“A holistic approach to (glyco)protein hormone doping analysis”

R. Gutierrez Gallego, J. Segura, J. Bosch, E. Llop (IMIM, Barcelona, Spain)

PROJECT REVIEW

Protein hormones represent an extremely challenging analytical problem in terms of anti-doping control. The main reasons are that most of these proteins are produced by humans and that the concentrations in body fluids, such as blood or urine, are very low. Whereas the second condition puts stringent demands on the analytical instrument in terms of sensitivity the first condition does similar to the scientist’s ingenuity in order to enable differentiation between like and non-like. Thus far, all protocols addressing protein hormone doping are based on immunological techniques only and suffer from the problems like the unknown specificity of the antibodies in the assays, the potential cross-reactivity under different conditions, and the fact that all sample handlings cannot be monitored and only an end-stage reading is provided. Still, regardless the analytical measurement immunoglobulins will be required to address specifically a particular category of proteins in complex mixtures.

This project aims at the development of a single step purification of all protein hormones from plasma through a multi-antibody platform followed by in-situ solid-phase proteolysis and nano-LC chip mass spectrometric identification and quantification. Three different phases can be distinguished for each protein hormone:

A- the characterisation of antibodies addressing a particular protein (or category) by means of surface Plasmon resonance (SPR). From this study the best immunoglobulin (in terms of surface bound properties, specificity and cross reactivity, thermodynamic parameters of the interaction, compatibility in a mixed antibody setting) will be selected.

B- B- extrapolation of the SPR results to a LC-compatible immunoaffinity stationary phase. For this purpose monoliths functionalised to use similar immobilisation chemistry as in SPR will be employed. Again, first single antibody monoliths shall be characterised and subsequently multi antibody monoliths will be build to finally address all protein hormones in a single step.

C- C- Identification and quantification of the IAC purified material. This will be accomplished by -1- eluting the IAC captured material onto a protease-containing stationary phase with a short (min) stop-flow setting to allow proteolysis -2- elution of the generated peptides into a reversed phase

column to allow conventional chromatography -3- identification of the peptides from each hormone. Quantification shall be achieved using diagnostic peptides with specific isotopic labelling that will be injected as internal standards in each analysis.

The outcome should be a “all-in-one” single injection system that reduces sample manipulation/loss and addresses all hormones employing the same sample, recovering the remainder of the sample for other analytical procedures.

Results and Conclusions

The project AntiProDo aimed at the development of a single instrumental set-up that included purification of four protein hormones from plasma through a multi-antibody platform followed by in-situ solid-phase proteolysis and nano-LC chip mass spectrometric identification and quantification.

Through a meticulous characterisation of the binding characteristics of multiple antibodies, one was selected for each of the four initial target analytes: hGH, EPO, hCG and IGF-I. Subsequently, customised monolith solid supports in silica capillaries were produced and functionalised with the antibodies. The functional behaviour was verified. Simultaneously, the same capillary support was developed to house proteolytic enzymes and the activity, efficiency and durability established. At another front, the target proteins were submitted to mass spectrometric analysis to establish the proteotypic peptides to target in a final setting. Heavy isotope labelled peptides were produced as internal standards and the analytical method based on nano-LC ms designed and validated. Ultimately, all elements were hyphenated to demonstrate the proof of concept for this approach.

Further optimisation of the individual steps, particularly at the reproducibility in the manufacture of the functionalised capillaries, is required before this approach can be taken further