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P. Hemmersbach*, K. Esbensen+, H. R. Norli*, K. I. Birkeland* and F. Westad+

CHEMOMETRIC EVALUATION OF URINARY STEROID PROFILES IN DOPING CONTROL

*) Hormone Laboratory, Section for Doping Analysis, Aker Hospital, N-0514 Oslo, Norway, +) SINTEF SI, Dept. of Chemical Information Technology, Box 124 Blindern N-0314 Oslo, Norway

Introduction

Today's doping definition of the International Olympic Committee (IOC) Medical Commission is based on the banning of pharmacological classes of agents.[1] The analytical challenge of this definition can be divided into three aspects:

1. For synthetic exogenous compounds the identification of the parent compound, or one or more diagnostic metabolites, in an athlete's urine sample is evidence for the administration of the banned drug.
2. For substances that are produced endogenously (f. ex. testosterone) analytical results such as the ratio testosterone/epitestosterone[2] or testosterone/luteinizing hormone[3,4] in combination with other endocrine parameters[5] or tests[6] - can reliably indicate an offence on the basis of normal ranges, based on one individuum or a population, and statistical probabilities.
3. For synthetic anabolic-androgenic substances the influence on the excretion of endogenous steroids (steroid profile) may indicate an offense even if the parent compounds and/or metabolites are no longer detectable in urine.[7-9]

The influence of anabolic-androgenic misuse on the production and excretion of endogenous steroids is well understood[10] and steroid profiling in urine has been used for endocrinological diagnostic means for many years[11]. Hence, considerations of ratios between different endogenous steroids could be a useful tool to detect the influence of anabolic androgenic misuse.

The use of multivariate data analytical methods (in particular Partial Least Squares (PLS) regression) is a powerful tool for analysis of complex data structures connected to

chromatographic separations[12,13]. In the present study we have used multivariate data analysis to evaluate the relative steroid profiles in a group of subjects testing positive for anabolic steroids in doping control and a large group of matched controls. We also evaluated the effect of testosterone administration and analyzed four unknown samples, suspected for AAS misuse but testing negative in routine tests, with these models.

Materials and methods

The study group consisted of 23 male athletes testing positive for a broad range of anabolic-androgenic substances (AAS) in the routine doping control. The control group consisted of 105 healthy male students between 22 and 26 years of age, all of them willing to give a statement that they had never used AAS. We also studied four individuals that were suspected of doping with AAS, but tested negative in the routine doping control, and five voluntary young men participating in an excretion study with 250 mg testosterone enanthate. All the urine samples were stored at 4° C in plastic containers until analysis.

The samples where cleaned up and analyzed according to procedures followed in our laboratory for the analysis of anabolic-androgenic steroids. They are based on a comprehensive and sensitive gas chromatographic / mass spectrometric screening procedure published by Donike et.al.[14]. The set of steroids analyzed by their prominent ion trace includes androsterone, etiocholanolone, testosterone, epitestosterone, 5α-androstan-3α,17β-diol, 5β-androstan-3α,17β-diol, 11β-OH-androsterone, 11β-OH-etiocholanolone, dehydroepiandrosterone, 4-androstene-3,17-dione and 17α-methyl-testosterone as internal standard. The peak areas were normalized to total equal area for each individual sample before data analysis.

The multivariate data analytical approaches used here encompass PCA (Principal Component Analysis)[15,16], PLS-regression[16,17] and the related PLS-DISCRIM analysis[17,18]. PCA results in a dimensionality reduction onto a low-order projected space, outlining the inter- object relationships in (far) fewer dimensions than in the original data matrix. PCA is often described as a variance-maximising transformation, but is used here in a typical chemometric, i.e. truncated fashion, focusing on the projected rendering of the original complex data structure. In the present study we especially make use of simple two-

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dimensional projection spaces, score plots, from the initial 10-dimensional space. The software program used was Unscrambler II from Camo AS, Trondheim, Norway.

Results

By use of the PLS-DISCRIM analysis of the measured and normalized peak areas of endogenous steroidal metabolites in the urine, we have created a two-dimensional score plot (Figure 1).

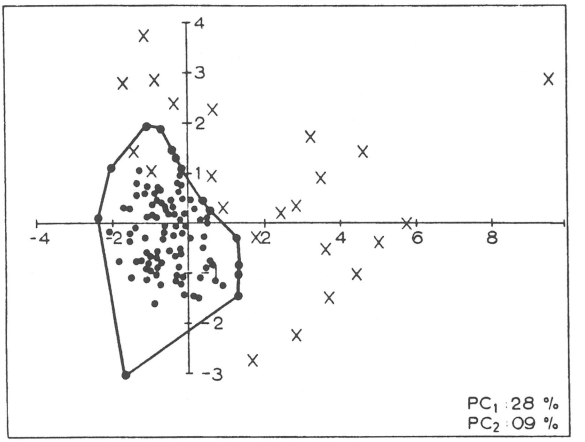

Figure 1 Control - Misuser Discrimination Plot (PLS-DISCRIM)
- • Control group
- × Misusers - Anabolic steroids detected in urine sample

We have delineated the periphery of the control class by a connecting envelope. This plot shows an almost complete separation between these two groups; only two misusers fall within this enclosed area. To visualize the relative impact of the various ratios between different metabolites, the accompanying loading plot (Figure 2), which relates to exact the same model. This plot indicates the ratios which of the parameters that mainly contribute to the observed discriminations. Steroids in different sectors and far from each other mean a great discrimination power of the respective ratio. In our model the ratios androsterone/etiocholanolone, testosterone/epitestosterone and also dehydroepi-androsterone/epitestosterone contribute significantly to discriminate AAS misusers from the control group.

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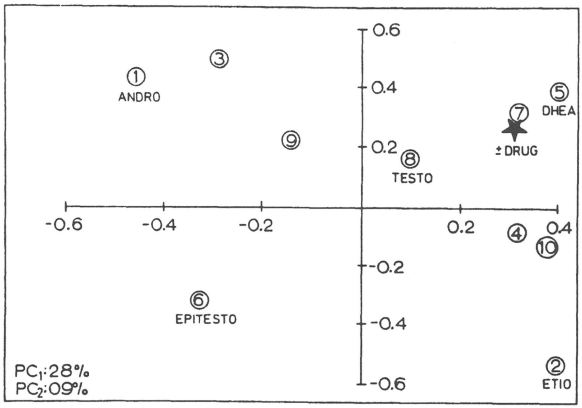

1 Androsterone, 2 Etiocholanolone, 3 5α- Androstan-3α, 17 β-diol, 4 5β -Androstan-3α, 17β-diol, 5 Dehydroepiandrosterone, 6 Epitestosterone, 7 Androstendione, 8 Testosterone, 9 11-OH-Androsterone, 10 11-OH-Etiocholanolone

Figure 2 Discrimination Variable Plot (PLS-DISCRIM)

We tested our PLS-DISCRIM model (Figure 3) by analyzing

A) an excretion study with 250 mg testosterone enanthate,

B) 4 urine samples found to contain anabolic steroids in routine analysis,

C) 4 urine samples from the Bodybuilding Federation not containing anabolic steroids,

D) 3 different control urines from randomly selected non AAS misusers (□).

While samples from the experiments A), B) and C) show a clear evidence of displaying a disturbed natural steroid profile the control samples are positioned inside our "normal range".

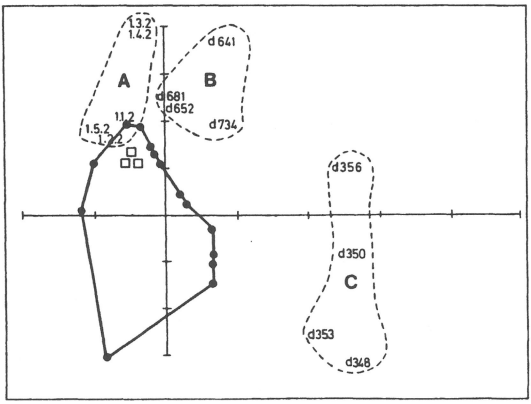

Figure 3 Projection of unknown samples into PLS-DISCRIM plot

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Discussion

The use of modern multivariate data analysis makes it possible to handle a large body of digital information simultaneously, and hence to simultaneously consider the ratios between all important steroids in the urine. This has been done in the present study, which for the first time present a PLS-discrimination plot based on a selected fraction of the total data variance (Figure 1). We also used this as a tool to visualize the relative importance of the different ratios measured (Figure 2). However, it is to be noted that almost 2/3 of the total data variance in the ten X-variables, is not correlated to the known misuser behaviour. This means that direct comparison of the ratios would not only have incorporated parts of this uncertainty but would have had to be carried out only on one ratio at the time. Neither of these approaches are fully satisfactory.
The score plot gives a rather good separation between the abusers and the controls, with only two individuals not correctly classified. These misusers could not be separated from the controls by any planar construction, indicating an asymmetrical classification problem.

In conclusion, we have presented a new methodology for AAS misuser discrimination based on a simultaneous multivariate PLS-discrimination analysis of the steroid profile in urine. With this it is possible successfully to discriminate 21 known misusers within this first data base consisting of 23 misusers and 105 controls. The basic PLS-DISCRIM plot from this approach also successfully discriminates four known misusers as well as substantiates four other suspected candidate misusers.
The study shows that chemometrics may be a valuable tool in evaluating a urinary steroid profile with respect to AAS misuse. The model has to be further developed in order to apply it routinely in doping analysis.

Acknowledgement

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