In: W Schänzer, H Geyer, A Gotzmann, U Mareck (eds.) Recent Advances In Doping Analysis (13). Sport und Buch Strauß - Köln 2005

V. Belalcazar[1,2] , J. A. Pascual[1,2] , S. Abanades[1,3], M. Farré[1,3], and R. de la Torre[1,2]

Stability of EPO in urine stabilized by sodium azide

1. Pharmacology Research Unit, Municipal Institute of Medical Research, Barcelona Spain.
2. Dept. Experimental and Health Sciences, University Pompeu Fabra, Barcelona, Spain.
3. Dept. of Pharmacology, Autonomous University of Barcelona, UAB. Barcelona, Spain.

Introduction

The issue of the stability of EPO (both natural and recombinant) in human urine has not been properly addressed in the literature. Apart from obvious effects like adsorption, thermal decomposition, etc., given the complexity of the urinary composition, the potential for the presence of residual enzymatic activities at the time of urine collection able to destroy or change the substance cannot be discarded. Those activities could potentially make EPO undetectable or even lead to a misinterpretation of results. In the absence of sufficient data on those effects, different (conservative) protocols have been proposed in doping control trying to ensure the best transportation and storage conditions.

The aim of the present study was to evaluate the stability of erythropoietin in urine samples under different usual laboratory or transportation temperature conditions. Sodium azide was added to the samples in order to avoid any bacterial growth. Hence other effects arising from the exposure to such temperatures, i.e. bacterial growth that may contribute to the enzymatic destruction or modification of EPO are out of te scope of the present work.

Experimental

Urine samples obtained after administration of 3 consecutive doses of Epoetin beta (Neorecormon, Roche) 20 IU/kg every 48h. Urine was collected also for the 48h subsequent to each dose (a total of 6 days) and finally pooled. Sodium azide (1%) was added as a preservative.

20 ml aliquots (in duplicate, indicated as A and B) of the pooled urine sample were submitted to the following storage conditions:

- 3 freeze/thaw cycles
- 4 days at 4ºC

- 4 days at room temperature (ca. 25ºC)
- 4 days at 37ºC

The method used for the detection of EPO is based on the protocol described by Lasne (Lasne et al 2002). In brief, the method consists of two big steps: The concentration of the urine sample and the immunodetection.
The urine sample was concentrated using filters with a molecular weight cut-off of 30.000 Da (Centricon plus-20™ and YM-30™). An isoelectric focusing technique was used to separate the proteins based on their pI. All samples and the reference material were loaded in a polyacrylamide gel (T=5%, C=3%, 7 M urea, 2-6 pH gradient) and run at 3600Vh, 1W/cm of the gel length, Vmax= 2000V. After the isoelectric focusing, the proteins were transferred to a polyvinilylidenfluoride (PVDF) membrane using a basic transfer buffer (25 mM Tris-192 mM glycine) under the following conditions: 1 mA/cm^2 during 30 min. After the blotting, the membrane was treated with DTT 5mM at 37ºC for 1 hour and then blocked with 5% low fat milk. EPO was detected using a monoclonal antibody (mouse monoclonal antibody-Mab AE7A5 anti-human EPO from R & D). The Mab was transferred by electro-blotting (1 mA/cm^2 membrane, 10 min) to another PVDF membrane using an acid transfer buffer (0.7% acetic acid solution). The membrane was blocked using 5% low fat milk to avoid high background. The transferred Mab was recognized by a polyclonal (Pab) biotinylated anti-species antibody (goat anti-mouse polyclonal antibody, IgG) linked to a Streptavidin-horseradish peroxidase complex. Finally the sandwich: Mab + Pab + Streptavidin-peroxidase was detected by the production of chemiluminescence when in contact with the appropriate reagent (Supersignal West Femto from Pierce) that was detected by a CCD camera (FUJIFLIM Luminescent Image Analyser LAS-1000).

Results

Urine samples fulfilled the “stability criteria” according to the WADA Technical Document TD2004EPO. The concentration of EPO (comparing the response obtained by chemiluminescence) seemed to be fairly stable in all conditions checked, even under the aggressive 4 days at 37ºC. A very slow decline (fainter bands) could be observed as the temperature increased. However, the limited data available, does not allow to extract further conclusions since those variations could be simply explained by the inter-asssay reproducibility of the procedure.

The evaluation of the results was carried out according to different approaches already described in the literature, i.e.:

% Basic isoforms: Defined as the quotient between the sum of the areas of the bands (integrated valley to valley) with pI values equal or more basic than those of the rEPO standard and the sum of areas of all bands (expressed as %). A sample will be interpreted as containing rEPO when the % basic isoforms is above 80%.

Quotient: Defined as the quotient between the area of the second most intense band of all (integrated valley to valley) and the area of the most intense band in the endogenous area.

A sample will be interpreted as containing rEPO when the two most intense bands of all are located in the recombinant area and the defined *quotient is higher than 2*.

WADA ((TD2004EPO): A sample will be interpreted as containing rEPO when:

1. In the basic area there must be at least 3 acceptable, consecutive bands assigned as 1, 2, 3 or 4 in the corresponding rEPO standard.
2. The two most intense bands in the basic area must be consecutive. The most intense band must be 1, 2 or 3.
3. The two most intense bands in the basic area must be *more intense* than any other band in the endogenous area.

Figure 1 shows the images obtained after chemiluminescence detection of the IEF profiles.

Table 1 shows the results obtained for those applicable markers (% basic isoforms and quotient) as well as the final evaluation (negative (-) when absence of rEPO or positive (+) when presence of rEPO.

The pooled urine sample used for the assay was part of an excretion study of EPO beta hence known as containing rEPO. The sample turned out to be "borderline" according to the criteria for evaluation of isoform profiles, meaning that depending on the criteria used, or the storage conditions applied, replicates were evaluated as negative or positive. On the other hand, quantitative criteria gave a good measure of the magnitude of any change in the profile. According to the results obtained, the sample showed to be stable under all conditions assayed. EPO itself did not show to be particularly sensitivity to the temperature in urine, provided that other factors like bacterial growth are avoided. It is true that there are small differences between the values for % basic isoforms or the quotients obtained with a certain trend (consistent with the expected results: 37ºC producing the larger changes or more

degraded profiles). Nevertheless those differences were too small and fairly consistent with the repeatability of the method.

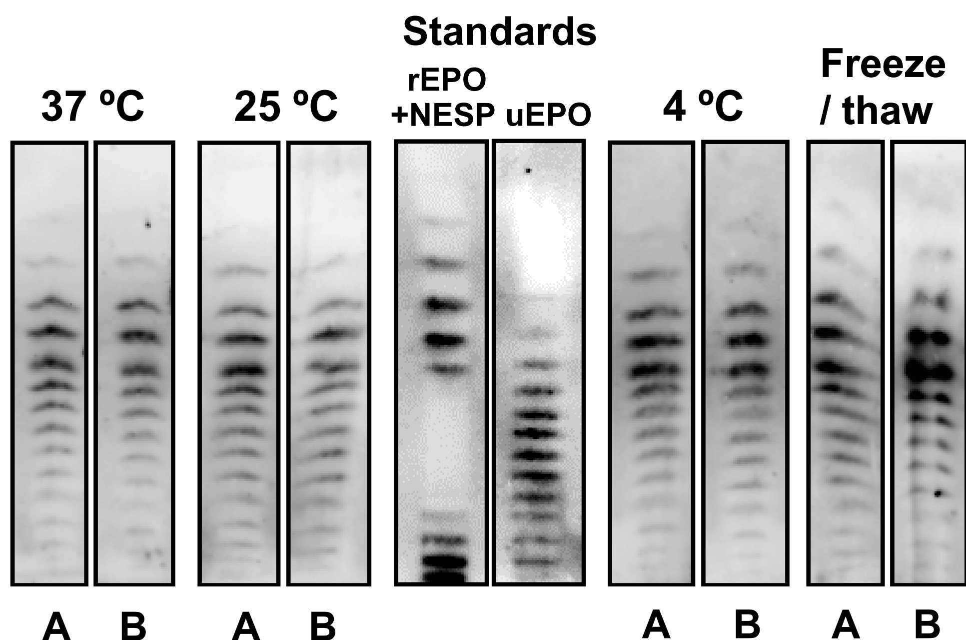

Figure 1. Isoform profiles obtained after submitting a urine sample to different experimental storage conditions. Duplicates for each condition are identified as A and B. The analysis for standard preparation of a mixture of rEPO, NESP and urinary EPO are also included.

		4 days, 37ºC	**4 days, 25ºC**	**4 days, 4ºC**	**3 freeze/thaw cycles**
% Basic Isoforms	**A**	58 (-)	58 (-)	69 (-)	64 (-)
	B	65 (-)	57 (-)	69 (-)	60 (-)
Quotient	**A**	1.8 (-)	2.2 (+)	2.9 (+)	2.1 (+)
	B	1.8 (-)	2.3 (+)	3.1 (+)	2.1 (+)
WADA (TD2004EPO)	**A**	(-)	(+)	(+)	(+)
	B	(-)	(-)	(+)	(+)

Table 1.- Results obtained after evaluation of samples following different criteria (% basic isoforms, quotient and WADA technical document TD2004EPO). Apart from the numerical value of the markers the final evaluation (negative (-) or positive (+)) is also included.

The situation would have been very different if the urine sample had failed the stability test, indicating the presence of a residual activity able to degrade EPO. Nevertheless, we should not forget that, according to the regulations, if a urine sample shows an unstable profile, the transportation conditions followed are then irrelevant since the sample is considered not valid for the test.

Conclusions

The samples were prepared under conditions routinely used for the preparation of reference materials, hence 1% sodium azide was added as a preservative. It must be highlighted that this is, of course, not the way the samples are collected for doping control since, at present, no additives are used at the moment of collection. Further studies should be performed in order to definitely ascertain the potential benefits of the presence of sodium azide on the stability of EPO apart from the obvious effect of preventing any bacterial growth that could result in an invalid sample.

Having those aspects into account, the following conclusions were extracted:

- Urinary EPO, itself, is sufficiently stable allowing the use of regular transportation conditions in doping control, i.e. ambient temperature.
- If a sample showed to contain any residual activity able to degrade EPO (i.e. failing the stability test according to TD2004EPO) the sample would not be considered valid. While residual activities present at the moment of collection are inevitable, further effects (i.e. due to bacterial growth) could be prevented under appropriate transportation and storage conditions. However, there is always a risk that those prevented effects show up once the sample is submitted to hours of incubation as part of the test.
- ▪ • There is no indication of the alteration of the profile towards the creation of a false positive.
- Although not significant, results seem to be a bit better (more intense bands) under storage at 4º C, avoiding either higher temperatures or freeze/thaw cycles.

Acknowledgements

The authors thank B. Cano for the contribution to the present work. This work was funded by WADA.

Bibliography

Lasne, F. J Immunol.Methods, 2001, (253), pg: 125-131.
Lasne, F. et al. Anal. Biochem, 2002, (311), pg: 119-126.
Pascual, J.A. et al. Recent advances in doping analysis, 2002, (10), pg: 135-144.
WADA Technical document TD2004EPO