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Sports drug testing for erythropoiesis-stimulating agents and autologous blood transfusion

With the rise of biotechnology during the 1980s, production of recombinant proteins also has also become a major driving force in pharmaceutical industry. After a long search for erythropoietin (EPO), the main protein factor responsible for human red cell production (erythropoiesis), and its successful isolation in 1977,[1] molecular biology made cloning of the EPO gene (1985)[2] and high-quality production of EPO for the treatment of primarily kidney-diseased persons (1989) possible.[3] In parallel, EPO also started to be misused by athletes for doping purposes, as an increase in red blood cell (RBC) mass led to improved performance characteristics especially in endurance sports. During the following years, variants of the first recombinant EPO pharmaceutical formulation (epoetin alfa) entered the market (epoetin beta, darbepoetin alfa, epoetin delta, methoxy polyethylene glycol-epoetin beta), with the ultimate goal to improve serum half-life of the protein. First EPO biosimilars (mostly of the epoetin alfa and beta type) already turned up during the early years of the new century.[4] In 2000, the first useful method for routine EPO doping testing became available[5] and since then has been extensively applied in anti-doping control. It uses the highly sensitive clone AE7A5 anti-EPO antibody in combination with an electrophoretic method called isoelectric focusing (IEF).[6]

Immunoaffinity enrichment of EPO from matrices used in doping control (urine, blood) gained importance with the introduction of another electrophoretic method, which was shown to be also capable of differentiating between endogenous and recombinant epoetins (sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE)). A recently developed protocol modified the original enzyme-linked immunosorbent assay (ELISA) microtiter plate method of SDS-PAGE and allows now the application of the same commercial ELISA for also preparing immunoaffinity-purified urine samples for IEF-PAGE.[7]

Affinity purification is also important when blood is used for recombinant EPO detection. The novel fusion protein EPO-Fc not only stimulates RBC production after subcutaneous or intravenous injection but also after pulmonary administration (inhalation). Two protocols were developed for the analysis of EPO-Fc in serum and plasma.[8]

Aside from EPO and its analogues, other substance classes may also act as erythropoiesis-stimulating agents (ESA). HIF (hypoxia-inducible factor) stabilizers are small molecules capable of stimulating endogenous EPO production. They inhibit an enzyme (HIF-prolyl hydroxylase), which under non-hypoxia conditions promotes degradation of the EPO gene transcription factor HIFα. For endogenous regulatory suppression of EPO gene transcription, the human body uses GATA transcription factors. So-called GATA inhibitors are able to abrogate this suppression and thus stimulate erythropoietin synthesis. Mass spectrometry is the method of choice for detecting potential abuse of these substances.[9]

The Athlete Biological Passport (ABP) and its haematological module aim to monitor erythropoiesis-relevant blood parameters (e.g. haemoglobin concentration, reticulocytes) in a longitudinal and athlete-specific way. Statistically unexplainable changes in these patterns can either be used as direct proof of doping or for targeting athletes in combination with conventional drug testing strategies.[10] The International Cycling Union (UCI) has been successfully using the ABP for detecting ESA-doping since 2008.[11]

While an application of EPO gene therapy for doping purposes (EPO gene doping) has not been reported so far, medical progress in this field necessitates the development of detection strategies for this kind of genetic intervention. A comprehensive review of methods qualified for tracing EPO gene doping in blood was prepared[12] as well as a protocol for a fast transgene detection by a one-tube nested PCR method.[13]

Oxygen transfer cannot only be enhanced by stimulating erythropoiesis, but also by direct transfer of RBC into the blood stream. While heterologous blood transfusion is detectable due to the presence of different cell-specific surface antigen populations in one and the same individual, no such direct method exists for autologous blood transfusion testing. Blood bags contain plasticizers and elevated levels of their metabolites are found in urine after transfusion, which was the basis for the development of an indirect marker test method.[14] Storage of whole blood at fridge temperatures leads to changes in RBC cytoskeleton and transmembrane proteins, which are traceable by proteomic methods (two-dimensional gel electrophoresis, stable isotope peptide labelling combined with mass spectrometry).[15] However, the direct detection of autologous blood transfusion still remains one of the hottest topics in sports drug testing research today.

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References

[1] T. Miyake, C.K. Kung, E. Goldwasser. Purification of human erythropoietin. *J. Biol. Chem.* **1977**, *252*, 5558.
[2] F.K. Lin, S. Suggs, C.H. Lin, J.K. Browne, R. Smalling, J.C. Egrie, *et al.* Cloning and expression of the human erythropoietin gene. *P. Natl. Acad. Sci. USA* **1985**, *82*, 7580.
[3] M.A. Foote, in *Erythropoetins, Erythropoietic Factors, and Erythropoiesis*, 2nd edition, (Eds: S.G. Elliott, M.A. Foote, G. Molineux), Birkhäuser, Basel, **2009**, pp. 77–85.

[4] H. Schellekens. Biosimilar epoetins: How similar are they? *EHLP J.* **2004**, *3*, 43.
[5] F. Lasne, J. de Ceaurriz. Recombinant erythropoietin in urine. *Nature* **2000**, *405*, 635.
[6] N. Debeljak, A.J. Sytkowski. Erythropoietin and erythropoiesis stimulating agents. *Drug Test. Anal.* **2012**, *4*, 805.
[7] P. Reihlen, E. Völker-Schänzer, B. Majer, W. Schänzer. Easy-to-use IEF compatible immunoaffinity purification of Erythropoietin from urine retentates. *Drug Test. Anal.* **2012**, *4*, 813.
[8] C. Reichel, M. Thevis. Detection of EPO-Fc fusion protein in human blood: Screening and confirmation protocols for sports drug testing. *Drug Test. Anal.* **2012**, *4*, 818.
[9] S. Beuck, W. Schänzer, M. Thevis. Hypoxia-inducible factor stabilizers and other small-molecule erythropoiesis-stimulating agents in current and preventive doping analysis. *Drug Test. Anal.* **2012**, *4*, 830.
[10] Y.O. Schumacher, M. Saugy, T. Pottgiesser, N. Robinson. Detection of EPO doping and blood doping: The haematological module of the athlete biological passport. *Drug Test. Anal.* **2012**, *4*, 846.
[11] M. Zorzoli, F. Rossi. Case studies on ESA-doping as revealed by the biological passport. *Drug Test. Anal.* **2012**, *4*, 854.
[12] E.W. Neuberger, M. Jurkiewicz, D.A. Moser, P. Simon. Detection of EPO gene doping in blood. *Drug Test. Anal.* **2012**, *4*, 859.
[13] D.A. Moser, E.W. Neuberger, P. Simon. A quick one-tube nested PCR-protocol for EPO transgene detection. *Drug Test. Anal.* **2012**, *4*, 870.
[14] J. Segura, N. Monfort, R. Ventura. Detection methods for autologous blood doping. *Drug Test. Anal.* **2012**, *4*, 876.
[15] Z. Nikolovski, C. De La Torre, C. Chiva, E. Borràs, D. Andreu, R. Ventura, *et al.* Alterations of the erythrocyte membrane proteome and cytoskeleton network during storage - a possible tool to identify autologous blood transfusion. *Drug Test. Anal.* **2012**, *4*, 882.