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Chemometric Evaluation of Urinary Steroid Profiles in Doping Control

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Ten endogenous steroid hormones and metabolites were determined according to the screening procedure for anabolic steroids in spot urine samples from 105 healthy young male athletes (control samples) and 23 males that tested positive for anabolic steroids in the doping control (positive samples). The GC–MS peak areas for each sample were normalized to total area. Multivariate data analysis by Partial Least Square Regression (PLSR) and using a coded Y-variable (positive samples: +1 and control samples −1) allows projection of the most systematic profile structures into a 2D plot revealing a clear distinction between the control and misuser groups. The most important determinants of the location in the loading plot were the ratios of testosterone to epitestosterone and androsterone to etiocholanolone. The ratio between 11-β-hydroxyandrosterone and 11-β-hydroxy-etiocholanolone was less important, in accordance with the fact that anabolic-androgenic steroid intake primarily affects the excretion of testosterone from the testis and to a much lesser degree adrenal steroid genesis. We present a preliminary validation of this model (PLS1-DISCRIM) for analysing steroid profiles in doping control samples from several categories of athletes, some of which are suspected for drug misuse, and results from a one dose excretion study in healthy volunteers. Our findings suggest that use of multivariate PLS-regression may give valuable information about anabolic androgenic steroid misuse in sport. When appropriately calibrated, this methodology may delineate drug misusers directly from the screening procedure for anabolic steroids in spot urine tests.

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INTRODUCTION

The misuse of anabolic androgenic steroids (AAS) in sports has necessitated the development of control procedures [1], including highly specialized laboratories using a combination of gas chromatography and mass spectrometry to detect AAS and/or their metabolites in urinary samples [2, 3]. However, paralleling the increasing efficiency of the doping control programmes, the pattern of AAS misuse has changed: xenobiotic anabolic steroids are mostly used during periods with low risk of being tested, and endogenous substances like testosterone, that are difficult to detect, are in widespread use [4–7]. In looking for methods to broaden the window of detection of AAS misuse [8], it has been suggested to consider the influence of AAS intake on the urinary steroid profile [9–13]. The fact that only a single, untimed urine sample is available for analysis in doping control, excludes the possibility of using normal excretion rates to prove misuse [14], and makes the interpretation of absolute quantitative values difficult.

The influence of AAS misuse on the production and exretion of endogenous steroids is well understood [12, 13, 15] and steroid profiling in urine has been used for endocrinological diagnostic means for many years [16]. Hence, considerations of ratios between different endogenous steroids could be a useful tool to detect the influence of AAS.

The use of multivariate data analytical methods (in particular Partial Least Squares (PLS) regression) is a powerful approach for analysis of complex data structures connected to chromatographic separations [17, 18].

In the present study we have used multivariate data analysis to evaluate the steroid profiles in urine samples from a group of subjects testing positive for anabolic

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steroids in doping control, and a large group of samples from matched controls. This is called the discrimination study below. The model that was developed was then tested on three other groups of AAS misusers.

MATERIALS AND METHODS

Study population

The misuser group consisted of spot urine samples from 23 athletic men, testing positive for a broad range of AAS in a routine doping control (Table 1), and urine samples from a control group of 105 healthy male athletic students between 18 and 35 years of age, all of them willing to give a statement that they had never used AAS.

To test the model developed we examined urine samples from three groups of individuals. Group A: five voluntary young men participating in an excretion study with 180 mg testosterone enanthate, injected intramusculary. Urine was collected 24 h after administration of the drug. Group B: four individuals testing positive for stanozolol, metandienone, nandrolone, and testosterone. Group C: four individuals that were suspected of doping with AAS, but tested negative in the routine doping control. All the samples where stored at 4°C in plastic containers until analysis.

Table 1. Some characteristic data concerning the positive samples in the discrimination study

Athlete	Drug (INN)	Sport
1	Methyltestosterone	Bodybuilding
2	Nandrolone	Powerlifting
3	Stanozolol, Nandrolone	Bodybuilding
4	Stanozolol	Bodybuilding
5	Stanozolol	Bodybuilding
6	Stanozolol	Bodybuilding
7	Testosterone	Bodybuilding
8	Drostanolone	Bodybuilding
9	Metanedienone, Nandrolone, Methyltestosterone	Bodybuilding
10	Mesterolone, Metenolone, Nandrolene	Bodybuilding
11	Stanozolol	Weightlifting
12	Nandrolone, Ephedrine	Bodybuilding
13	Nandrolone	Powerlifting
14	Metenolone	Powerlifting
15	Stanozolol	Powerlifting
16	Metandienone	Powerlifting
17	Stanozolol	Powerlifting
18	Metandienone	Powerlifting
19	Testosterone	Bodybuilding
20	Testosterone	Bodybuilding
21	Testosterone	Bodybuilding
22	Testosterone	Bodybuilding
23	Testosterone	Athletics

The nomenclature of anabolic-androgenic steroids in this table is according to World Health Organization, International Nonproprietary Names (INN) for Pharmaceutical Substances, Genève (1982).

Steroids and reagents

All chemicals not specified were purchased from Sigma Chemical Co., St Louis, MO, U.S.A.

Sample preparation

Sample preparation was done according to Donike *et al.* [2,2a] with some modifications: 1 ml of urine was added to 250 ng methyltestosterone (17α-methyl-17β-hydroxy-4-androsten-3-one), Steraloids Inc., Wilton, NH, U.S.A.) as an internal standard. A clean up on Sep-Pak(R)-C18 cartridge (Waters Millipore, U.S.A.), was done prior to enzymatic hydrolysis. The urine extract was hydrolysed with β-glucuronidase from *E. coli* (Boehringer Mannheim, Germany) in 1 ml 0.2 M sodium phosphate buffer, pH 7.0, overnight at 37°C. The hydrolysed urine was then extracted with 10 ml of pentane and evaporated under a stream of nitrogen. The dry residue was dissolved in 30 μl of *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) (Machery-Nagel, Düren, Germany)/trimethyl-iodosilane (ITMS), the MSTFA–ITMS ratio being 500:2 (v/v), containing 0.2% 2,3-dihydroxybutane-1,4-dithiol (dithioerythritol), and heated for 30 min at 70°C.

GC–MS determination

The instrument used was a Hewlett-Packard GC 5890 II/MSD 5970 with electron impact ionization at 70 eV. Column: fused silica capillary column, cross-linked methyl silicone (HP, Ultra 1), 12 m; i.d. 0.2 mm; film thickness, 0.33 μm; carrier gas, helium 1 ml/min; splitless injection; computer, HP 59944C MS ChemSystem.

Data acquisition

Detection of steroids was done in selected ion monitoring (SIM)-mode with a dwell time of 70 ms. The entire set of variables analysed for is listed in Table 2. After acquisition the peaks in the ion chromatograms were automatically integrated, manually checked and then transferred to a database. In order to control

Table 2. Mass spectrometric and chromatographic data

Compounds (variables)	Ion trace m/z	RT min*	Rsd of RT†
1 Androsterone	434	18.87	0.46
2 Etioholanolone	434	19.26	0.40
3 5α-Androstane-3α,17β-diol	256	19.74	0.19
4 5α-Androstane-3α,17β-diol	256	20.01	0.14
5 Dehydroepiandrosterone	432	22.26	0.06
6 Epitestosterone	432	23.47	0.05
7 4-Androstene-3,17-dione	430	24.61	0.04
8 Testosterone	432	25.39	0.04
9 11β-Hydroxyandrosterone	522	26.03	0.06
10 11β-Hydroxyetiocholanolone	522	26.50	0.03
17α-Methyltestosterone‡	301	28.14	0.01

*Absolute retention time, calculated as the mean of ten samples.
†Relative standard deviation of the retention time.
‡Internal standard.

hydrolysis and chromatographic performance, by means of stable retention time and sensitivity, each sample set-up contained a blank urine and a calibration mixture containing 200 ng/ml androsterone, 200 ng/ml etiocholanolone, 3 ng/ml 5α-androstane-3α,17β-diol (Mann Research-Lab. Inc., NY, U.S.A.), 3 ng/ml 5β-androstane-3α,17β-diol, 5 ng/ml dehydroepiandrosterone, 1 ng/ml epitestosterone, 3 ng/ml 4-androstene-3,17-dione, 3 ng/ml testosterone, 30 ng/ml 11β-hydroxyandrosterone and 30 ng/ml 11β-hydroxyetiocholanolone.

Data analysis

The multivariate data analytical approaches used here encompass Principal Component Analysis PCA [19, 20], PLS-regression [21, 22] and the related PLS-DISCRIM analysis. PCA results in dimensionality reduction into a low-order projected space, outlining the interobject relationships in fewer dimensions than in the original data matrix. In the present study we make use of simple two-dimensional projection spaces, score plots, from the initial 10-dimensional X-space.

The peak areas were normalized to total equal area for each individual sample before data analysis. The X-block used in the PLS-regression analysis consisted of the normalized data which matched the variables listed in Table 2. The discriminating Y-block consisted of only one variable, coded to +1 for positive sample and −1 for control sample. This is the simplest possible form of the PLS-DISCRIM dummy regression model [21–22].

We first established the PLS-DISCRIM model on the 23 + 105 sample calibration dataset.

When testing our model in experiments A, B and C, the test datasets were subsequently projected passively into the basic PLS-DISCRIM two-dimensional score space, i.e. the test data were not included in the discriminating Y-block together with the basic misuser/control set.

RESULTS

By use of the PLS-DISCRIM analysis of the measured endogenous steroidal metabolites in the urine, we have created a two-dimensional score plot (Fig. 1). In Fig. 1 we have delineated the perifery of the control samples by a connecting envelope. This plot shows an almost complete separation between these two groups of samples; only two positive samples fall within the control group. The PLS-DISCRIM analysis shows that only 28 + 9% = 37% of the total variance is captured in this plot. Another way of phrasing this would be that almost 2/3 of the total data variance in the X-matrix is not related to the discriminating Y-information, yet the powerful supervised PLS-projection technique manages to extricate the embedded, critical 37% that do correlate.

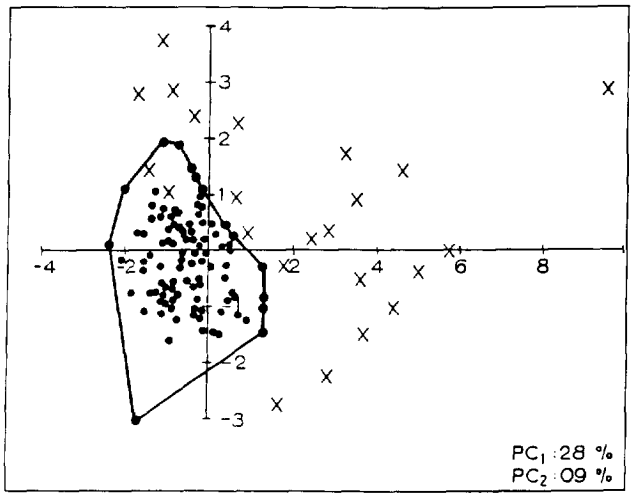

Fig. 1. Score plot from the control-misuser discrimination study. Control group (●), misuser group (×).

To visualize the relative impact of the various ratios between different metabolities, the accompanying loading plot is shown in Fig. 2, which relates to the same modelling variance fractions (28 and 9%). This plot shows that the most important ratio to distinguish between the misuser group and the control group is the androsterone/etiocholanolone ratio. Powerful information is also to be found in the testosterone/etiocholanolone ratio, and the dehydroepiandrosterone/epitestosterone ratio. The 11β-hydroxyandrosterone/11β-hydroxyetiocholanolone ratio seems to be less important. To test the reproducibility of the model we reanalysed the 16 samples that delineate the perifery of the control samples area in Fig. 1. Figure 3 shows that the new envelope created by this second analysis nearly superimposes on the first, with a slight overall

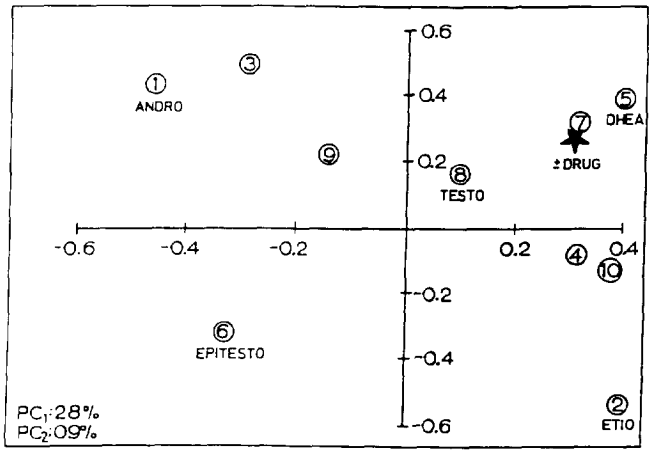

Fig. 2. Loading plot from the control-misuser discrimination study. Androsterone (①), etiocholanolone (②), 5α-androstane-3α,17β-diol (③), 5β-androstan-3α,17β-diol (④), dehydroepiandrosterone (⑤), epitestosterone (⑥) 4-androstene-3,17-dione (⑦), testosterone (⑧), 11β-hydroxyandrosterone (⑨), 11β-hydroxyetiocholanolone (⑩) ± drug (✱).

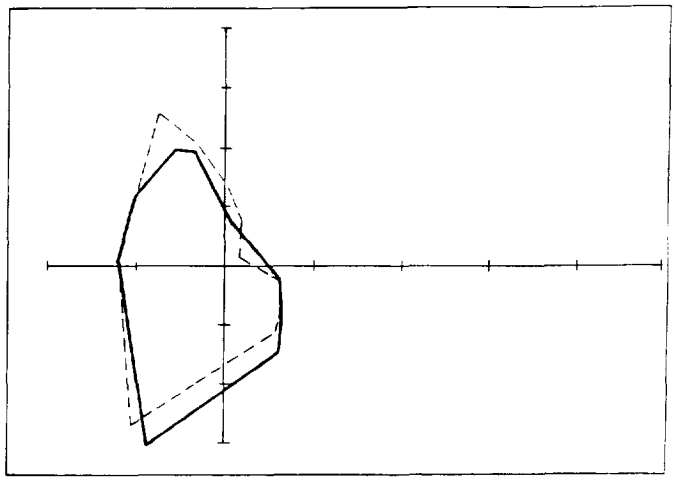

Fig. 3. Score plot showing the reproducability of the control group. Second analysis of the 16 samples that delinated the perifery of the control group in Fig. 1.

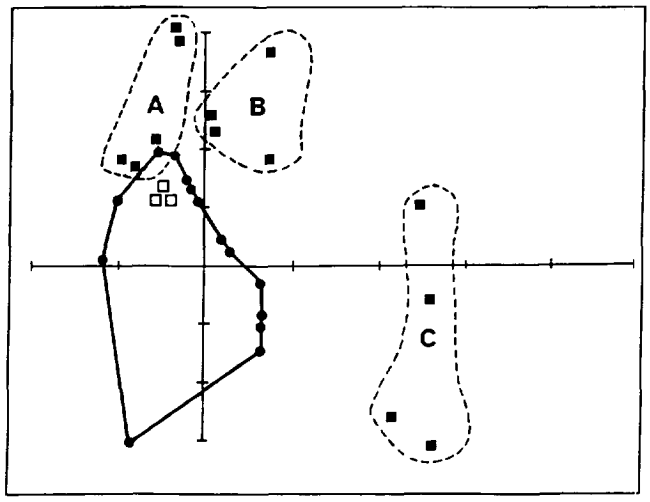

Fig. 5. Score plot showing the model tested on samples from three different groups. (A) Excretion study with 180 mg testosterone enanthate. (B) Samples tested positive for anabolic steroids. (C) Samples from bodybuilders with supressed steroidprofile, without any finding of forbidden substances. □, blank urines.

translation in the PC-2-direction. Figure 4 shows the result of a similar re-analysis of some positive samples from the AAS-misusers. They show some differences with respect to the first analyses, but are all located clearly outside the envelope delineating the control sample area.

Figure 5 shows the results from testing this model in three different experiments. (A) After injection of 180 mg testosterone enanthate, two of five individuals were clearly located outside the control sample area. (B) Samples from four additional individuals testing positive for AAS were also clearly located outside the control sample area. (C) Samples from four bodybuilders which tested negative in doping control, but nevertheless were suspected for AAS misuse, because of the extreme low amount of excreted endogenous steroids, showed a severely disturbed steroid profile. These samples were located outside the control sample area.

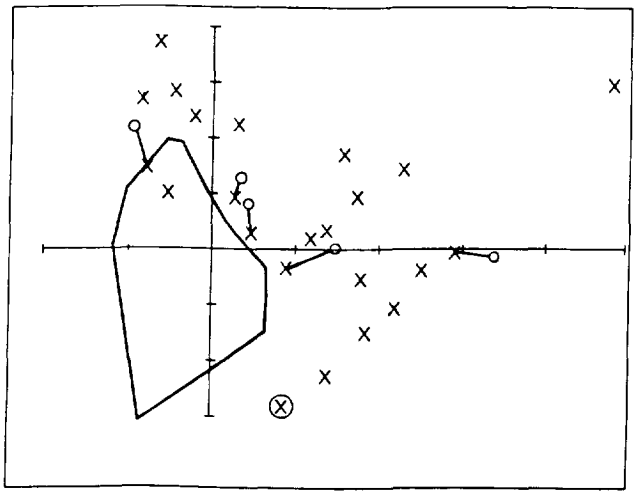

Fig. 4. Score plot showing the reproducability of the misuser group. Misuser group (×), second analyses of 6 samples in the misuser group (○).

DISCUSSION

The influence of AAS misuse on the excretion of endogenous steroids is well known. The typical pattern of steroid excretion after AAS misuse is elevated ratios between testosterone/epitestosterone and between androsterone/etiocholanolone, together with a low total excretion of testosterone and metabolities. It has been suggested to make use of this type of information in doping control, when a sample shows a clear disturbance in the excretion pattern of endogenous steroids. To take into account natural variations in steroid profile, instrument variations and large amounts of data, the use of modern multivariate data analysis (PCA and PLS-regression) makes it possible to handle a large body of digital information simultaneously. It is now possible simultaneously to consider the entire set of ratios between all important steroids in a urine sample.

This has been done in the present study, which for the first time presents a PLS-discrimination plot which enhances the misuse fraction of the total data variance. In addition, we have used this as a tool to visualize the relative importance of the different ratios measured (loading plot, Fig. 2). However almost 2/3 of the total data variance in the ten X-variables, is not correlated to known misuser behaviour. This means that direct comparison of the ratios would not only have incorporated very large parts of this uncertainty, but would have had to be carried out only one ratio at a time. Neither of these approaches can be considered satisfac-

tory. The score plot in Fig. 1 on the other hand, gives a clear separation between the samples from AAS misusers and the control samples. Only two individuals were not correctly classified, i.e. two AAS misusers not discriminated.

The samples from control and the misuser groups could not be separated with any planar construction, indicating an asymetrical classification problem. This was the reason why we rejected the possible use of linear discriminant analysis (LDA).

The present discrimination procedure will exclude all possible type I misclassifications (i.e. never accepting known control group members in the misuser field). Therefore any new control group sample that falls outside the present field, will automatically result in an enlargement of the control sample area, at the expense of the misuser boundary. This is a deliberate aspect of our PLS-DISCRIM design philosophy.

Figure 2 shows that the ratio between androsterone/etiocholanolone and testosterone/epitestosterone as well as dehydroepiandrosterone/epitestosterone are the most important ratios that discriminate between misusers and the control fields. This is not surprising since AAS affects the production of testosterone in the testis directly and indirectly through suppression of pituitary LH secretion. The ratio of 11β-hydroxyandrosterone/11β-hydroxyetiocholanolone is not so important, for the discrimination, which is in accordance with the fact that adrenal steroidogenesis is to a much lesser degree affected by AAS misuse.

Application of multivariate evaluation of steroid profiling in routine laboratory practice must be easy to use. It must be easy to include this type of data analysis as an integral part of the routine screening for anabolic steroids, because it will be too time consuming to do a new injection. By only using normalized peak areas in the present study, we circumvent all problems associated with absolute quantification, but miss some of the possibilities to take into account historical data based on absolute data.

The differences in steroid concentrations cover a wide range, and it is therefore important not to overload the GC-column. Some results seem to indicate that a normal steroid profile may drop into the abuser area if this is the case. In Table 2 the relative standard deviation of the retention time for androsterone and etiocholanolone is several times the relative standard deviation for testosterone and epitestosterone, which may indicate column overload. Even though this is only a possibility at this stage, clearly this uncertainty needs our further attention.

The replicate analysis presented in Fig. 3 also needs to be established on a larger data base. At present there is no universal agreement upon a statistical method for testing the overall discriminating strength when more than one discriminant index is used, as when using to PLS-components in the present case. This is worked upon by our group as well as others. We have instead here presented a sensitivity analysis of the discriminating boundary between the misuser group and the control group based on a second analysis from a new aliquot of the sample, that shows quite satisfactory stable relationships, especially along the major PC-1 discrimination direction. In Fig. 4 the inclusion of the four body-builders in the misuser-group is due to the fact that among analysts in doping laboratories they are often recognized as having abnormal urinary steroid profiles, although testing negative for anabolic steroids. It is widely assumed that this is due to the fact that they have stopped taking drugs before announced testing. It is therefore interesting that they fell in the misuser group when tested in our model.

A methodology for AAS misuser discrimination based on a simultaneous 10-AAS multivariate PLS-discrimination analysis has been developed. It is possible to successfully discriminate 21 known misusers within this first data base consisting of 23 misusers and 105 controls. Testing the model in small groups of misusers gives interesting findings, but needs to be confirmed in larger groups.

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