Application Note

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A fast preparative method for detection of recombinant erythropoietin in blood samples

Françoise Lasne,* Laurent Martin and Jean Antoine Martin

Introduction

While first- and second-generation recombinant erythropoietins (EPOs) are detectable in urine, we showed that analysis in blood is more appropriate for the third-generation, continuous erythropoietin receptor activator (CERA), due to its poor excretion in urine. However, isoelectric focusing (IEF) of EPO in serum or plasma is not as simple as in urine since it requires an initial preparative step by immunoaffinity chromatography. That is why enzyme-linked immunosorbent assay (ELISA) and IEF are used as screening and confirmation tests for CERA detection, respectively. Due to the observation of false negative results of ELISA, we have developed a fast method for preparing plasma or serum samples, making IEF as easy to use in blood as in urine. While preparation by immunoaffinity is kept for confirmation analysis, this convenient method is proposed for screening analysis. In addition to CERA, Darbepoetin alfa is very easily identified in blood due to its typical IEF profile. Identification of first-generation EPO drugs will require criteria specific for blood. In comparison with urine, EPO analyses in blood appear beneficial in terms of sensitivity, stability during physical exercise, and prevention of sample adulteration at collection.

Anti-doping control of erythropoietin (EPO) is based on the differentiation of natural endogenous from recombinant hormones by their isoelectric profiles.[1] SDS (sodium dodecyl sulfate) electrophoresis is a useful complementary method.[2] Both methods were developed for urine samples. While first- and second-generation recombinant EPOs are detectable in urine, we showed that analysis in blood is more appropriate for the third-generation, continuous erythropoietin receptor activator (CERA), due to its poor excretion in urine.[3] However, isoelectric focusing (IEF) of EPO in serum or plasma is not as simple as in urine since it requires an initial preparative step by immunoaffinity chromatography.[4] We thus developed a convenient method (more than 20 samples can be treated in parallel) for preparing plasma or serum samples, making IEF as easy to use in blood as in urine.

Material and Methods

The principle of this purification method relies on the high stability of EPO at acidic pH due to its heavy glycosylation.[5] Perchloric acid is used to precipitate most of the plasma or serum proteins, while EPO is not affected. For this, 1 volume of serum (or plasma) is diluted with 9 volumes of 0.9% NaCl and 5 volumes of 1.8 M perchloric acid are then added. Most of the proteins are immediately precipitated and the supernatant is collected after 10 min of centrifugation at 2700 g. Perchloric acid is then neutralized by slowly adding 5 volumes of 1.8 M $KHCO_3$. A second precipitation occurs due to formation of insoluble $KClO_4$ according to the reaction: $HClO_4 + KHCO_3 \rightarrow H_2O + KClO_4 + CO_2$. After a second centrifugation, the supernatant containing EPO is collected and submitted to the usual process of ultra filtration and IEF as described for urine [6] and immunopurified EPO from blood.[4]

Since recombinant EPO molecules are identified by their isoelectric profiles, it was essential to ensure that the latter were not modified by the acidic extraction. This was demonstrated by adding different types of EPO to serum samples that were then submitted to acidic precipitation. As shown in Figure 1, the isoelectric profile of every type of EPO present in blood (natural, recombinant Epoetins, NESP- novel erythropoiesis stimulating protein, CERA) was recovered unchanged after acidic extraction.

Discussion

This method is especially useful for CERA detection in blood. Anti-doping control of CERA currently relies on screening by enzyme-linked immunosorbent assay (ELISA) test [7] and confirmation analysis by immunoaffinity and IEF.[3] During our experience with this process, we became aware of false negative results with ELISA that were clearly positive with IEF. We thus advise IEF for both screening and confirmation, as is the case for detecting other EPOs in urine. Preparation by perchloric precipitation is convenient, making screening by IEF quite practicable, whereas preparation by immunoaffinity may be used for confirmation analysis.

In contrast to ELISA, this method can also screen blood samples for other types of EPO, which may be advantageous in terms of sensitivity. For instance, we reported the presence of NESP from only 0.5 mL of plasma, whereas the image from 40 mL of urine taken at the same time was too faint to be reported. In this case, the athlete had ingested enough water to greatly dilute the urine sample (specific gravity 1.002). The expansion of plasma volume was not sufficient to mask the presence of NESP in blood.

It must be noted that CERA and NESP are easily identified in blood or urine due to their very typical profiles. Identification of

* *Correspondence to: Françoise Lasne, Agence française de lutte contre le dopage (afld), département des analyses, 143, avenue Roger Salengro, 92290 Châtenay-Malabry, France. E-mail: f.lasne@afld.fr*

Agence Française de Lutte contre le Dopage (afld), Département des Analyses, France

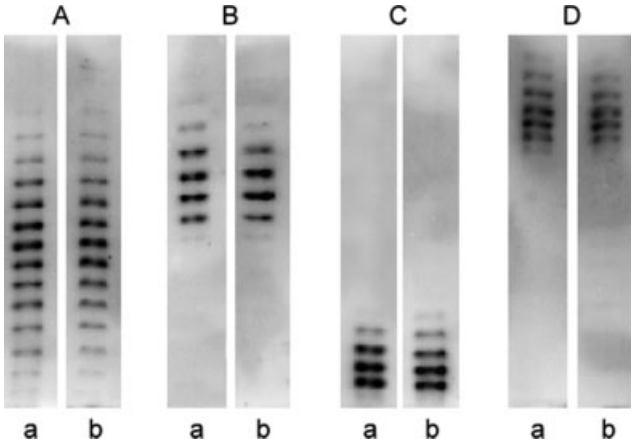

Figure 1. Isoelectric patterns of EPOs. Four types of EPO: natural urinary (A), Epoetin alfa (B), Darbepoetin alfa (C) and CERA (D), were introduced (b) or not (a) into plasma samples. The IEF patterns observed after perchloric precipitation of proteins (b) were quite identical to those obtained from the same products directly analysed out of plasma without any preparative step (a). Note that the concentrations of the EPOs introduced into plasma were chosen here to make the endogenous EPO from plasma not visible. Anode is at the bottom of the figure.

first-generation EPO drugs (including biosimilars) requires more attention in blood. We previously showed that natural EPO is clearly acidified when excreted in urine while recombinant EPOs are much less transformed, probably due to a preferential reabsorption of the most basic isoforms by tubular cells.[6] The differences in the isoelectric patterns of natural (more acidic) and first-generation recombinant EPOs (more basic) are thus reinforced in urine. The identification criteria for differentiating these hormones in urine cannot be transposed to blood and specific criteria will have to be established. On the other hand, these criteria will not have to take into account the effects of physical exercise on IEF profiles, as is the case in urine, since these profiles are not affected in blood.[7]

Last, EPO analyses in blood appear beneficial in terms of sensitivity, stability during physical exercise, and prevention of sample adulteration at collection. This convenient preparative method now makes screening quite feasible, at least for NESP and CERA, and opens interesting perspectives for the first-generation recombinant EPOs.

References

[1] F. Lasne, J. de Ceaurriz, *Nature* **2000**, *405*, 635.
[2] C. Reichel, R. Kulovics, V. Jordan, M. Watzinger, T. Geisendorfer, *Drug. Test. Anal.* **2009**, *1*, 43.
[3] F. Lasne, L. Martin, J. A. Martin, J. de Ceaurriz, *Haematologica* **2009**, *94*, 888.
[4] L. O. Narhi, T. Arakawa, K. H. Aoki, R. Elmore, M. F. Rohde, T. Boone, T. W. Strickland, *J. Biol. Chem.* **1991**, *266*, 23022.
[5] F. Lasne, L. Martin, N. Crepin, J. de Ceaurriz, *Anal. Biochem.* **2002**, *311*, 119.
[6] F. Lasne, L. Martin, J. A. Martin, J. de Ceaurriz, *Int. J. Biol. Macromol.* **2007**, *41*, 354.
[7] S. Lamon, S. Giraud, L. Egli, J. Smolander, M. Jarsch, K. G. Stubenrauch, A. Hellwig, M. Saugy, N. Robinson, *J. Pharm. Biomed. Anal.* **2009**, *50*, 954.