (hours): -24,-22,-19,-17,-2, 0, 2, 4, 6, 8, 10, 12, 14, 24, 32, 36, 48 and 72. The samples were immediately frozen (-20°C) until analysis.

Urinary Steroids

Urinary unconjugated steroids plus steroid glucuronides were determined by gas chromatography mass spectrometry after hydrolysis of the conjugates with β -glucuronidase as previously described [2]. Classical steroid profile includes: testosterone, epitestosterone, androsterone, etiocholanolone, 5a-androstandiol, 5b-androstandiol and the metabolite ratios. For the hydroxylated steroids the following steroids were considered: 16a-OH-androstenedione, 16a-OH-etiocholanolone, 16a-OH-androsterone, 6b-OH-androsterone, 6b-OH-etiocholanolone, 6a/b-OH-androstenedione, 6a/b-OH-testosterone, 16a-OH-DHEA and some metabolites ratios.

IRMS Analysis

The IRMS analysis was performed combining HPLC purification and subsequent analysis with GC/C/IRMS without any derivatization. The method is highly specific as described [3]. Testosterone, epitestosterone, androsterone, etiocholanolone, 5a-androstandiol, 5b-androstandiol and DHT ^{13}C d values were measured.

Data analysis

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

For the prediction we used the Coomans' plot to highlight the graphical differences between two groups (positive and control). While the **DModXPS+** (Distance to the model in the X space, use to detect moderate outliers) was selected to define if a sample belongs or not to the control group and determine the subject detection time (windows detection).

Control group: samples before time 0 were included.

Positive group: samples with $T/E \geq 4$, atypical profile or $\Delta\delta^{13}_{(ERC-Target)} > 3$

Prediction group: samples with $T/E \leq 4$ after oral treatment.

Results and Discussion

Oral Testosterone Undecanoate

Preliminary PCA analysis were performed on all samples divided by control and positive groups. The score plot of the first two PC's demonstrated that subjects are different and behave dissimilar to oral testosterone undecanoate (Figure 1). Then the subjects were analyzed separately using the samples before administration as their individual own controls. After testosterone administration two separated clusters in every subject can be identified (Figure 2). The positive samples do not belong to control class. An improvement of the separation between classes from "Classic" to IRMS values is always observed.

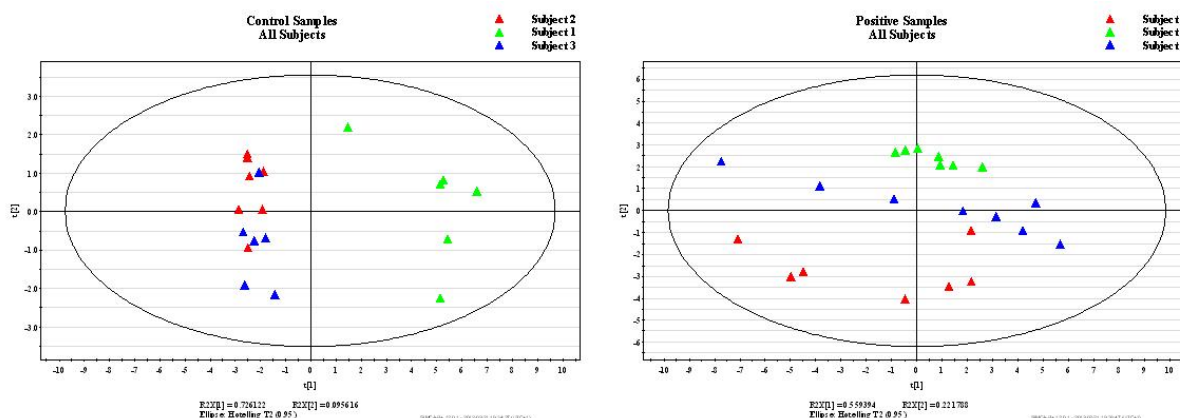


Figure 1: Preliminary PCA analysis showing all samples divided by control group (left) and positive (right) after oral testosterone undecanoate administration

In the IRMS model the control group is less dispersive which allow us to get a more accurate model and in consequence more efficient in the prediction. In fact, the differences between positive and control group are more pronounced and the distribution of the prediction samples describes the kinetic excretion of the administered substance.

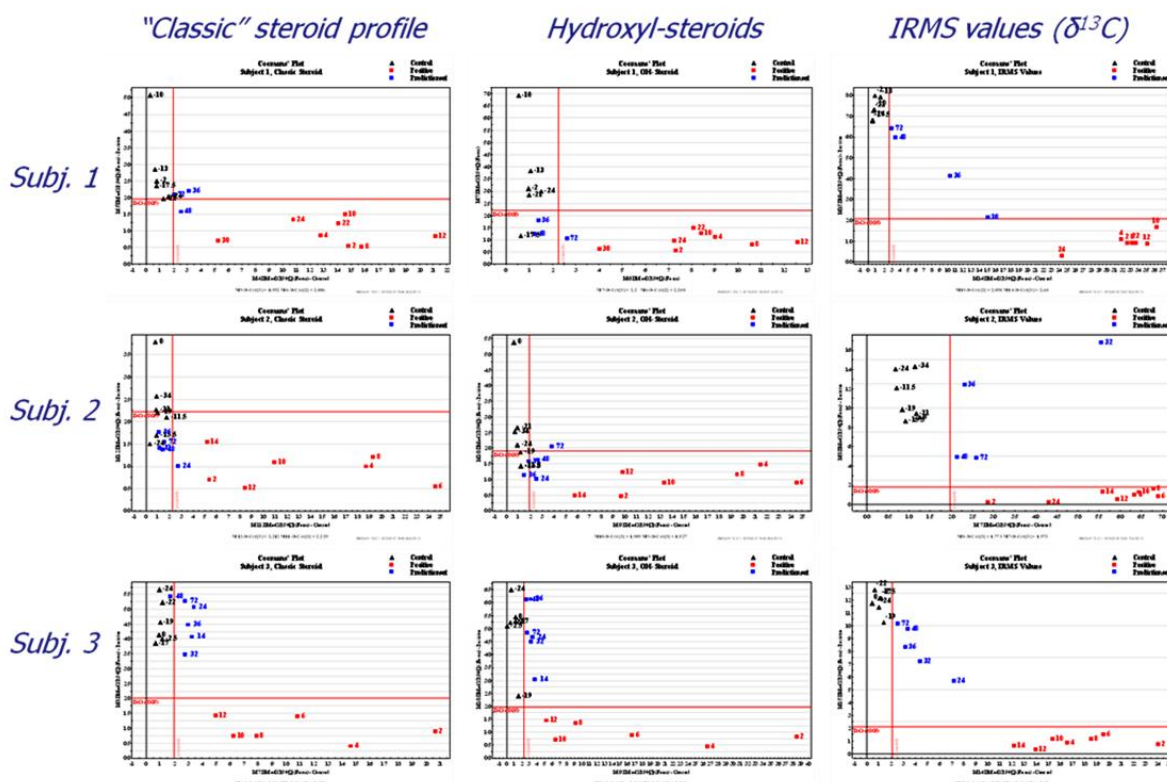


Figure 2: MVA by variables blocks and subjects

In Table 1 the detection windows by the approach here proposed are summarized and compared to the reference approach based on the current WADA criteria. More in details, Table 1 shows some limits of the current approach. In subject 1, T/E is greater than 4 until 30 h, but the IRMS analysis is negative. At the contrary for the other two subjects, after 14 and 12 h T/E is less than 4 but the IRMS analysis is still positive until 24 h and 14 h, respectively.

Subject	WADA				MVA (Distance to Model, SIMPCA)					
	T/E		IRMS		Classic profile		Hydroxy-steroid		IRMS values	
	t (h)	N°	t (h)	N°	t (h)	N°	t (h)	N°	t (h)	N°
1	30	8	24	7	48	11	30	10	72	12
2	14	7	24	8	24	8	24	10	72	12
3	12	6	14	7	36	11	72	12	72	12

Table 1: Summary of time window detection (t) and number of samples (N°) that were detected as abnormal after the application of WADA and MVA approaches

The prediction with the MVA is always better than classical approach for all subjects. Inside of the MVA and comparing the two variables blocks, the best predictions occurred from IRMS values, in number of samples and window time detection (see also Figure 3). The prediction achieved with the IRMS variables demonstrated to be more stable and have a less dispersive distribution, helping to improve the prediction of the model.



Figure 3: Graphical representation of the DModXPS+ for every model and subject.

Conclusions

Considering these preliminary studies we suggest an extension of the variables for the longitudinal studies evaluation including the hydroxylated steroids as proposed also by Van Renterghem et al.[4] and IRMS data, maintaining a PCA as MVA method. Between the three variables blocks: the “classic” variables and the hydroxy-steroid variables are quite similar in the prediction, while using the IRMS values the predictions improved with a lower occurrence of false negative results, in this way the approach becomes more powerful. Finally, we propose that IRMS values and some selected hydroxy steroid to be evaluated for the future development of the endocrinological passport.

References

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