"Ultra-Sensitive Mass Spectrometric Detection of a rEPO Specific O-glycopeptide as an Unambiguous Proof of Doping—Follow-up (GOpep2)"

Dr. J. Pascual, Dr. J. Segura (Fundacio IMIM (FIMIM), Spain)

PROJECT REVIEW

A project (acronym GOpep) was approved by WADA with the objective of detecting a Neu5Gc containing EPO O-glycopeptide using latest generation MS instruments (i.e. AB Sciex Qtrap 6500).

The EPO O-glycopeptide shows the lowest glycan heterogeneity, thus being the best choice to reach the necessary MS sensitivity. Results obtained showed that the glycopeptide isoform containing Neu5AcNeu5Gc was found to be the most abundant form with its triply charge species at m/z 810.3 giving the best signal. A limit of detection of around 2 IU EPO/L, from a standard preparation was achieved, compatible with the expected concentrations in human urine. An antibody against the peptide was also developed and initial results show that it also recognizes the glycopeptide. However, matrix effect when spiking real samples at very low concentrations, as well instrumental conditions to speed-up the analysis are still to be solved. The hypothesis of this project is that MS sensitivity has reached a status in which EPO glycopeptides, particularly O-glycopeptides as they present lower heterogeneity, are detectable in urine or blood samples. Using a proper combination of specific peptide immunopurification with other desalting techniques, matrix effects can be avoided and the required sensitivity for the EPO O-glycopeptide containing the non-human tag (Neu5Gc) reached.

Objectives:
1.- To improve sample clean-up by using the already developed polyclonal antibody against the peptide backbone and other desalting techniques.
2.-. To improve the nanoLC-MS/MS set-up using monolithic columns for high throughput and on-line sample clean-up.
3.- To develop monoclonal antibody for future use of the methodology if the use of the polyclonal proves successful.
4.- To validate the procedure in urine and serum samples

Results and Conclusions:

The main objective of the project was to develop an MS-based analytical procedure for the detection of an EPO O-glycopeptide containing the non-human monosaccharide N-glycolyl-neuraminic acid (Neu5Gc) as an unambiguous proof of the exogenous origin of the hormone (i.e. rEPO or analogues). The trypsin released EPO O-glycopeptide T13: (E117-R131) shows the lowest glycan heterogeneity, thus maximizing signal sensitivity while the peptide backbone will make it unique for EPO.

Through the method development, it was found that the formation of ubiquitous ammonium adducts was unavoidable, even under conditions where no ammonium salts were used.

The ammonium adducts of the doubly and triply charged species of the “endogenous” species (T13 O-2Neu5Ac) have masses identical (within 0.5 m/z) to the non-adduct forms of the “exogenous” species (T13 O-1Neu5Ac-1Neu5Gc) containing a 13C atom. The potential risk of having false positives forced the development of a method in which there was a complete chromatographic separation between these two very similar species.

The overall results demonstrated that the “exogenous” glycopeptide (T13 O-1Neu5Ac-1Neu5Gc) could be detected in rEPO using both a QTRAP6500 (low resolution) and an Orbitrap Fusion Lumos (high resolution). This approach includes the development of an MRM and PRM method respectively and the enrichment of the target EPO T13 O-1Neu5Ac1Neu1Gc using the anti-EPO T13 antibodies.

The method developed in this project is relatively straightforward, however the LOD using the most sensitive instrument in the market is still far from its applicability for real biological samples.

Still, as opposed to SAR-PAGE method, the proposed strategy may have the potential to unequivocally identify rEPO by detecting the 1-2% of T13 O-1NeuAc1NeuGc present in the sample using the signal of T13 O2Neu5Ac, which is present in all forms of EPO (exogenous and endogenous), as its own internal standard and quality control. The peptide chosen (T13) is unique for EPO, so it might allow the development of specific immunopurification techniques.