Article scientifique concernant les performances de l'analyse discriminante pour l'interprétation des résultats de l'analyse EPO

Françoise Lasne[1*]
Jean Thioulouse[2*]
Laurent Martin[1]
Jacques de Ceaurriz[1]

[1]Agence française de lutte contre le dopage, Département des analyses, Châtenay-Malabry, France
[2]Université de Lyon, Université Lyon I, Laboratoire de Biométrie et Biologie Evolutive, Villeurbanne, France

Received June 13, 2006
Revised December 14, 2006
Accepted December 15, 2006

Research Article

Detection of recombinant human erythropoietin in urine for doping analysis: Interpretation of isoelectric profiles by discriminant analysis

The detection in urine of recombinant human erythropoietin (rHuEPO), a hormone misused by endurance athletes as a doping agent, is based on the differentiation of its isoelectric pattern from that of the corresponding natural hormone. Different empirical criteria have been proposed for discriminating the images of the patterns but none of them have been elaborated from a rational statistical approach. Discriminant analysis was applied to a dataset of profiles defined as positive (116 profiles from 26 subjects) (presence of rHuEPO and possibly residual natural endogenous hormone) and negative (131 profiles from 131 subjects) (presence of natural endogenous hormone only). The different bands were numbered according to a template of 16 possible positions and their relative intensities constituted the 16 variables of the statistical analysis. This method was then tested with data from an administration trial of low doses (6.7–10 IU/kg) following high-dose (265 IU/kg) injections (71 profiles from one subject). The analysis of the dataset clearly separated the negative and positive profiles. A cross-validation procedure confirmed that the analysis was extremely stable: with ten-fold cross-validation, no false positives were observed even with 100 000 simulations. Furthermore, the detection of rHuEPO in the profiles from the low-dose trial was greatly improved in comparison with a previously validated empirical criterion.

Keywords:
Discriminant analysis / Doping / Erythropoietin / Isoelectric profiles
DOI 10.1002/elps.200600363

1 Introduction

Erythropoietin (EPO), a glycoproteic hormone produced by the adult human kidney, stimulates red blood cell production. The recombinant form of this hormone quickly became misused by endurance athletes as a doping agent to improve aerobic performances, and the International Olympic Committee officially prohibited it in 1990. The first antidoping control for this hormone was performed only in 2000, however, at the Sydney Olympic Games. At this time, both indirect blood parameters showing a stimulation of red cell production and a direct detection of rHuEPO in urine were required to report an adverse finding in antidoping control. Since 2002, the direct detection of rHuEPO in urine is the necessary and sufficient condition for this.

Detection relies on the differentiation of natural endogenous and recombinant exogenous EPO by their isoelectric profiles. Whereas the former is composed of a great number of bands located in the pH range of 3.7–4.7, the latter is composed of six bands located in the pH range of 4.4–5.1 and 3.7 – 4 for epoetin α or β and darbepoetin α (a hyperglycosylated analogue of epoetin), respectively. Since the injected recombinant hormones are recovered in urine with a practically unmodified isoelectric profile, they give rise to typical patterns that signal their presence. The images corresponding to the EPO banding patterns are thus the basis for characterizing this hormone in the analyzed urine samples [1]. An initial visual evaluation of the image permits an efficient diagnosis; however, in order to objectify the attributes of the profiles, different numerical data analyses have been proposed and have led to several 'criteria for positivity' to ascer-

Correspondence: Dr. Françoise Lasne, Agence française de lutte contre le dopage, 143, avenue Roger Salengro, F-92290 Châtenay-Malabry, France
E-mail: f.lasne@afld.fr
Fax: +33-1-46603017

Abbreviations: EPO, erythropoietin; **rHuEPO,** recombinant human erythropoietin

* These authors contributed equally to this study.

tain the presence of recombinant hormone in a urine sample. All of them must guarantee an infallible identification of any 'negative' profiles (*i.e.* corresponding to natural endogenous hormone) to prevent 'false positive' misinterpretation. On the other hand, they aim to be as sensitive as possible for detecting recombinant hormones in nonpatent cases (low-dose injections and/or progressive disappearance from urine with time). Till now, the numerical criteria have been empirically established from the observation of a great number of profiles corresponding to excretion studies of recombinant hormones and to investigations of various control populations. In order to optimize their numerical characterization, we submitted different types of profiles to discriminant analysis.

2 Materials and methods

2.1 Sample analysis

The isoelectric profiles of EPO in urine were monitored through the IEF and 'double blotting' methods previously described [2]. Briefly, urine samples (20 mL) were submitted to ultrafiltration using membranes with a molecular mass cutoff of 30 000 Da in order to concentrate the hormone in the retentate. This retentate was assayed for its EPO level by ELISA (human EPO Quantikine IVD from R&D Systems) and submitted to IEF (pH gradient of 2–6). The catholyte used for the IEF run, 2% w/v 6–8 carrier ampholytes, was enriched with 2% w/v methyl red. This dye migrated during the run and was finally located in the position of its apparent p*I* (4.3) in the 7 M urea gels used, as a red line reflecting all the possible disturbances of the pH gradient encountered in the different lanes of the gel. A digital photograph of this red line was stored. The EPO isoforms were specifically revealed by the double-blotting method [3] using monoclonal antihuman EPO AE7A5 from R&D Systems. The final result was a chemiluminescent image of these isoforms obtained with a LAS-1000 plus a CCD camera (Fuji).

Two reference preparations, Biological Reference Preparation (BRP batches 1 and 2a) from the European Pharmacopoeia Commission (an equimolecular mixture of EPO (rHuEPO): epoetin α and β) and darbepoetin α (NESP) from Amgen were mixed and systematically included in the IEF runs so that they were present in all of the analysed images as position markers.

2.2 Establishment of the profiles

The profiles resulted from the evaluation of the luminescent images by the AIDA (Advanced Image Data Analyser) 1D (v. 3.44) software from Raytest.

A densitometer window (5 mm width) was drawn so that it included the most basic band of BRP and the most acidic band of NESP and was positioned on each of the different lanes.

Baselines of the different lanes were drawn valley-to-valley for each peak in a profile, and the percentage of the peak integral reduced by the background intensity in comparison with the sum of all the peaks of the profile was recorded as its 'relative intensity' (RI).

Numbers were assigned to the bands from band 0, corresponding to the most acidic band of BRP. The numbers from 1 to 6 were assigned to the more basic bands and from −1 to −9 to the more acidic ones (Fig. 1). In the cases of curved bands in a lane due to disturbance of the pH gradient by the corresponding sample, the photograph of the methyl red line was used to identify band 0 (Fig. 2).

For each of the patterns, the relative intensities corresponding to each of the 16 possible bands (from −9 to 6) were stored in 16 columns of an Excel file, one row corresponding to one profile.

All the profiles were first evaluated for their so-called 'percentage of basic isoforms', which was the numerical parameter initially validated to detect the presence of recombinant Epoetin in urine when the method was developed. The 'basic isoforms' were from 1 to 6 and corresponded to the bands colocalised with the BRP bands apart from band 0. The % of basic isoforms was thus the sum of the relative intensities of bands from 1 to 6. From previous statistical analysis of control populations, a cutoff value of 80% for this parameter excluded the possibility of natural endogenous EPO and was validated as a criterion for positivity (unpublished data).

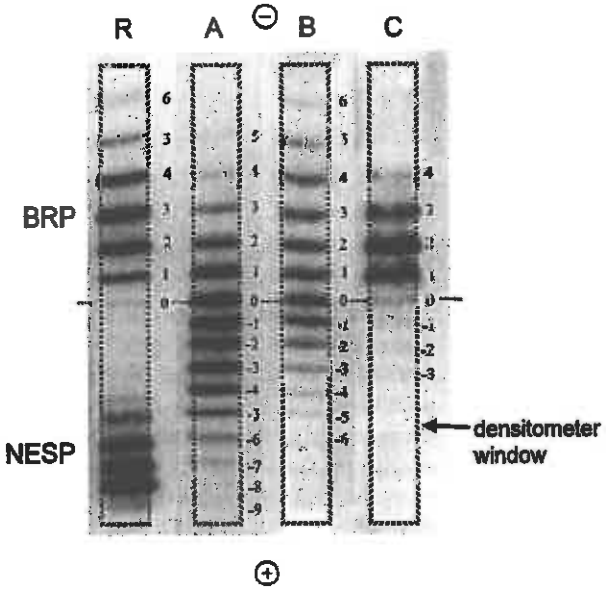


Figure 1. Assignment of band numbers. Bands were numbered from band 0, corresponding to the most acidic band of BRP in the reference lane (R), with positive and negative values for the more basic and more acidic bands, respectively; lane A: negative pattern with typical natural urinary endogenous EPO, B: negative pattern with atypical aspect as observed after some particularly strenuous physical exercise, C: positive pattern corresponding to the urinary excretion of epoetin α.

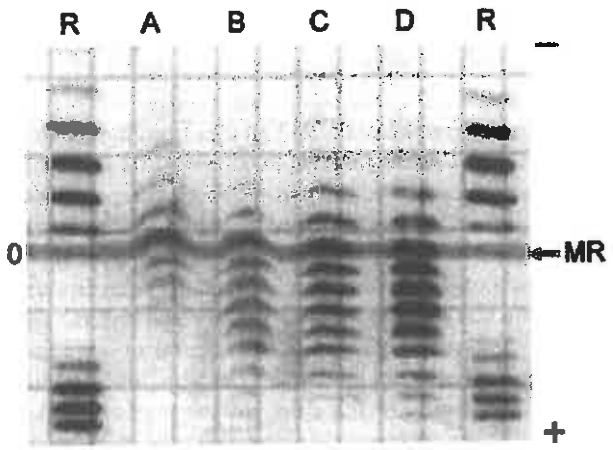


Figure 2. Use of methyl red for the identification of band numbers. The photograph of the red line corresponding to methyl red (MR) in the IEF gel at the end of the run is superimposed with the EPO banding patterns. This photograph is positioned so that the red line goes across the most acidic bands (band 0) of BRP in the different reference lanes (R). This line automatically connects all the bands 0 of the different lanes whether the migration has been disturbed (A, B) or not (C, D).

2.3 Selected profiles for the discriminant analysis

This statistical study was first conducted on a reference dataset corresponding to 247 profiles previously identified as positive ($n = 116$) or negative ($n = 131$) for the presence of rHuEPO.

Out of the 116 positive profiles, 37 were observed in urine samples from seven healthy volunteers during 4 wk of treatment (50 IU/kg, three times *per* week) followed by 2 wk of treatment at a lower dose (20 IU/kg, three times *per* week), 61 were from one healthy volunteer during 10 days of treatment (250 IU/kg, six times) followed by 2 wk of treatment at a lower dose (13 IU/kg, four times *per* week), and 18 were from antidoping control analysis having been classified as positive by the percentage of basic isoforms criterion. The different administration trials had been approved by the ethics committee of Montpellier hospital (France) and all the subjects had granted written informed consent before study initiation.

Negative profiles ($n = 131$) were obtained from 131 individual subjects. They were composed of 50 typical natural urinary endogenous EPO profiles (referred to as 'typical') and 81 profiles presenting some shift towards basic pH, as observed in urine samples collected after some particularly strenuous physical exercise (referred to as 'atypical').

All of the typical and atypical profiles were classified as negative for the presence of rHuEPO by the percentage of basic isoforms criterion. Examples of a 'positive' pattern and the two types of negative pattern are given in Fig. 1.

The specificity of the method was illustrated using an additional set of 105 profiles obtained from 56 subjects who had never received recombinant EPO injections.

The capability of the discriminant analysis to detect the presence of low rates of recombinant EPO in a profile (sensitivity) was tested by comparison with the percentage of basic isoforms criterion on 71 profiles from an administration trial composed of a first set of five 'high-dose' (265 IU/kg) injections followed by a second set of seven 'low-dose' injections (5–10 IU/kg) of epoetin α in one subject. These profiles corresponded to urine samples collected during periods ranging from 2 to 5 days following the different injections.

2.4 Discriminant analysis

The datasets were submitted to discriminant analysis [4] using the linear discriminant analysis (lda) function of the R statistical software environment [5]. The aim of this method is to best separate two or more classes of objects, and then to classify new objects into these classes. The use of the lda function is explained thoroughly by Venables and Ripley [6], and only a brief explanation is given here. The data table is made of p variables (columns) measured on n objects (rows). These objects belong to g classes. Let W be the within-class covariance matrix, and B the between-class covariance matrix. Let M be the $g \times p$ matrix of class means, and G be the $n \times g$ matrix of class indicator variables ($g_{ij} = 1$ if and only if case i is assigned to class j). Let $\bar{x}$ be the means of the variables over the whole sample.

$$W = \frac{(X - GM)^{\mathrm{T}}(X - GM)}{n - g} \tag{1}$$

$$B = \frac{(GM - 1\bar{x})^{\mathrm{T}}(GM - 1\bar{x})}{g - 1} \tag{2}$$

Linear discriminant analysis seeks linear combinations xa of the p variables that have a maximal ratio of the separation of the class means to the within-class variance, *i.e.* maximising $\frac{a^{\mathrm{T}}Ba}{a^{\mathrm{T}}Wa}$. These (3) combinations are called linear discriminant functions, and they can be used both to represent the original dataset and to assign new objects to the classes.

The 247 observations in the reference table were initially classified into three groups corresponding to positive profiles ($n = 116$) and the two negative sub-groups of typical ($n = 50$) and atypical ($n = 81$).

The analysis of this known reference table allowed the calibration of the method and the construction of a model to which unknown profiles could be compared. The percentage of misclassifications was computed by bootstrap simulation [7] and the validity of the model, particularly the absence of false-positives, was tested by ten-fold cross-validation, as follows: one-tenth of the data were drawn at random from the full dataset and used to compute a new model. The remaining nine-tenths were used as 'new' profiles to validate this model, and all the misclassifications were recorded. The

whole procedure was performed a great number of times (drawing one-tenth of the data each time), and the total number of misclassifications was noted. These bootstrap and cross-validation procedures were performed both considering or not that some of the positive profiles were not derived from different individual subjects. The results were identical, which means that the possible bias introduced by the fact that several samples came from the same individual was negligible.

A classification procedure was then designed to allocate new profiles to a group with a known probability, using the 'predict' function of the MASS package for R [5]. Since this function of classification was intended to be used for antidoping control, specific requirements were taken into account. In particular, in order to prevent any false-positives, a high probability threshold (probability less than 1/10 000 to be negative) was programmed to validate a positive classification. This threshold was not applied for the validation of negative cases. Since there is no need to differentiate typical and atypical groups in antidoping control, these groups were pooled together as negative profiles in the final result.

In addition, the procedure provided graphical outputs illustrating the classification of the profiles. All these procedures were developed for the R statistical software environment, and they are freely available from the authors.

3 Results

3.1 Reference dataset analysis

As shown by the plot of the 247 profiles from the reference table on the two linear discriminant axes (LD1 × LD2), the positive profiles on the left part of the plot were very well separated from the two other groups, while typical (bottom) and atypical (top) profiles slightly overlapped (Fig. 3). The percentage of misclassification computed by the bootstrap procedure was equal to 2.5% for typical *versus* atypical negative profiles, but no false positives (typical or atypical *vs.* positive profiles) could be found, even with 10 000 random draws. The results of the cross-validation procedure confirmed that the analysis was extremely stable: with ten-fold cross-validation, no false-positive was observed with 100 000 simulations.

3.2 Classification procedure

Figure 4 gives an example of the allocation procedure outputs. The report first indicates the probabilities of the allocations (A). In this example, the probability that profile 1 belongs to the positive group is 0.998563 and the probability

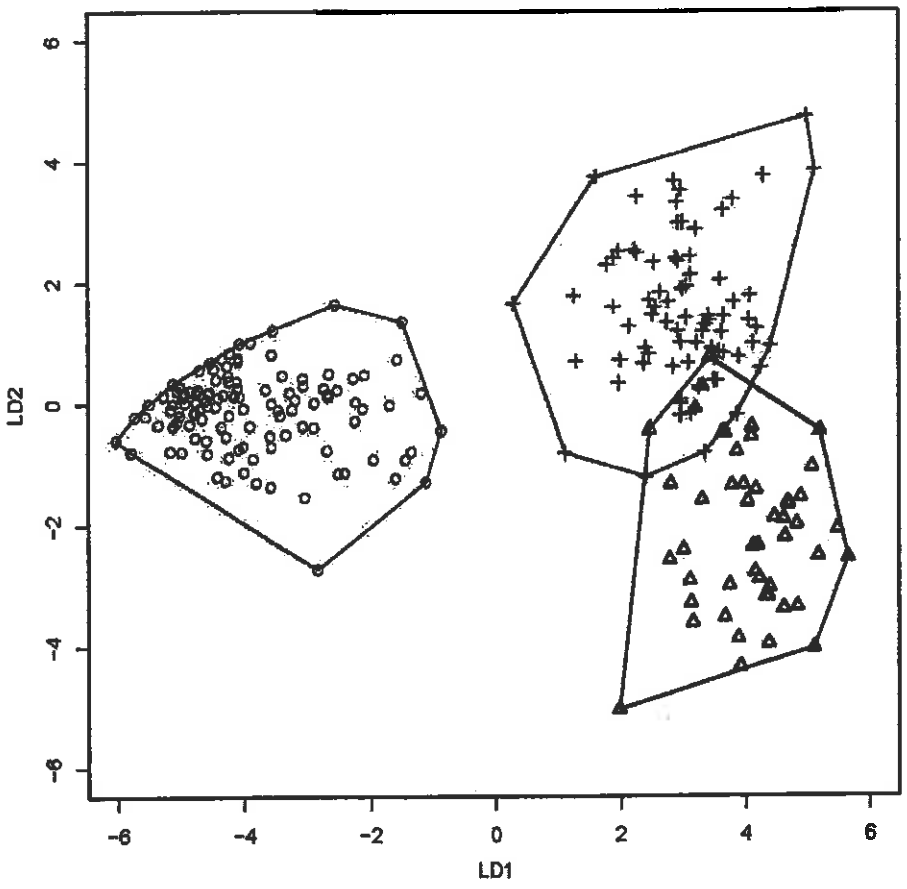


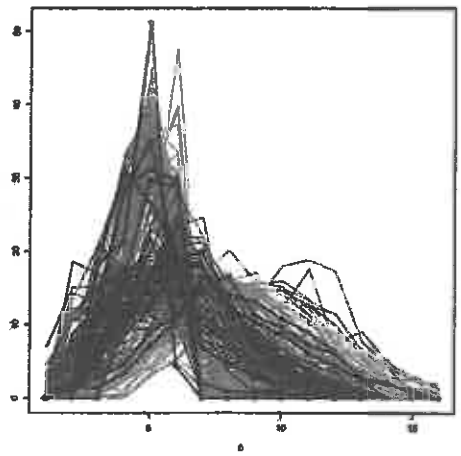
Figure 3. Plot of the 247 samples of the reference table on the two linear discriminant axes (LD1 × LD2). (○): positive; (△): negative typical; (+): negative atypical.

A

```
Number of unclassifiable samples : 1
              Prob(+)          Prob(-)
N01           0.9986           0.0014368
------------------------------
Number of positive samples : 5
              Prob(+)          Prob(-)

N02           1                1.722e-11
N03           1                2.590e-12
N04           1                2.214e-14
N05           1                1.546e-08
N06           1                4.697e-20
------------------------------
Number of negative samples : 8
              Prob(+)          Prob(-)
N07           0.002030         0.9980
N08           0.000286         0.9997
N09           2.674e-12        1
N10           4.188e-08        1
N11           1.032e-11        1
N12           9.328e-16        1
N13           1.427e-16        1
N14           1.043e-12        1
```

B



C

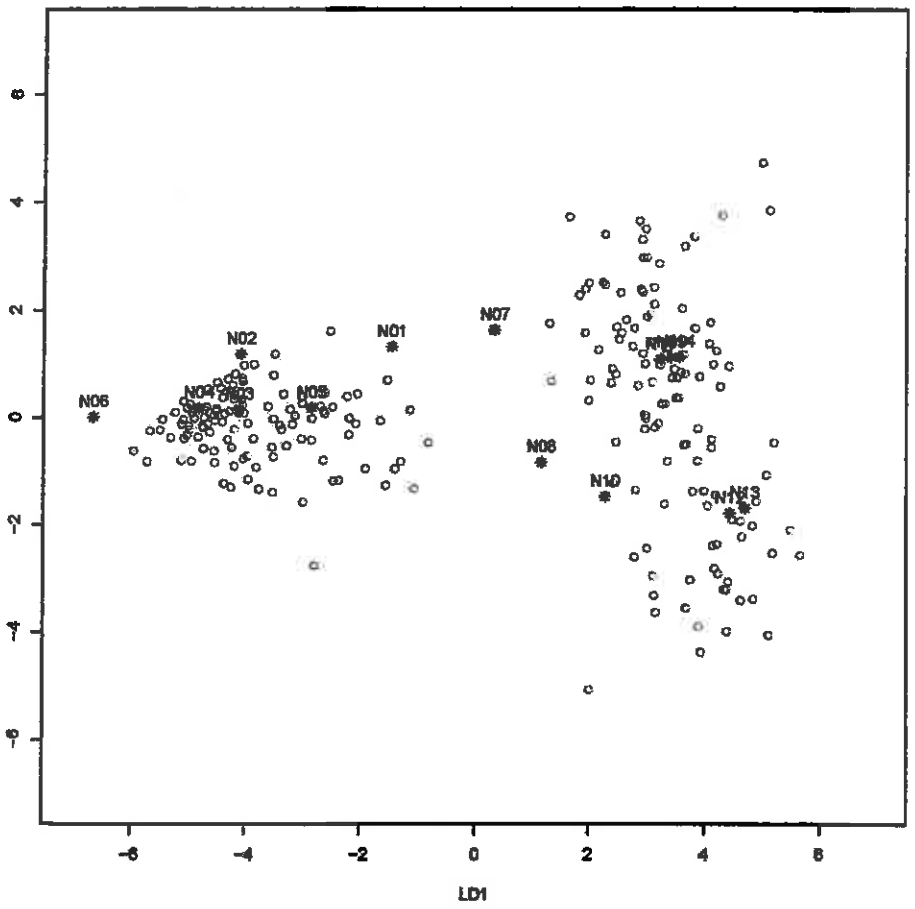


Figure 4. Outputs of the procedure to allocate interpreted profiles to the groups of negative or positive. (A) Probabilities of the allocations, (B) Curves corresponding to the profiles of interpreted profiles (black) superimposed to the reference curves (red: positive, green: 'typical negative', blue: 'atypical negative'), (C) Projection of the interpreted profiles on the two linear discriminant axes.

that it belongs to the negative group is 0.001437 (the sum of probabilities is equal to 1). Since the probability to be negative (0.001437) is more than 1/10 000, this profile is said to be 'unclassifiable'. Profiles from 2 to 6 are classified as positive as they have probabilities of being negative of less than 1/10 000. Profiles from 7 to 14 are classified as negative since the probabilities of their being negative are greater than of being positive. In this group, no additional threshold is imposed for acceptance of the classification, as is the case in the group of positive profiles: 7 and 8 are classified as negative even though their probability of being positive is more than 1/10 000.

Two graphical outputs illustrate the allocation of the interpreted profiles. In the first one, the profiles are displayed as curves connecting the 16 points corresponding to the relative intensities of the 16 possible bands. The curves corresponding to the interpreted profiles are superimposed on the collection of the curves corresponding to the reference profiles (B). The second graphic is a projection of the interpreted profiles on the linear discriminant (LD) axes (C). These graphics allow the consistency of the allocation of the interpreted profiles to the two groups to be checked visually.

3.3 Specificity of the classification

The specificity of the discriminant analysis was demonstrated by the cross-validation procedure of the reference dataset analysis. On the other hand, as an illustration of the specificity, all of the 105 profiles obtained from the additional set of 56 subjects safe from any injection of recombinant EPO were reported as negative by the classification procedure.

3.4 Sensitivity of the detection of rHuEPO

The classification of the profiles observed during the 2–5 days period following the different injections of the administration trial was conducted using both the percentage of basic isoforms criterion and discriminant analysis. Of the 71 observed profiles, 34 *versus* 50 were classified positive, 13 *versus* 5 were unclassifiable and 24 *versus* 16 were classified negative by the percentage of basic isoforms criterion and discriminant analysis, respectively, thus demonstrating the greater sensitivity of the classification by the latter method (Fig. 5).

4 Discussion

Since natural endogenous EPO is physiologically present in urine, an absolute differentiation of this hormone from recombinant exogenous EPO is a requisite for any analysis used in the context of antidoping control. The method validated by the International Olympic Committee (IOC) and the World Anti Doping Agency (WADA) authorities for antidoping control relies on the characterization of the isoelectric profile of this hormone in urine. The result of the analysis is an image composed of several bands. This image is converted into numerical data corresponding to the positions and relative intensities of the bands (profile). Although in most of the cases an obvious interpretation is suggested by the image, objective criteria using numerical data of the profile are required to ensure the reliability of interpretation.

Initially, a ratio referred to as the percentage of basic isoforms was empirically established from the observation of typical images and was proposed to differentiate natural and recombinant EPO profiles. A threshold value calculated from the statistical analysis of data from control populations were validated as the criterion for positivity (*i.e.* excluding the possibility of natural EPO). Other combinations of numerical data have since been empirically developed, such as a 'two-band ratio', and proposed to characterize the profiles [8].

The approach related here used discriminant analysis of typical profiles in order to optimize their differentiation. The aim of this statistical method is to describe the differences between groups of observations (discrimination), and to use this description to allocate new observations to the groups (classification). For this, all the variables of the dataset (and not only a few of them) are used and a linear combination of these variables is computed. This linear combination possesses the mathematical property of a maximal ratio of between-group to within-group variance. Discriminant analysis requires a sufficient number of observations compared with the number of variables. In the present study, 247 observations (profiles) of 16 variables (relative intensities of the 16 possible bands) were chosen as a reference dataset ensuring good numerical conditions.

Care was taken in the selection of the negative (absence of rHuEPO) profiles to include the cases of both typical natural EPO and atypical profiles. It should be noted that the proportions of typical and atypical profiles selected for the negative dataset do not reflect their occurrence in real con-

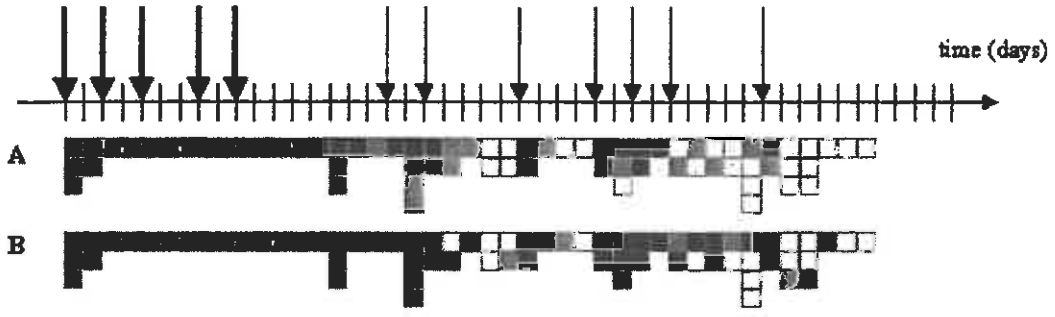


Figure 5. Classification of profiles observed during the administration trial. The thick arrows indicate injections of 265 IU/kg and the thin ones, injections from 5 to 10 UI/kg. The rectangles below the axis correspond to the profiles classified by % of basic isoforms (A) and discriminant analysis (B) Black Square, positive; gray square, unclassifiable; white square, negative. Some urine samples were taken at different times on the same day.

ditions of antidoping control analysis. Most of the urine samples, though taken in competition just after a physical exercise, give rise to typical natural EPO profiles. Only a few of them produce profiles referred as to atypical in this article. The appearance of such profiles in some particular conditions of strenuous exercise deserves additional study. Whatever the origin of this phenomenon, it was essential to ensure that atypical profiles, though presenting more overlapping bands with the recombinant hormone than typical natural ones, were clearly differentiated from positive profiles (presence of rHuEPO) by discriminant analysis, as was the case with the previously validated percentage of basic isoforms criterion. The stability of the results demonstrated by cross-validation of the reference data was highly satisfactory.

The program developed from these reference data appears to be a reliable and sensitive tool for interpreting new profiles. The use of a conservative threshold of probability for the validation of a positive result is a guarantee against false-positives. On the other hand, the greater sensitivity of this classification in comparison with the previously validated criterion ensures a better detection of low doses of rHuEPO. This is of particular interest in view of the allegation that athletes escape detection by reducing the injected doses [9].

The proposed program has been elaborated for detecting epoetin α and β but it may be expanded for the detection of other types of EPO drugs. A small number of profiles observed following the injection of darbepoetin α (NESP) were tested with the present version of the program (data not shown). When recovered in the urine from injected subjects, this hyperglycosylated rHuEPO analog presented a clearly more acidic isoelectric profile (Fig.1). The clearcut isolation of these profiles on the linear discriminant axes, away from the groups of negative and 'positive to epoetin α and β', suggests very easy identification. The reference dataset is going to be completed with such profiles. Another recombinant hormone, epoetin ω, and a different type of EPO that is produced by gene activation (epoetin δ, Dynepo) will be the subjects of a study in the near future. Broadly speaking, the diversification of EPO drugs requires adapted criteria for antidoping control analysis. The statistical approach presented here appears to be a convenient and powerful response to this problem.

The program proposed for interpreting the profiles was developed from the R statistical software environment [5] which is freely available under the terms of the GNU General Public License as published by the Free Software Foundation (GNU General Public License http://www.gnu.org/copyleft/gpl.html). The procedures described here are also freely available, and can therefore be used by all antidoping control laboratories. Of course, the interpreted data must first be comparable with the reference dataset. This implies perfect standardization of the images and of corresponding integrated profiles.

From our data, the discriminant analysis appears to be a very high-performance method for classifying EPO profiles, preventing false-positive interpretations, and improving the detection of rHuEPO in not obviously patent cases.

EPO administration trials were partially supported by grants from the World Anti Doping Agency (WADA) and were conducted as a collaborative study from the Science and Industry Against Blood doping (SIAB) research consortium. The authors wish to thank Professor Michel Audran and Professor Christian Préfault from University Montpellier 1 as supervisors of the trials and Dr. Michael Ashenden from SIAB as a project coordinator.

5 References

[1] Lasne, F., de Ceaurriz, J., *Nature* 2000, *405*, 635.

[2] Lasne, F., Martin, L., Crepin, N., de Ceaurriz, J., *Anal. Biochem.* 2002, *311*, 119–126.

[3] Lasne, F., *J. Immunol. Methods* 2001, *253*, 125–131.

[4] Fisher, R. A., *Ann. Eugen.* 1936, *7*, 179–188.

[5] R Development Core Team, *R: A Language and Environment for Statistical Computing*, R Foundation for Statistical Computing, Vienna, Austria. ISBN: 3-900051-07-0, 2005; http://www.r-project.org.

[6] Venables, W. N., Ripley, B. D. (Eds.), *Modern Applied Statistics With S*, 4th Edn., Springer, New York 2002, pp. 331–351.

[7] Tomassone, R., Danzard, M., Daudin, J., Masson, J. P. (Eds.), *Discrimination et Classement*, Masson, Paris 1988, pp. 80–90.

[8] Breidbach, A., Catlin, D. H., Green, G. A., Tregub, I. *et al.*, *Clin. Chem.* 2003, *49*, 901–907.

[9] Ashenden, M., Varlet-Marie, E., Lasne, F., Audran, M., *Haematologica* 2005, *91*, 1143–1144.