"Optimization of SAR-PAGE for inclusion of peginesatide (Omontys, Hematide) in comprehensive electrophoretic ESA doping testing"

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Project Overview

Currently, comprehensive initial testing for erythropoiesis stimulating agents (ESAs) is done by electrophoretic methods (IEF-, SDS-, SAR-PAGE) according to WADA TD2013EPO and the forthcoming TD2014EPO. However, these protocols only comprise endogenous and recombinant erythropoietins and their analogues (e.g. darbepoetin alfa). ESAs, which lack the primary structure of EPO and in particular the first 26 amino acids of the N-terminus, cannot be detected due to non-interaction with the monoclonal anti-EPO antibody used for Western blotting (clone AE7A5). Peginesatide (Omontys; formerly known as Hematide) is a so-called EPO-mimetic peptide, which is structurally unrelated to the amino acid sequence of EPO. Two methods (SDS-PAGE, electrospray mass spectrometry) were developed and published in 2011 in order to overcome this situation. However, both methods are unable to simultaneously detect epoetins (EPOs) - the majority of misused ESAs. By modifying Sarcosyl (SAR)-PAGE - one of the main electrophoretic methods used in EPO anti-doping testing - peginesatide will now also become part of routine ESA doping testing with SAR-PAGE. Despite Omontys was recalled in 2013, misuse by athletes cannot be excluded.

Results and conclusions

An electrophoretic method was successfully developed in order to get Peginesatide, a PEGylated EPO-mimetic peptide dimer, migrate in SAR-PAGE, which is one of the main methods used in EPO-testing. Due to its short amino acid chain and the two PEG-groups, sarcosyl cannot sufficiently solubilize Peginesatide for running it on SAR-PAGE. Upon replacement of 30% of the SAR in the running buffer by SDS (resulting in a final composition of 0.03% SDS and 0.07% SAR), Peginesatide migrates into the stacking gel without significantly broadening the CERA-band. With the optimized "SDS-modulated" SAR-PAGE method (mSAR-PAGE), Peginesatide can also be differentiated from CERA, since both bands are well separated from each other.

The new method also allows the simultaneous detection of Peginesatide and all EPO-based ESAs on one blot. By duplexing the two primary antibodies (clones 11 F9 and AE7A5) followed by incubation with an HRP-labelled anti-mouse secondary antibody, which binds to both antibodies, single blotting can be applied for detection.

However, the modification of an already published method for immunoaffinity purification of Peginesatide from serum and plasma samples could not be solved so far. The published protocol used covalently immobilized clone 1G9 antibody and released bound Peginesatide by heating the beads in SDS-

sample buffer. Despite double-blotting, bands from the light antibody chain remained visible on the blot. Hence, this approach was not applicable for the simultaneous detection of Peginesatide next to epoetins by single blotting. In search of a solution, three alternative methods were tested: (1) covalent immobilization of clone 1G9 antibody on agarose beads followed by acidic elution; (2) immunoprecipitation using anti-antibody coated magnetic beads; (3) immunoprecipitation using biotinylated clone 1G9 antibody and streptavidin-coated magnetic beads. However, they did not yet lead to useful results. Despite the project has ended, we will continue trying to find a solution.