Project Review

"Development of a method for the detection of doping with all forms of recombinant erythropoietins in human blood"

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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. The WADA-accredited method for the detection of doping with recombinant human erythropoietins (rhEpo) is based on isoelectric focusing (IEF).

Our laboratory and the anti-doping control laboratory of Montreal developed – independently of each other – an SDS-PAGE method which serves as an additional confirmation tool for the worldwide practiced Epo-IEF method. The advantages and additional benefits of this method were presented at various scientific meetings in 2007 and 2008 (e.g. the capability to distinguish between Epo-biosimilars and effort urines, no interference by active urines). By applying this strategy we were able to clearly and multiply demonstrate the abuse of Dynepo by athletes – which is difficult to uncover with the IEF-method alone.

Howewer, both the SDS-PAGE method and the Epo-IEF method use urine as sample matrix. Unfortunately, one of the latest generation Epo-pharmaceuticals (MIRCERA, a PEGylated Epoetin beta) is hardly excreted in urine due to its prolonged serum half-life and molecular mass (ca. 60 kDa). An ELISA test will be offered by the manufacturer for quantifying MIRCERA in blood. The consequences will be that in the future THREE methods will have to be performed in order to unambiguously detect the misuse of recombinant eyrthropoietins and analogs, i.e. the Epo-IEF method, the SDS-PAGE method for additional evidence (e.g. Dynepo, effort urines, biosimilars), and the serum/plasma ELISA for detecting MIRCERA abuse. Two different matrices will have to be used: blood and urine. The aim of this project is to develop a method which is capable of detecting doping with all forms recombinant erythropoietins in blood and in a single experiment.

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Results and Conclusions

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 similar 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 specifically enhanced the sensitivity for MIRCERA. SARCOSYL, 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) [1], Neville (borate-sulfate boundary) [2], and Allen-Moore (e.g. borate-citrate boundary) [3] 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.

The developed method is suitable for blood and urine, is not prone to „active“ and „effort urines“, is highly sensitive (down to femtogram-level, i.e. ca 10 amol) and with enhanced sensitivity compared to the traditional SDS-PAGE method for MIRCERA. The criteria of positivity (qualitative criteria, relative mobility values) are

simpler since only one band instead of a series of isoforms and their distribution has to be evaluated. Of special importance is the fact that only one matrix and only one method are necessary for the detection of doping with all forms of recombinant erythropoietins (**one matrix – one method approach**) – instead of currently 4 methods and two matrices. Also, the sensitivity of SAR-PAGE for MIRCERA is higher than the sensitivity of IEF-PAGE (this was independently shown by the anti-doping control laboratory in Lausanne) – which is especially important for the screening. Hence, we also recommend the usage of SARPAGE as a screening procedure, because otherwise cases of low dosed MIRCERA would be missed by the IEF-PAGE method (false negatives). And finally, the required sample volume for SAR-PAGE is very low: 200 µL of serum are sufficient for the detection of shEPO and all forms of recombinant EPO.

Publications:

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 1, 494-504