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Validation of a steroidomic model and its application in doping control

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Abstract

In doping control, steroid profiles have greatly evolved since the introduction of the T/E ratio as more endogenous steroids hit the market and more insights into the steroid metabolism were gained. With the introduction of a Bayesian adaptive model to establish individual thresholds to circumvent large inter-individual variations of steroid metabolites, a big leap in steroid profiling was made. However, as different confounding factors of the steroid profile were investigated in doping analysis (e.g. genetic polymorphisms, alcohol, ...), the interpretation of steroid profiles became more complicated in order to detect atypical findings for misuse with endogenous steroids.
A statistical discrimination algorithm based upon Support Vector Machines was developed for evaluation of longitudinal steroid profiles which are stored in the biological passport. This steroidomic model allows a quick and accurate evaluation of comprehensive steroid profiles including minor steroid metabolites. The validity of this model was tested upon a database containing longitudinal data from 47 volunteers as well as excretion urines after administration of various endogenous steroids: testosterone undecanoate, testosterone gel, dehydroepiandrosterone, dihydrotestosterone gel, 4-androstenedione, 4-androstenediol, 5-androstenediol, 6-oxo-androstenedione and 7-keto-dehydroepiandrosterone. The model was further optimised for feasible application in a doping control setting using sensitive steroid metabolites. The model is applied to different kinds of phenotypic steroid profiles and results were verified with those of a confirmatory IRMS method.

Introduction

Recently, a support vector machine model [1] was applied to improve the feasibility of interpreting extended steroid profiles in doping analysis [2]. This statistical discrimination approach combines information of previously stored steroid profiling results in a biological passport, with a lateral evaluation of 24 steroid metabolites to yield an Abnormal Steroid Profile Score (ASPS), facilitating their interpretation.
The preliminary results of this steroidomic approach indicated its effectiveness and sensitivity towards the detection of administration of endogenous steroids in urine. Diagnostic accuracy of the method was better than any other known steroid profiling approaches. Detection times were found to be up to 5 days whereas previous longitudinal biomarker strategies demonstrated maximal detection windows of 3 days [3]. Long detection times can be explained by the recognition of changes in the steroid profile when returning to androgen homeostasis.
The performance of the SVM model is first investigated when operated with less steroid profile metabolites as variables. It is desirable to reduce the original number from 24 steroid profile markers to a ten-some in order to maintain excellent quantification standards for all monitored metabolites over time. Therefore, the best selection of input variable for the steroidomic model should be found.
Additionally, the model was validated for a large cohort of individual steroid profiles obtained over a period of 5 months.
Finally, the results of the steroidomic model were coupled to the results of IRMS to evaluate their relative performance.

Experimental

Analytical. All analyses were performed using a GC/MS method monitoring 24 steroid metabolites [4]. Several datasets containing extended steroid profiles were constructed to train and test the SVM model:

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Dataset1: A population study with 2027 negative urines of healthy male athletes was performed. Population statistics are used in the adaptive model [5].
Dataset2: A long-term follow-up study of 47 healthy male volunteers (aged 32.6±12.1 and weighted 75.9±9.4 kg) was conducted. This sample collection study was approved by the ethical committee of the Ghent University Hospital (B670201110846). All volunteers provided 10 blank urine specimens during a 5-month period under written consent. Genotyping was performed for UGT2B17. In total, this dataset contained 470 blank steroid profiles.
Dataset3: Six healthy male volunteers were administered with single small therapeutically recommended doses of 40 mg testosterone (T) undecanoate, 100 mg T-gel, 250 mg dihydrotestosterone (DHT)-gel and 50 mg dehydroepiandrosterone (DHEA). All participants gave written consent and the study was approved by the Ethical Committee of the Ghent University Hospital (B67020064707). One week before administration, three blank urine samples per volunteer were collected each day. Then, in a 4-week period, the steroid formulations were administered to the volunteers in the above mentioned order at the beginning of a new week. In total, dataset3 consists of the steroid profiles of 110 blank urines and 565 post-administration samples. Detailed information on the administration and urine collecting protocol can be found elsewhere [4].
Dataset4: Previously produced excretion urines were analysed with the same analytical method [4]: one subject was administered independently with 100 mg DHEA, 50 mg androstenedione (Adion) and 50 mg 7-keto-DHEA. Two other volunteers were administered with 100 mg Adion, 100 mg 4-androstenediol (4Aediol) and 100 mg 5-androstenediol (5Aediol). All administration studies were approved by local ethical committees. These substances were taken by other three volunteers than those who participated on the experiments with T undecanoate, T-gel, DHT-gel and DHEA in dataset3. Dataset4 consisted out of 114 steroid profiles.

IRMS-analysis was performed on target compounds androsterone (andro), etiocholanolone (etio) and the androstanediols with pregnanediol as a reference compound according to the recently described method [6].

Data analysis. In a two-step procedure, the steroidomic model is trained to learn the difference between basal and abnormal steroid profiles. First, the monitored steroid profile values of an individual sequence are normalised with individual statistics of blank profiles using the adaptive Bayesian model presented by Sottas *et al.*[7]. Then, the normalised steroid profiles are classified as negative (blank and pre-administration profiles) and positive (post-administration profiles). Using a kernel trick, the SVM constructs a separating hyperplane defining the difference between both classes. Once the model has been trained, its can be applied to unknown profiles where an ASPS quantifies its distance to the separating hyperplane. The software used was the independent SVM-Kernel Methods Toolbox for Matlab version 7.6.0 [8]. Classification accuracy was assessed with Receiver Operating Characteristics (ROC) analysis. The model's performance was assessed using leave-one-subject-out cross validation (LOSOCV).

Results and Discussion

Input variable reduction. The initial model was developed using dataset1 and dataset3 to enhance the instant interpretation of steroid profiles containing 24 metabolites. In comparison with the T/E ratio the diagnostic properties of the steroidomic model are presented in Figure 1 (green). Various combinations of steroid metabolites were tested as input variable for the SVM. Based upon the best biomarkers proposed in previous work [3], a selection of 11 steroid metabolites was found that had similar diagnostic properties (Figure1, magenta ROC-curve) compared to the 24 metabolite SVM model:
- T
- Epitestosterone (E)
- 5α-androstane-3α,17β-diol (5ααβ-Adiol)
- 5β-androstane-3α,17β-diol (5βαβ-Adiol)
- DHT
- 3α-5-cyclo-5α-androstan-6β-ol-17-one (5cyclo)
- 7β-OH-DHEA
- 6α-OH-Adion
- 4-OH-Adion
- 16α-OH-DHEA
- 16α-OH-Adion

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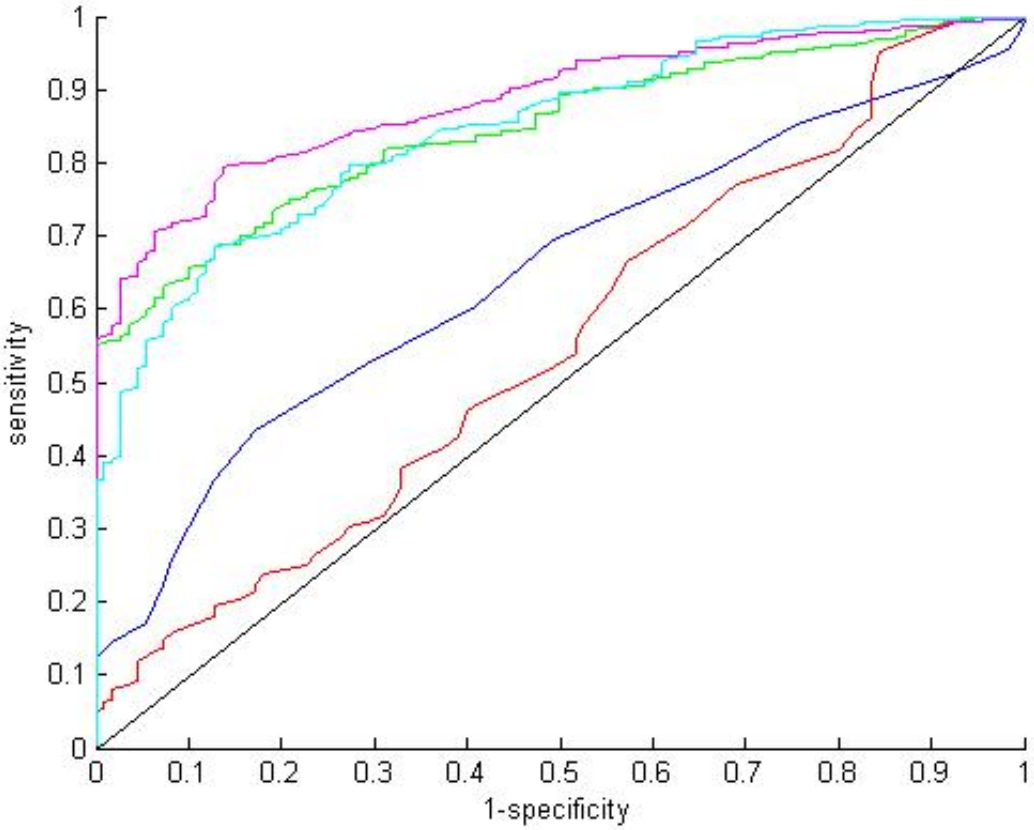

Figure 1: ROC-plot of the SVM-model on 24 (green) and 11 (magenta) steroid profile metabolites T/E ratio evaluated with population statistics (red) and with subject-based limits (blue) established with dataset3. Additional training on dataset3 and dataset2 resulted in the SVM-model with ROC-curve in turquoise.

Maximal diagnostic accuracy of 88.6% was achieved. Interestingly, andro and etio are not featured in this selection in favour of the androstanediols. This can be explained as these latter are preferred due to better diagnostic properties caused by closer metabolic relation to the administered parent compounds relative to androsterone and etiocholanolone. It can also be noted that 5cyclo is accounted rather than the parent compound as marker for the detection of DHEA.

Validation with additional longitudinal data. So far the SVM-model was operated using the short-term longitudinal steroid profiles of 6 subjects. Full validation was conducted in LOSOCV manner with an additional dataset2 for long-term evaluation of individual statistics of 48 volunteers. When increasing the training set of 6 to 6+48=54 subjects a small loss of 3% in detection accuracy was observed. Finally, a sensitivity of 38% was reached at a level for which no false positives were found (Figure 1 turquoise). This loss of sensitivity and accuracy can be justified by a gain in generalization capability of the model as larger variances are accounted. In Figure 2, an evolution towards stable detection accuracy is demonstrated when the initial training set (consisting of dataset3) is replenished with additional subjects from dataset2. After addition of three extra volunteers, stable detection accuracies were obtained. The constant detection accuracy indicates that the generalization capability of the obtained model is good.
The current SVM model now accounts for long-term intra-individual variations. This also prevents serial dependence i.e. resemblance of two steroid profiles due to short collection intervals. Moreover, the added dataset2 encompassed a wide range of genetic variation of which polymorphism of UGT2B17 was proven to be important for the urinary values of the T/E ratio [9]. In dataset2 8% del/del 46%ins/del and 46%ins/ins were found over 470 steroid profiles. Both the T/E ratio as the resulting ASPS of these steroid profiles were plotted in Figure 3. It can be noticed that the (inter-individual) variation of the ASPS is far less compared with the T/E ratio.
Preliminary data on alcohol consumption were recorded. 25 samples claimed to be provided within 48h after (uncontrolled) alcohol consumption. Since it is known that alcohol influences the glucuronidation of androgens like T, the samples were not

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considered for reference population. The blank steroid profiles gave rise to a normal distribution of the ASPS values presented in the histogram in Figure 3.
This allowed a parametric determination of the 99.9% reference limit (RL)=0.61. This threshold is also presented in red in Figure 3. Although none of the ASPS corresponding to post-alcohol steroid profiles exceeds the established RL, a significant difference (p=0.025, T-test) in mean in was found for blank ASPS's and ASPS obtained after alcohol consumption; here a maximal ASPS was observed of 0.20 after drinking of 5 glasses of wine.

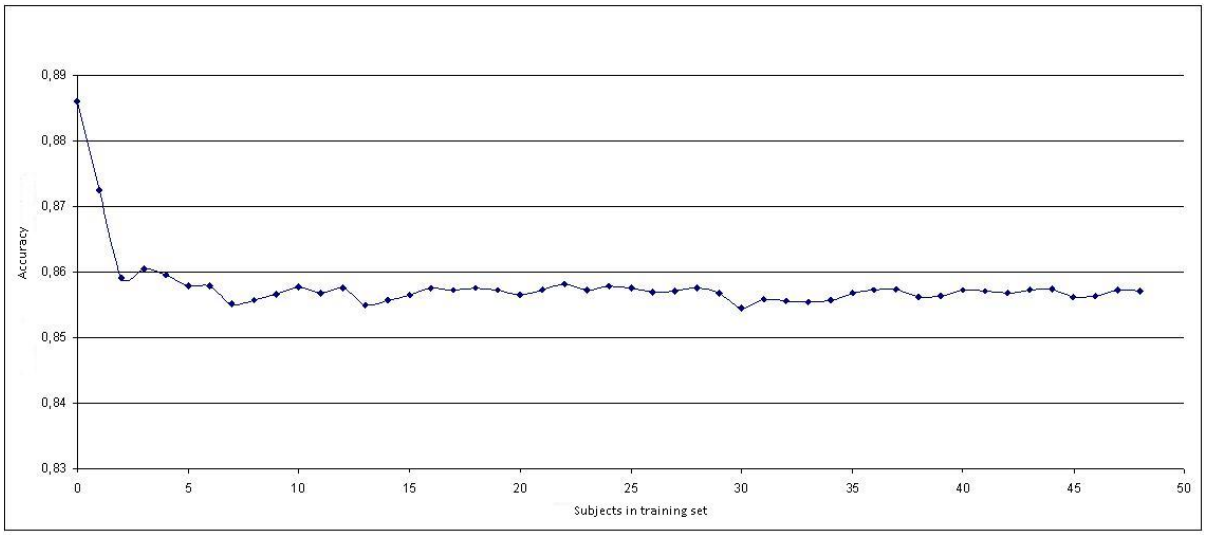

Figure 2: Evolution of the detection accuracy as more subjects of dataset2 are encompassed in the training set, which consists of the entire dataset3 with 6 subjects.

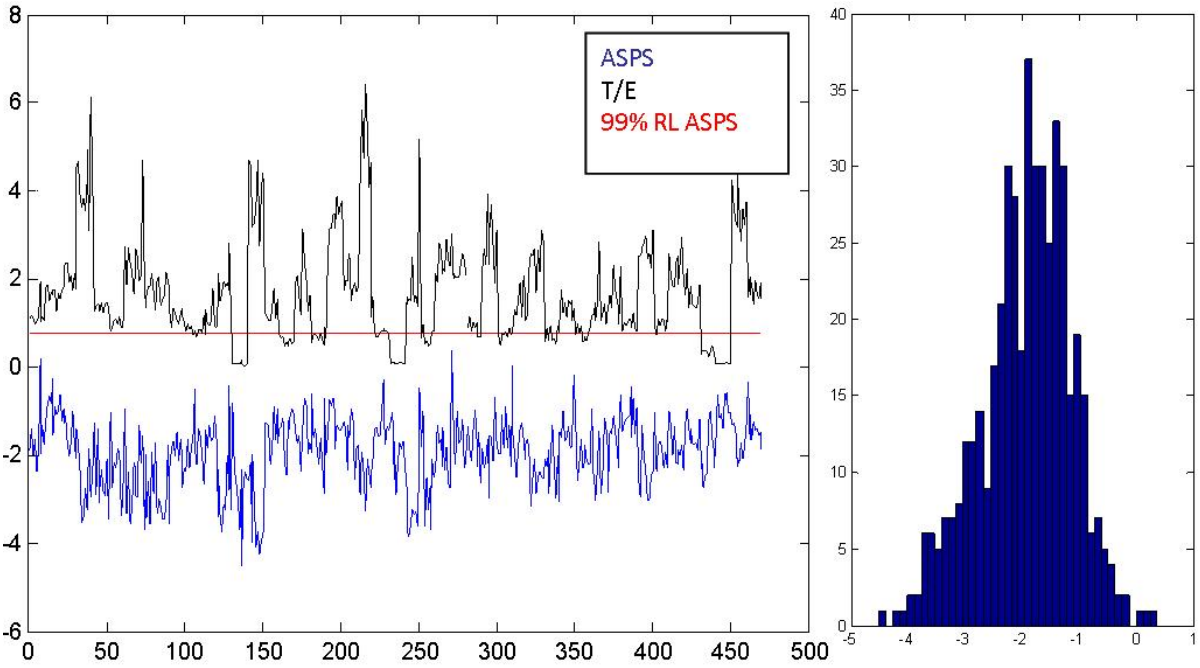

Figure 3: Representation of the T/E ratio (black) and the ASPS (blue) of the 470 steroid profiles in dataset2 (n=47).

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Post-administration profiles. Figure 4 presents the pre- (blue) and post- (black) administration profiles of the ASPS for several endogenous steroids and the 99.9%RL (red). For the various kinds of endogenous steroids used in the administration trials, the post-administration profiles show increases up to 10 units. The plots A-D in Figure 4 originate from dataset3, which was used for cross-validation. Here, the administration of oral steroid formulation T (Figure 4A) and DHEA (Figure 4B) and topical applications of T (Figure 4C) and DHT (Figure 4D) indicate that the detection times can be prolonged to seven days after administration, contrasting to previous detection times between 30 and 84h found with individual biomarkers strategies [3]. The plots E-H of Figure 4 show post-administration profiles of data featured in the independently tested dataset4. Again, here all blank steroid profiles are below the established threshold limit whereas a substantial number of samples collected up to 4 days.

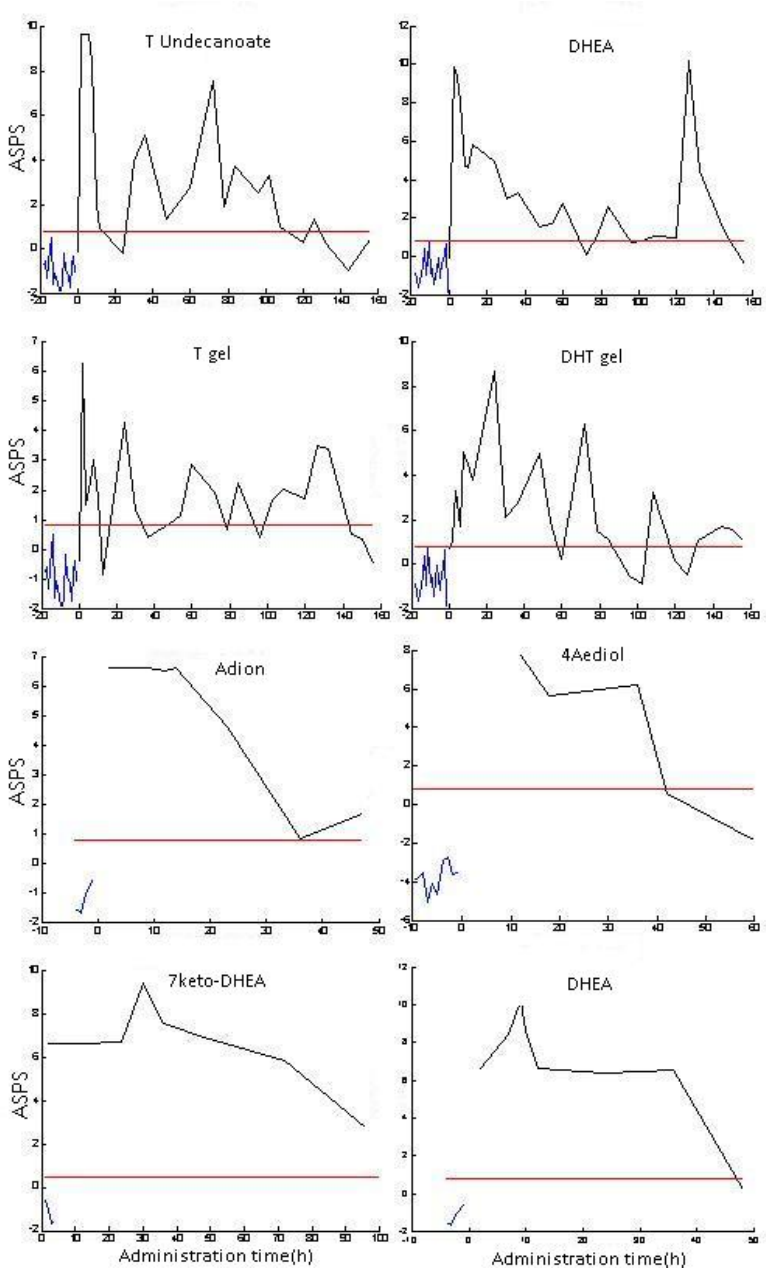

Figure 4: ASPS profiles before (blue and negative time indication) and after (black line) administration of different endogenous steroids (A: T undecanoate, B: DHEA, C: T-gel, D: DHT-gel, E: Adion, F: 4Aediol, G: 7-keto-DHEA, H: DHEA). The red line indicates the 99.9% reference limit.

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IRMS control. The detection times obtained by IRMS using in-house established compound specific thresholds for (Δ)δ13C values [6], were retrospectively applied onto the results of the steroidomic model. Hence, of all positive values of the (n=255) found by the steroidomic model, 64% could be confirmed by IRMS.

Conclusions

The steroidomic model is capable to successfully evaluate the biological fingerprint of 11 markers demonstrating excellent diagnostic properties. Interpretation is straightforward: comparison of the ASPS with one threshold of 0.61 is sufficient to evaluate the entire steroid profile.
The model was validated with the longitudinal profiles of 48 volunteers, ensuring the generalizing properties of the model. Independent tests were conducted with excretion urine from other administration studies proving its performance and robustness to detect misuse with various kinds of endogenous steroids. Steroid preparations and gel formulations could be detected up to seven days after application.
IRMS analysis resulted in a 64% confirmation rate of the positive output of the steroidomic model.

References

1. Vapnik V.N., *Statistical Learning Theory*. 1998, New York: Wiley-Interscience.
2. Van Renterghem P., Sottas P.-E., Saugy M., Delbeke F.T., Van Eenoo P. (2011) Statistical discrimination of steroid profiles. In:Schänzer, W., Geyer, H., Gotzmann, A., and Mareck, U. (eds.) *Proceedings of the 29th Workshop on Dope Analysis* (19), sportverlag Strauss. pp 63-72.
3. Van Renterghem P., Van Eenoo P., Sottas P.-E., Saugy M., Delbeke F.T. (2011) *Clin. Endocrinol.* **75**, p. 134–140.
4. Van Renterghem P., Van Eenoo P., Delbeke F.T. (2010) *Steroids* **75**, p. 1047–1057.
5. Sottas P.-E., Saudan C., Schweizer C., Mangin P., Saugy M. (2008) *Forensic Sci. Int.* **174**, p. 166-172.
6. Van Renterghem P., Polet M., Brooker L., Van Gansbeke W., Van Eenoo P. (2012) *Steroids* **77**, p. 1050-1060.
7. Sottas P.-E., Baume N., Saudan C., Schweizer C., Kamber M., Saugy M. (2007). *Biostat.* **8**, p. 285-296.
8. Canu S., Grandvalet Y., Guigue V., Rakotomamonjy A., *SVM and Kernel Methods Matlab Toolbox*, in *Perception Systèmes et Information*. 2005, INSA de Rouen: Rouen, France.
9. Jakobsson Schulze J., Lundmark J., Garle M., Skilving I., Ekström L., Rane A. (2008). *J. Clin. Endocrinol. Metab.* **93**, p. 2500-2506.

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