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Project For Inter-Laboratory Comparison of the Method for the Detection of rhEPO in Human Urine. Results, Conclusions and Current Status.

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Introduction

Early in 2000, a method for the detection of recombinant erythropoietin (rEPO) in human urine was first introduced by F. Lasne et. al.(1) . The method is based on the separation of the different isoforms of EPO (both endogenous and exogenous) using isoelectric focusing (IEF) followed by a double blotting process(2) and chemiluminescent detection.

After using the method for the analysis of more than 600 samples, its selectivity and specificity was checked by blind analysis of known negative (population) and positive samples from EPO administration studies before the Sydney 2000 Olympic Games. As a result, the method was used during those Games in combination with another complementary screening method based on the analysis in blood and serum of different haematological parameters affected by the administration of rEPO(3).

Because the urinary method is complex, it has required considerable training and practice to successfully establish the method in other doping control laboratories. However once the method was operational in several laboratories it was possible and desirable to carry out a full validation of the method including both intra and inter-laboratory assays. Such study will assist in fully characterising the method by determining the set of parameters which can affect the results.

A protocol was written from the coordinating laboratory and submitted to those laboratories having the method already running in their facilities. At that time (January, 2001), four

laboratories had already been trained by the developing laboratory (LNDD, Châtenay-Malabry, France) and had been checking the method in their own facilities. Thus the laboratories, able to participate in a potential validation protocol were:

LAB	DESCRIPTION
PARIS	Laboratoire National de Depistage du Dopage (LNDD) Châtenay-Malabry, France
BARCELONA (coordinator)	Doping Control Laboratory, Pharmacology Research Unit Institut Municipal d'Investigació Mèdica (IMIM) Barcelona, Spain
SYDNEY	Australian Sports Drug Testing Laboratory (ASDTL) Pymble, Australia
OSLO	Hormone Laboratory. Section for Doping analysis. Aker hospital Oslo, Norway
LAUSANNE	Laboratoire Suisse d'Analyse du Dopage. Institut Universitaire de Médecine Légale. Lausanne, Switzerland

The protocol was submitted to the Medical Commission of the IOC and funding was granted. A final report was prepared and submitted to the sponsor [4].

Experimental

The protocol was divided in two major steps:

- During the first step, a common description of the standard operating procedure for the method was agreed. The identification of reagents considered as critical for the performance of the method was also carried out. As part of that step, further decisions were taken, like the establishment of the rEPO reference standard, the definition of the "marker" that was going to be used to draw conclusions on the presence of rEPO, etc. Also the already existing data gathered by the team developing the method[1] was also evaluated in order to reach final conclusions regarding selectivity and specificity. In addition, a protocol for a simple intra-laboratory validation protocol was also proposed in order to check for intra-laboratory reproducibility and limit of detection.
- During the second step, the inter-laboratory comparison was carried out using the following scheme:

  Reference urine samples were prepared by the National Analytical Reference Laboratory (NARL) of Australia. A pool of blank urine (not containing rEPO) with an amount of EPO, as measured by immunoassay around 3 IU/L was prepared. Samples with increasing concentrations of rEPO were then prepared by spiking the blank urine so that the final rEPO concentrations were: 0 (blank urine), 1.5, 3. 10 and 20 IU rEPO/L.

Reference urines were divided in 40 ml aliquots. 5 replicate aliquots of each urine sample were sent to each participating laboratory (5 x 5 = 25 urine samples), blind coded.

Results and discussion

rEPO Reference Standard

The agreed rEPO Reference Standard was:

Erythropoietin BRP (Batch No.1, reference number E1515000), European Pharmacopoeia Commission. E-mail: **CRS@mail.pheur.org**. Website: http:www.pheur.org.

The quality of the standard is recognised by the European Directorate for the Quality of Medicines and the Council of Europe.

Each vial, of a freeze-dried preparation of erythropoietin, contains 250 μg and 32,500 IU of biological activity.

The Marker

The need for a surrogate marker (magnitude to be measured) as a result of the application of the procedure was recognised. The same "marker" as used during the application of the method so far was agreed. The marker, **"% of basic isoforms"** is defined s follows:

$$\textbf{\% basic isoforms} = \frac{\textbf{sum of areas of basic isoforms}}{\textbf{sum of areas of all isoforms}} \cdot 100$$

where:

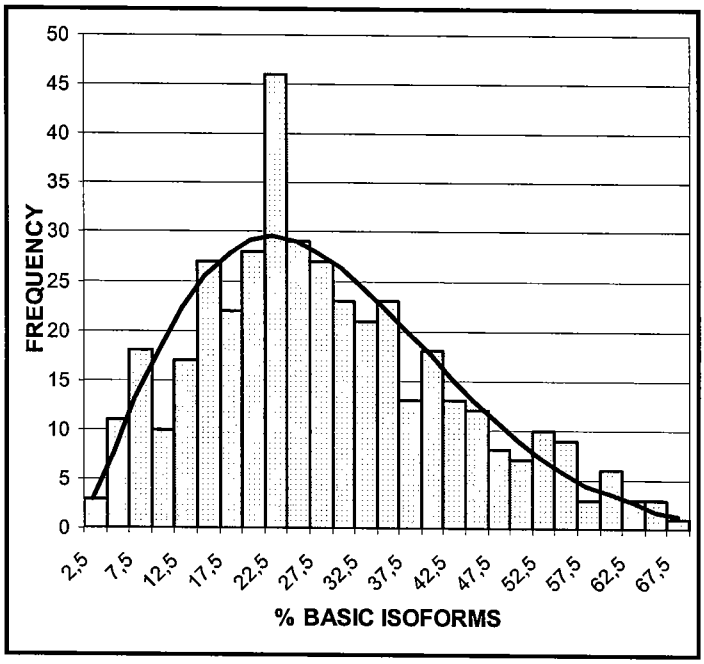
sum of areas of basic isoforms: is the sum of the areas of all bands appearing at isoelectric point (pI's) values above the position defined by the less basic isoform of the reference rEPO standard.

sum of areas of all isoforms: is the sum of the areas of all bands detected along all the pI range.

Evaluation of population data: criterion for positivity

For the establishment of the criterion for positivity, a set of population data was used. The population (N=411, including those samples already studied prior to the Sydney Olympic Games) consisted of samples from all around the world corresponding to different ethnic groups, sex and sports or training conditions (including altitude, hypobaric chamber, etc.) The samples belong to a total of 264 different subjects, some of them tested at different times. The original data was obtained from F. Lasne (LNDD, Châtenay-Malabry, France). The population

data were fitted to a number of distributions, with and without prior transformation. Figure 1 shows the results when a beta distribution was used, giving an excellent score for the Kolmogorov-Smirnov test for goodness of fit.



*BETA distribution**	
N	411
alfa	2,65
beta	6,7
mean	0,283422
S.D.	0,140082
mode	0,224490
Kolmogorov-Smirnov (p)	**0,4307**

* The population values for the distribution fitting model in the table are expressed as a fraction rather than as a percentage."

Figure 1. Beta distribution fitting for the evaluation of blank population data for the values of the rEPO marker (%basic isoforms).

Depending on the fitting model and the statistical risk assumed, values of the marker from **80% to 86%** were found as the possible criterion for positivity for the chosen marker.

Analysis of the reference samples from the protocol of the study

One of the first things evidenced by the study was the potential risk for void tests due to the presence of spurious electrophoretic artefacts (spots, smear, etc.). These situations were identified as the major source of variation of the values obtained for the marker among the laboratories suggesting that stringent criteria for the acceptance of an image should be imposed before issuing a result. Figure 2 shows some of the results obtained when the blank urine was analysed. Apart from considerations regarding the differing resolutions obtained by the laboratories and signal to noise ratios of the images, a very similar result was obtained from the point of view of how the results "look like" from an overall evaluation of the image.

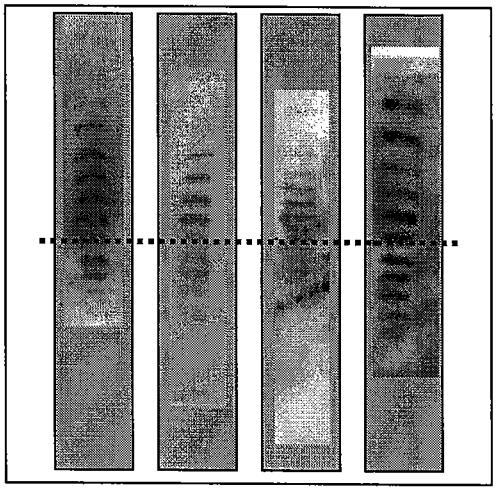


Figure 2. Example of the results obtained by the participating laboratories for the analysis of the pooled blank urine. The line indicates the position of the less basic isoform of the corresponding rEPO standard.

As higher amounts of rEPO were present, better performance was observed, from the sensitivity point of view, and all laboratories gave clear positive results. Figure 3 shows the results obtained by different laboratories for the sample containing rEPO at 3 and 10 IU/L.

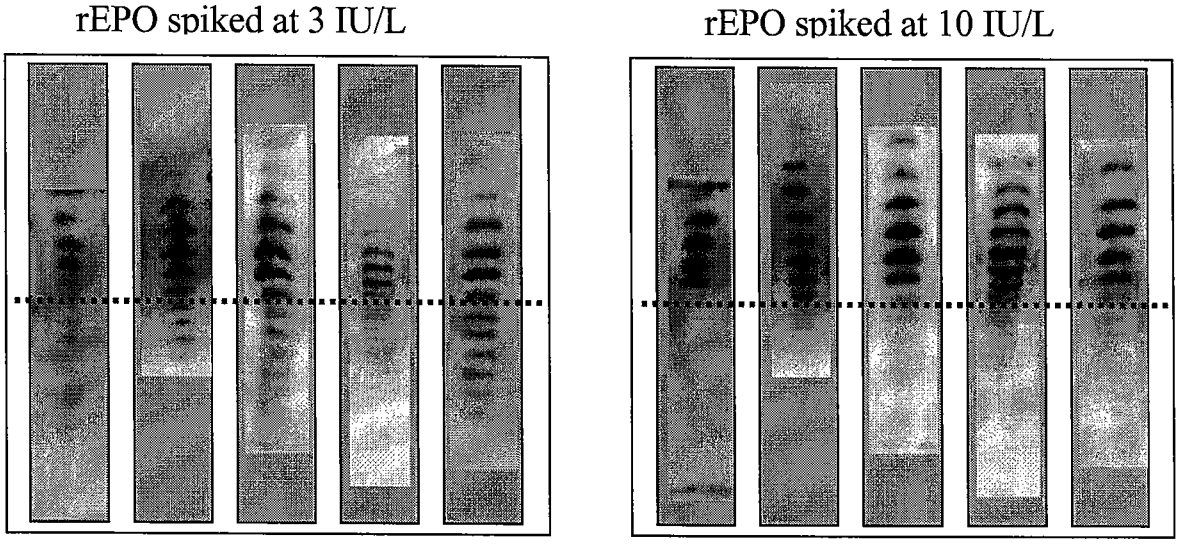


Figure 3. Example of the results obtained by the participating laboratories for the analysis of the urine samples spiked with reference rEPO at concentrations of 3 and 10 IU/L respectively. The line indicates the position of the less basic isoform of the corresponding rEPO standard.

When the images were re-examined and samples not complying with minimal quality criteria regarding signal to noise ratio as well as absence of artefacts casting doubt on the integrity of the result, the results for inter-laboratory reproducibility summarised in Table I were obtained:

Table I. Inter-laboratory reproducibility obtained for some of the samples analysed as part of the current protocol.

BLANK URINE		3 IU/L rEPO		10 IU/L rEPO	
N	10	N	17	N	25
Mean	**73.4**	**Mean**	**82.2**	**Mean**	**97.3**
s.d.	**4.7**	**s.d.**	**5.4**	**s.d.**	**1.6**
Range	**13.8**	**Range**	**15.9**	**Range**	**7.2**
CI95 mean*	2.9	CI95 mean*	2.6	CI95 mean*	0.6
CI95 value*	9.2	CI95 value*	10.6	CI95 value*	3.1

* Confidence Interval (α=0.05)of the mean or a value.

As can be observed, the blank urine chosen (pooled sample) resulted in a high percentage of basic isoforms. This is a very unusual circumstance that, on the other hand, has served to investigate its influence on any marker or positivity criterion to be developed.
From the results, it became clear that as the % basic isoforms becomes higher (a positive case) the standard deviation of the results is greatly reduced. Thus when facing a real positive case, it may be expected that the results will be potentially reproducible in any laboratory.

Another important element that came to the attention of the group when analysing the results was the fact that the marker (% basic isoforms) contained only a minor portion of the information contained in the image. Hence, improving the marker by adding other qualitative criteria (e.g. number of visible bands, position of each band, etc.) would probably result in more consistent results. In the end, it would be ideal if such an approach could result in a robust qualitative evaluation just of presence or absence of rEPO.

A possible approach (under evaluation) for a new way to analyse the images including also qualitative criteria is shown in Table II. Two different lists of requisites are listed. The first related with the acceptability criteria for the image obtained. This list is meant to be used in confirmation analysis. Less stringent criteria can be used for screening purposes triggering confirmation of suspicions samples. The second list corresponds to the criteria for positivity.

For the moment, a certain quantitative criterion of comparison between bands in each area of the gel ("so called basic and acidic) seems unavoidable.

Table II. Draft proposal for criteria of evaluation of results: acceptability of images and criterion for positivity.

CRITERIA FOR ACCEPTABILITY	
1	At least 3 bands must be sufficiently intense and resolved to be clearly visible and their area quantified.
2	Relevant areas, spots or smear on a lane that may be part of IEF bands and could significantly affect the consideration or integration of the 3 more intense bands, invalidate the lane.
3	A differences in the position of equivalent bands between two consecutive standards that may cast doubt on the homogeneity of the performance of the gel or the identification of equivalent bands, invalidates all lanes in between.

CRITERIA FOR POSITIVITY (for those lanes fulfilling the criteria for acceptability)	
I	2 out of the 3 more intense bands must be co-localised in the area defined by the bands of the corresponding standards.
II	The 2 more intense bands must show an intensity (integrated area valley to valley) at least double than the most intense band (if any) not co-localised in the area defined by the bands of the standards.

This criteria constitute the essence of the way we examine the overall image obtained. The maximum of the intensity must be in an area "corresponding to an exogenous standard". There is no need for a great number of bands to be clearly visible providing the particular zone on the image is free of artefacts. And finally the intensity should be sufficiently higher than any "endogenous" band to ensure that we are facing a case in which apart form the appearance of the exogenous substance, we observe the depletion of the endogenous production. The additional advantage is that the criteria are general and do not necessarily depend on the specific standards (they may be alpha or beta rEPO (as they are part of the rEPO standard used in this work) or potentially other preparations of rEPO, as well as for NESP.

Using such an approach the qualitative results obtained for the samples of the present protocol are listed in table III.

Table III. Re-evaluation of results obtained for the samples of the study when using the criteria listed above in Table II.

LAB 1	LAB 2	LAB 3	LAB 4	LAB 5
BLANK URINE				
N.S.	NEG	NEG	NEG	void
void	NEG	NEG	void	void
void	void	NEG	void	NEG
void	NEG	NEG	void	NEG
void	void	NEG	void	NEG
1.5 IU/L rEPO				
N.S.	NEG	NEG	**POS**	void
void	**POS**	NEG	void	void
void	**POS**	NEG	**POS**	void
void	**POS**	NEG	**POS**	void
void	void	**POS**	void	NEG
3 IU/L rEPO				
POS	**POS**	**POS**	void	**POS**
POS	**POS**	**POS**	**POS**	**POS**
POS	**POS**	**POS**	**POS**	**POS**
void	**POS**	**POS**	**POS**	void
void	void	**POS**	void	void
10 IU/L rEPO				
POS	**POS**	**POS**	**POS**	**POS**
POS	**POS**	**POS**	void	**POS**
POS	**POS**	**POS**	**POS**	**POS**
POS	void	**POS**	void	**POS**
void	void	**POS**	void	**POS**
20 IU/L rEPO				
POS	void	**POS**	void	**POS**
POS	**POS**	**POS**	**POS**	**POS**
void	void	**POS**	**POS**	void
void	void	**POS**	void	void
void	void	**POS**	void	**POS**

N.S.: No Sample, void: fail acceptability criteria. NEG: Negative , **POS**: Positive.

As can be seen, no false positive results were obtained. Despite the peculiar profile of the pooled blank urine, all results of the only real blank urine were negative (NEG). The sample with an spiked amount of 1.5 IU/L of rEPO (on a sample already containing almost double the

amount of endogenous EPO) has behaved as being in the range of the *"decision limit"* of the method, as defined by its capacity to differentiate the presence of rEPO in the presence of endogenous EPO (both in specific relative amounts). It should not be forgotten that the sample was, strictly speaking, positive since it contained rEPO. So it is consistent, as it is in all analytical methods having a decision or cut-off limit, that near the decision limit results will be inconclusive. The rest of the samples, seemed to be clearly above the decision limit, thus giving all consistent positive results (POS). The results suggest the importance of carrying out an screening and confirmation assays so that the risk of being near the decision limit is avoided.

Those criteria have to be checked against all the population data to ensure that no false positives are obtained regardless of the origin of the sample or its transportation or storage particularities. Furthermore, re-evaluating the results obtained for sensitivity when real positive samples from excretion studies were analysed, should show complete consistent 100% specificity results as well as equal or even better retrospectivity. As more data is gathered by the different laboratories applying the method, criteria may probably be refined to cope with those situations or new EPO preparations, unexpected today.

Conclusions

The method showed that require extensive training was required for it be successfully applied. Unexpected technical problems during the application of the procedure may result in samples that cannot be evaluated (void tests) more often than with other regular doping analyses.

There should be sufficient evidence of homogeneous performance along the sample lane (no artefacts potentially affecting the result should be acceptable). Stringent requirements must be established before any evaluation is performed.

Results are far more reproducible as the sample contains higher ratios of EPO basic isoforms. This fact will imply a better performance for positive samples and thus greater consistency of the results in an eventual counter-analysis.

The marker used (%basic isoforms) is too simplistic and does not have into account most of the information contained in the images. New criteria should be developed, both for acceptability of results as well as for positivity including other qualitative aspects of the image. The draft criteria presented here is an attempt of rationalisation of what an "expert would consider" when evaluating an EPO image. They may be a good starting point for a final consensus approach for the widespread application of the method.

References

1. Lasne F. and de Ceaurriz J. *Nature*; 2000; 405: 635
2. Lasne F. J. Immunol. Methods, 2001; 253: 125-131
3. Parisotto R, Gore C.J, Emslie K,R, Ashenden M.J, Brugnara C, Howe C, Martin D.T, Trout G.J, Hahn A.G. *Haematologica*, 2000;85:564-572.
4. J.A. Pascual and V. Belalcazar. Final Report: 2nd step: Inter-laboratory comparison report. Medical Commission, IOC.

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