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Detection of Epo-doping in blood

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Abstract

The detection of doping with recombinant peptide and protein hormones (e.g. erythropoietin – EPO, human growth hormone - hGH) is one of the most challenging analytical problems in doping control [1]. The World Anti-Doping Agency (WADA) accredited method for the detection of doping with recombinant human erythropoietins (rhEPO) is based on isoelectric focusing (IEF) [2]. In addition, SDS-PAGE has proven a valuable tool for detecting Dynepo-doping with increased sensitivity and is also useful for ruling out active and effort-type urines [3-6]. However, both the SDS-PAGE and the IEF-PAGE methods use urine as sample matrix for the majority of EPO-tests performed. Unfortunately, one of the latest generation of EPO-based pharmaceuticals (Continuous Erythropoietin Receptor Activator (C.E.R.A.), RO0503821, or pegzyrepoetin alfa; marketed under the brand name MIRCERA - a PEGylated epoetin beta) is hardly excreted in urine due to its prolonged serum half-life (ca 130 h) and molecular mass (ca 60 kDa) and hence has to be preferably tested in blood [7]. In consequence, *up to four methods* have to be performed in order to unambiguously detect and confirm the misuse of recombinant erythropoietins (either the original innovator products or biosimilar epoetins or (the currently worldwide ca 80) “copy” epoetins [8]), i.e. the EPO-IEF method for urine and the SDS-PAGE method for additional evidence (e.g. Dynepo [3-4], effort urines [4], degraded human urinary EPO (uhEPO [4]), some biosimilars [6]), as well as an ELISA [9] and/or the IEF-method for detecting the abuse of MIRCERA in blood [7]. *Two different matrices* have to be used, blood and urine. Due to the lower molecular mass of serum and plasma EPO (shEPO) compared to most recombinant erythropoietins [10] SDS-PAGE holds the potential of detecting doping with the majority of rhEPO-forms in blood and *in a single experiment*. Contrary to that, the isoform distribution of shEPO is less acidic than the distribution of uhEPO [11]. Unfortunately, SDS-PAGE is less sensitive for MIRCERA than for other rhEpos. We identified the reason for this altered behaviour and developed a new electrophoretic method with specifically enhanced sensitivity for MIRCERA. The

method (SARCOSYL-PAGE) is based on the theory of SDS-PAGE and thus is also capable of detecting doping with most rhEpos in blood [12].

Results and Discussion

Recombinant erythropoietins perform with different sensitivity on SDS-PAGE after Western blotting. While the sensitivity of the majority of epoetins (e.g. epoetins alfa, beta, delta, omega; darbepoetin alfa) is somewhat comparable on SDS-PAGE, the sensitivity of MIRCERA (PEGylated epoetin beta) is drastically decreased. Redesigning SDS-PAGE by exchanging the SDS for SARCOSYL in the sample and running buffers solved the problem. SARCOSYL (SAR), a methyl glycine-based anionic surfactant with slightly higher CMC but much lower aggregation number than SDS, is not capable of solubilizing PEGs under PAGE-conditions - regardless of their polymerization degree (PEGs 1500 to 35000 were tested). Instead, SARCOSYL is only binding to the protein-part of MIRCERA leading to a sharp band on SAR-PAGE. SDS, on the other hand, is binding to both the PEG- and protein-chains of MIRCERA, which leads to band broadening on SDS-PAGE. As a result, the monoclonal anti-EPO antibody (clone AE7A5) is no longer binding to the fully - i.e. PEG- and protein-chain - solubilized MIRCERA-molecules, but only to those molecules which contain only SDS bound to the protein-chain. Naturally, these molecules are located on top of the band, since their charge density is reduced and their migration behaviour decreased. Because these molecules resemble only a small fraction of the MIRCERA-molecules originally loaded on the gel, a decrease in sensitivity is observed. SARCOSYL, on the other hand, leads to a sharp MIRCERA-band, since no solubilization of PEG-chains occurs. Consequently, the antibody is able to bind to all MIRCERA molecules and no loss in sensitivity is observed after Western blotting. Besides, SARCOSYL-PAGE detects non-PEGylated epoetins with the same sensitivity and resolution as SDS-PAGE. The applicability of SAR-PAGE for detecting MIRCERA, recombinant epoetins, and endogenous EPO in blood and with high sensitivity could be demonstrated by performing single dose excretion studies. Besides, SAR-PAGE is not restricted to electrophoretic separations using the BisTris buffer system (e.g. MOPS-chloride boundary) but is fully compatible with other discontinuous buffer systems, namely the standard Laemmli (glycine-chloride boundary) [13], Neville (borate-sulfate boundary) [14], and Allen-Moore (e.g. borate-citrate boundary) [15] stacking systems – also indicating that the net-charge of the SARCOSYL-protein (i.e. erythropoietin, MIRCERA) micelles is stable within the pH-range of ca. 7-10. In a typical SAR-PAGE experiment, 200 µL of serum

are used, which allow the direct detection of MIRCERA, recombinant epoetins (e.g. NeoRecormon, Dynepo, NESP), and shEPO in a single experiment and with high (i.e. femtogram (low amol)) sensitivity (Fig. 1). The method can be used for screening and confirmation purposes.

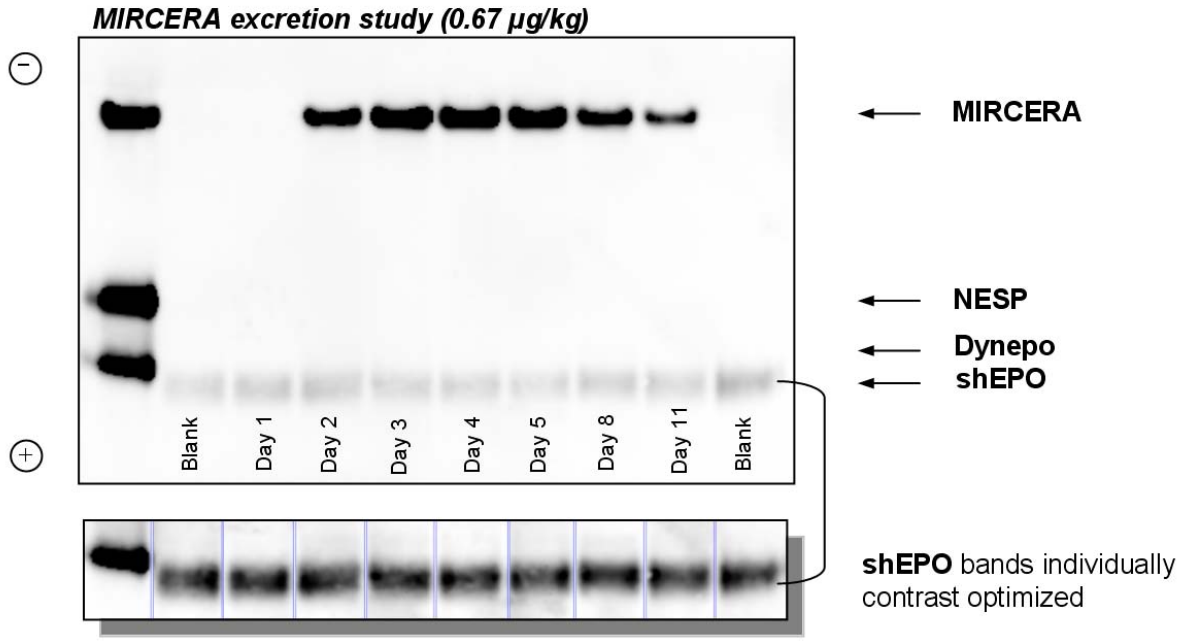

Figure 1: Detection MIRCERA by SARCOSYL-PAGE in 200 µL of serum and after a single dose subcutaneous application of 0.67 µg/kg.

References

[1] Jelkmann W. (2009), Erythropoiesis stimulating agents and techniques: a challenge for doping analysts, *Curr. Med. Chem.* **16**, 1236-1247.

[2] Lasne F, de Ceaurriz J. (2000) Recombinant erythropoietin in urine. *Nature* **405**, 635.

[3] Kohler M, Ayotte C, Desharnais P, Flenker U, Lüdke S, Thevis M, Völker-Schänzer E, Schänzer W. (2008) Discrimination of recombinant and endogenous urinary erythropoietin by calculating relative mobility values from SDS gels. *Int J Sports Med* **29**, 1-6.

4] Reichel C, Kulovics R, Jordan V, Watzinger M, Geisendorfer T. (2009) SDS-PAGE of recombinant and endogenous erythropoietins: benefits and limitations of the method for application in doping control, *Drug Test. Analysis* **1**, 43-50.

[5] Desharnais P, Ayotte C. (2007) Towards the screening of urinary EPO by SDS-PAGE. In: Schänzer W, Geyer H, Gotzmann A, Mareck U. (eds.) *Recent Advances in Doping Analysis (15)*, Köln, p 303.

[6] World Anti-Doping Agency. Harmonization of the method for the identification of

recombinant erythropoietins (i.e. epoetins) and analogues (e.g. darbepoetin and methoxypolyethylene glycol-epoetin beta). TD2009, Montreal (2009) http://www.wada-ama.org/rtecontent/document/td2009eop_en.pdf (access date July 16, 2009).

[7] Lasne F, Martin L, Martin JA, de Ceaurriz J. (2009) Detection of continuous erythropoietin receptor activator in blood and urine in anti-doping control, *Haematologica* **94**, 888-890.

[8] Macdougall IC, Ashenden M. (2009) Current and upcoming erythropoiesis-stimulating agents, iron products, and other novel anemia medications, *Advances in Chronic Kidney Disease* **16**, 117-130.

[9] Lamon S, Giraud S, Egli L, Smolander J, Jarsch M, Stubenrauch KG, Hellwig A, Saugy M, Robinson N. (2009) A high-throughput test to detect C.E.R.A. doping in blood, *J. Pharm. Biomed. Anal.* **50**, 954-958.

[10] Skibeli V, Nissen-Lie G, Torjesen P. (2001) Sugar profiling proves that human serum erythropoietin differs from recombinant human erythropoietin, *Blood* **98**, 3626-3634.

[11] Lasne F, Martin L, Martin JA, de Ceaurriz J. (2007) Isoelectric profiles of human erythropoietin are different in serum and urine, *Int. J. Biol. Macromol.* **41**, 354-357.

[12] Reichel C, Abzieher F, Geisendorfer T. (2009) SARCOSYL-PAGE: A new method for the detection of MIRCERA- and EPO-doping in blood, *Drug Test. Analysis* (accepted).

[13] Laemmli UK. (1970), Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature* **227**, 680-685.

[14] Neville DM. (1971) Molecular weight determination of protein-dodecyl sulfate complexes by gel electrophoresis in a discontinuous buffer system, *J. Biol. Chem.* **246**, 6328-6334.

[15] Allen RC, Moore DJ. (1971), Electrophoretic System, *U.S. Patent* **3620947**, 1-9.