"Ultra-sensitive mass spectrometric detection of an rEPO specific O-glycopeptide as an unambiguous proof of doping (acronym: GOpep)"

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Project Overview

Detection of rEPO and analogues and their differentiation from endogenous EPO has been hampered by two major reasons: similarity and sensitivity. Regarding similarity, we already evidenced, through glycan analysis, the presence of minor amounts of N-glycolyl-neuraminic acid (Neu5Gc, MW: 325.27 u) in the recombinant preparations, while in the endogenous hormone, N-acetyl-neuraminic (Neu5Ac, MW: 309.27 u). The direct analysis of sialic acids had the drawback of losing the information of what protein were they attached to. The analysis of glycopeptides where the exogenous moiety is present and the aminoacid sequence unequivocally corresponds to EPO will be a perfect solution. However, sensitivity, particularly for glycopeptides, is greatly reduced given their microheterogeneity. O-glycopeptides, being far less heterogeneous, would the obvious choice. New instrumentation (AB Sciex 6500 QTRAP IonDrive) is able to detect as little as sub-attomol amounts of material. This sensitivity is, at last, compatible with the amounts present in urine of the precious non-human EPO glycopeptides. When combined with advances in immunopurification developed these years, the resulting method for detection of the presence of rEPO or NESP in human urine or serum/plasma will be quite straightforward, as any other doping control screening method and will offer unequivocal evidence.
Thus, the hypothesis of this project is that MS sensitivity has reached a status in which EPO glycopeptides, particularly O-glycopeptides are detectable in urine or blood samples. The MS differences between endogenous and recombinant EPOs (including NESP) will provide specific detection of the recombinant species as proof of doping.
The objective of the project are:
To develop an optimized method of immunopurification and enzymatic digestion of rEPOs.
To optimize separation and MS conditions for each O-glycopeptide and scale-up sensitivity using the latest state-of-the-art MS instrumentation from AB Sciex.

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-glycolylneuraminic acid (Neu5Gc) using latest generation QTrap-MS instruments as an unambiguous proof of the exogenous origin of the hormone (i.e. rEPO or analogues).

The EPO O-glycopeptide resulting from a tryptic digest (EAISPPDAAS*AAPLR) was chosen as it is unique for EPO and shows the lowest glycan heterogeneity, thus maximizing signal sensitivity while the peptide backbone will make it unique for EPO.

A method was developed based on pre-concentration by immunopurification using MAIIA cartridges followed by tryptic hydrolysis and LC-MS analysis in MRM mode. The peptide glycoform containing two sialic acids was found to be most abundant; the endogenous-like glycoform containing two N-acetyl-neuraminic acids (Neu5Ac) and its non-human counterpart containing one Neu5Ac and one N-glycolyl–neuraminic acid (Neu5Gc). The triply charged species ([M+3H]+3) at m/z 805.0 and 810.3 respectively were therefore selected as the precursor ions for target detection. Scale-up sensitivity was evaluated comparing standard, micro and nano-LC-QTrap6500-MS systems from AB Sciex with a ca. 10 fold increase in sensitivity between each step. The LOD achieved in nano-LC-Qtrap-MS for the rEPO standard was 80 attomol rEPO/□L, equivalent to the final concentration of extracted EPO expected from 20 mL of urine sample of ca. 2 IU/L. However, when urine samples were spiked with low concentrations of rEPO and taken through the procedure, the matrix effect prevented the detection of those low amounts. For that reason different approaches were tested to circumvent the problem. On one hand, a polyclonal antibody was raised against the EPO peptide backbone (EAISPPDAASAAPLRC), modified with a terminal cysteine residue for synthetic reasons. The polyclonal antibody proved to recognize both the peptide and the EPO-O-glycopeptide, making it ideal for further purification of the sample before instrumental analysis. In parallel, magnetic beads coated with titanium dioxide were tested, as they are able to selectively bind acidic residues, as the sialic acids present in the O-glycopeptide. The approach proved to be successful in quantitatively binding the O-glycopeptides and elute them under conditions compatible with MS analysis.

A final sample preparation method needs to be developed taking advantage of those tested methodologies making the unambiguous detection of a non-human rEPO O-glycopeptide amenable to MS detection.