Project Review

"Identification and mass spectrometric characterization of urinary proteins other than Epo binding to clone AE7A5 anti-human EPO antibody"

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Non-specific binding of the monoclonal anti-human EPO antibody (clone AE7A5; R&D Systems, Inc.) used for the detection of doping with recombinant human erythropoietin by the worldwide practised isoelectric focusing (IEF) and Western double blotting method has been in discussion for several years. Publications in peer-reviewed journals addressed this subject in 2005 and 2006 and led to some scientific argumentation on the specificity of the employed detection antibody. However, under experienced users of the Epo-method several additional bands (usually two to four) in the basic region of the pH 2-6 IEF-gel caused scientific interest, because these bands could only be occasionally detected among the tested urinary samples. Their relationship to Epo remained unclear for years. Nevertheless, it was obvious that these bands did not interfere with the endogenous urinary and recombinant Epo-IEF profiles and thus the evaluation of the profiles was not disturbed or questioned at all. We were able to identify the protein causing these basic IEF-bands by means of carrier ampholyte IEF-PAGE, SDS-PAGE, Western blotting, and nano-ESI high resolution mass spectrometry and presented the results at the 2007 Cologne Workshop on Dope Analysis. The name of the protein is Zinc-alpha-2-glycoprotein. It is a high abundant urinary protein.

The purpose of this project is a detailed study on the specificity of the clone AE7A5 antibody. According to the WADA technical document on the "Harmonization of the method for the identification of epoetin alfa and beta (Epo) and darbepoetin alfa (NESP) by IEF-double blotting and chemiluminescent detection" the IEF-method "relies on the particular specificity of the monoclonal antibody with which it was developed (clone AE7A5)" and "this antibody is considered a critical reagent and shall not be changed". However, during SDS-PAGE experiments we were able to show that AE7A5 also binds to urinary proteins other than erythropoietin and Zinc-alpha-2-glycoprotein. These proteins might interfere with the interpretation of the Epo-IEF profiles.

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Results and Conclusions:

A comprehensive study on the non-specific binding behaviour of monoclonal anti-EPO antibody clone AE7A5, which has to be used according to the technical document on EPO-analysis of WADA (TD2009EPO) for the detection of EPO-doping, was performed. Human urine and serum were used as matrices, since both are also used for EPO-testing. Additionally, E. coli and S. cerevisiae lysates were investigated. Aside from *zinc-alpha-2-glycoprotein (ZAG)* a weak interaction with *transferrin* was detected (urine, serum). However, the non-specific interaction of the four proteins, which were mentioned by Khan et al. (2005) [1] in the context of 2D-PAGE (Tamm Horsfall glycoprotein, alpha-1-antichymotrypsin, alpha-2-thiol proteinase inhibitor, alpha-2-HS-glycoprotein precursor), could not be confirmed – neither on carrier ampholyte IEF- nor SDS-PAGE. Strong binding was observed for *thioredoxin reductase* of *E. coli*, but no interaction occured with human thioredoxin reductases 1 and 2. Another strong non-specific binding was found for *enolase* from *S. cerevisiae*. Contrary to E. coli thioredoxin reductase, *human enolases* (tested were two enolases from human *brain*) were also non-specifically detected by the antibody. E. coli thioredoxin reductase as well as human and S. cerevisiae enolases are also non-specifically bound on IEF-PAGE. However, due to their higher isoelectric points (pI) these proteins focus outside the region of the gel used for EPO-evaluation and therefore have no influence on the analysis results.

Due to the observed non-specific interactions of the clone AE7A5 anti-EPO antibody with proteins other than EPO, immunoaffinity purification is mandatory for SDS-PAGE analyses. This pre-cleaning step has already been performed in the past, since the high protein content of urinary retentates and serum samples impedes the accurate detection of EPO by SDS-PAGE. This step is mandatory due to the non-specific binding of the antibody to thioredoxin reductase of E. coli. The molecular mass of this protein is within the range of rhEPOs on SDS-PAGE. An interference with the detection of rhEPO-doping

is excluded by the combined usage of (1) two monoclonal antibodies directed against different epitopes for the immunoaffinity purification step, (2) the Western double-blotting procedure, and (3) the usage of IEF- and SDS-PAGE for the interpretation of analysis results. This strategy has already been implemented in TD2009EPO.

Reference:

[1] Khan A, Grinyer J, Truong ST, Breen EJ, Packer NH (2005) New urinary EPO drug testing method using two-dimensional gel electrophoresis. Clin Chim Acta 358:119-30.