"Detection of recombinant human erythropoietins by liquid chromatography mass spectrometry"

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Project aims:

For sports drug testing, the errorless discrimination of rEPOs from endogenous human EPO is important. The current gel electrophoretic methods are also labour-intensive for anti-doping laboratories.

Mass spectrometry-based procedures provide strong identification power, robustness and reproducibility in sports drug testing. Recently, we have developed a high-sensitivity and high-throughput qualitative detection method for human urinary NESP using LC-MS/MS. This was the first report of successful practical application of the mass spectrometric identification of NESP in human urine.

Following this outcome, the aims of this study are to optimize the NESP detecting method for a robust implementation in WADA accredited doping laboratories, and further to establish a novel detection method for other rEPOs, such as CERA in human matrices by LC-MS/MS for doping control purposes.

CERA is a long-acting erythropoietin derivative with methoxy polyethylene glycol butanoic acid linking to the N-terminal amino group or the ε-amino group of any lysine, predominantly Lys45 and Lys52, in the protein molecule. The structure of CERA is different from that of human EPO and epoetins. After enzymatic digestion, the target Pegylated peptides will be searched by the LTQ Orbitrap high resolution hybrid mass spectrometer for discriminating between CERA and endogenous EPO. Furthermore mass spectrometric glycoform profiling of rEPOs (e.g. epoetins) will be performed by LC-ESI-MS/MS. After enzymatic digestion of core protein and glycan of rEPOs, the target digested glycosylated peptides will be identified by the LTQ Orbitrap high resolution hybrid mass spectrometer (Thermo) and SynaptG2 HDMS TOFMS (Waters) for discriminating between epoetins and endogenous EPO. We focus the differences of syalylation, N-glycosilation, O- acetylation of sialic acids and O-glycosilation between epoetins and human EPO, and we have an optimized CID technique for discriminating N-glycopeptides by LC-MS.

Results and Conclusions:

The current analytical methods for recombinant human erythropoietin (rEPO) are mainly gel electrophoretic methods. Mass spectrometry is necessary for

the reliable identification of rEPOs in doping control. The aim of this research project is to develop the mass spectrometric detection method for human sports drug testing.

The research comprises three topics, i.e., 'Mass spectrometric characterization of rEPOs and their biosimilars', 'Improvement of the LC-MS/MS method for detecting darbepoetin alfa in human urine', and 'Mass spectrometric approach for detecting CERA'.

It has been recognized that EPO biosimilars typically have the same amino acid sequence as the innovator product, and that they have slight differences in overall chemical structure. As expected, the biosimilars of darbepoietin alfa have different glycan distributions compared with the originator. Interestingly, the biosimilars of darbepoietin alfa and a biosimilar of epoetin alfa contain not only des-arginine product comprising 165 amino acids, but also a C-terminal arginine product comprising 166 amino acids, in contrast to the results in which the originator recombinant EPO products. darbepoetin alfa and CERA contain only des-arginine EPO comprising 165 amino acids. The presence of C-terminal arginine EPO in human matrices might be evidence of administration of EPO biosimilars.

We established a high-quality and more robust UPLC-MS/MS method for detecting darbepoetin alfa using a deutrated internal standard. The lower detection limit was 1 pg/ml. The detection window in urine from patients treated with NESP was estimated to be up to 16 days after administration. The present method could be a useful tool for detecting the biosimilars of darbepoetin alfa for doping control testing because the method does not depend on the glycan profiles. CERA and epoetin beta representing human EPO could be successfully discriminated using LC-MS/MS with in-source CID. However, this mass spectrometry-based technology needs to be improved in terms of sensitivity for detecting CERA in human biological fluids.