PROJECT REVIEW

"Unequivocal confirmation of recombinant erythropoietin (rhEPO) in human urine through structural evidences of specific glycosylation"

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The abuse of recombinant hormones, as erythropoietin (EPO), in the world of sport is of special relevance. For long time, the absence of a methodology able to differentiate the recombinant pharmaceutical hormone (rhEPO) from its natural engonenic counterpart neither in blood nor in urine has created the suspicion of its widespread use in some sports.

Recently, two methods with completely different approaches have been proposed.
- The first one, using blood, is based on monitoring some *'indirect haematological parameters"* affected by the administration of EPO (soluble transferrin receptors, hematocrit, reticulocite, etc.). It is an indirect method that allows suspecting the misuse of EPO, but that needs a confirmation procedure to draw a definitive case.
- The second one, using urine is based on the ability of isoelectric focusing (IEF) in gel to separate between the different glycoforms of the protein based on their charge. Ultimately, the method uses a chemiluminescent detection system that allows visualising the spots corresponding to the different separated glycoforms.

Up to now, however, there is no definitive confirmation showing structural differences in the more basic isoforms characteristic of the rhEPO.

The aim of the present study is to identify the precise carbohydrate structure that is unique for the recombinant hormone (rhEPO) so being able to, in an absolute way, differentiate between endogenous and exogenous EPO.

The study will be based, primarily on the more promising glycoforms already separated by the existing IEF method to do:
- Analysis of the N-glycans using HPLC and fluorescent detection.
- Analysis of the peptide fingerprint using last generation mass spectrometric techniques.
(MALDI-TOF, nanoLC-ESI-Q-TOF).

Once the differences have been identified and characterized, research will be conducted for the use of those techniques on the direct urine analysis making the whole method applicable for high throughput analysis, as required by in-competition doping control.

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Results and Conclusions

The project “Unequivocal confirmation of recombinant erythropoietin (rhEPO) in human urine through structural evidences of specific glycosylation” (acronym:RHEPOSE) had as its central objective the structural elucidation of the glycans present in rEPO and NESP. The ultimate goal was obtaining the information that will lead to the establishment of the differences between those recombinantly produced materials and the endogenous urinary EPO.
We developed and applied all necessary tools for the structural analysis of the Nglycans present in each single IEF band and characterise the microheterogeneity of rEPO and NESP (E. Llop et al. Proteomics, 2007; 7: 4278-4291). The results showed that EPO alfa and beta as well as NESP, although very heterogeneous, have been purified to contain a great proportion of tri and tetraantenary structures most of them fully sialylated. No charges other than sialic acids were found in the carbohydrates of those recombinant species while there were present in endogenous urinary EPO. Some enzymatic activities potentially present in urine would justify the behaviour described for EPO in some samples. The addition of inhibitors or competitive substrates of such enzymes was shown to be a potential solution to those situations (V. Belalcazar et al. Electrophoresis, 2006; 27(22): 4387-4395).
The presence of N-glycolyl-neuraminic acid, a sialic acid not produced in humans was found in NESP, and confirmed in rEPO (BRP standard), in both cases in amounts of ca. 1% of the total sialic acid content. The presence of such monosaccharide in EPO may be used as an unequivocal proof of its exogenous origin.
The lack of a pure urinary EPO standard prevented us from achieving the same structural analysis with the endogenous protein. An appropriate purification procedure is still being developed, although the trace amounts of EPO in urine and the need to isolate its glycans from other glycoproteins, make it particularly challenging.
In a complementary approach, polyclonal antibodies were developed using short peptides containing the differing aminoacid sequences of rEPO and NESP. Those antibodies were able to differentiate between both species, although the sensitivity achieved prevented its use in routine analysis (E. Gimenez et al. Anal Bioanal Chem 2007; 388: 1531-1538)

Publications

- Variables affecting EPO detection by isoelectric focusing in human urine samples. (submitted).

- Potential isoform discrimination during immunoaffinity purification of epoetin and darbepoerin. 15th IFCC-FESCC European Congress of Clinical Chemistry and Laboratory Medicine.

- Anti-epo and nesp specific antibodies raised against synthetic eptides reproducing minimal differences in amino acid sequence. E. Gimenez, de Bolos, V. Belalcazar, D. Andreu, E. Borras, B. G. de la Torre, J. Barbosa, J. Segura, J.A. Pascual. Anal Bioanal Chem 2007; 388: 1531-1538.

- Assessing the instability of the isoelectric focussing patterns of erythropoietin in urine. V. Belalcazar, R. Gutierrez-Gallego, E. Llop, J. Segura, J.A. Pascual. Electrophoresis, 2006; 27(22): 4387-4395.

- Stability of EPO in urine stabilized by sodium azide. V. Belalcazar, J.A. Pascual, S. Abanades, M. Farre, R. de la Torre. In: Schänzer W, Geyer H, Gotzmann A, Mareck U, eds. Recent Advances in Doping Analysis (13). Köln: Sportverlag Straub, 2005: 427-431.

- Evaluation of protein N-glycosylation in 2-DE: Erythropoietin as a study case. E. Llop, R. Gutierrez-Gallego, V. Belalcázar, G.J. Gerwig, J.P. Kamerling, J. Segura, J.A. Pascual. Proteomics, 2007; 7: 4278-4291.

- Recombinant erythropoietin and analogues, a challenge for doping control. J.A. Pascual, V. Belalcazar, C. de Bolos, R. Gutierrez-Gallego, E. Llop, J. Segura. Ther Drug Monit, 2004; 26(2): 175-179