PROJECT REVIEW

"Development of a Proteomic Technology Platform for the Generic Detection of Protein Drugs in Sport"

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As the use of protein drugs for enhancing athletic ability becomes more prevalent it is essential to have a general detection technology able to be used to detect the protein drugs available now and any new protein variants that will be used in the future. This proposal is for the development of such a generic protein analysis platform based on protein concentration procedures, gel electrophoresis separations and in the first instance, antibody detection. Specifically, methods for the improved detection of the endurance drug, erythropoietin in urine will be developed, as well as testing the feasability of a new detection process for human growth hormone in plasma.

When proteins are produced for medical uses they almost invariably change their properties of isoelectric point (p1) and/or molecular mass (MW) or are metabolized differently from the native protein in the body. The pharmaceutical drug companies usually have no need to make proteins identical in structure and/ or composition to the native protein if the efficacy of the protein drug is not affected. Synthetic proteins are difficult and costly to "humanize" and are sometimes actually deliberately modified in the production process in order to affect their delivery and/or their time in the body. These characteristics can be exploited experimentally to enable the drug forms to be differentiated from the native human forms of the protein.

Proteome Systems has a proven track record in proteomic analysis and in the development of technology to characterize proteins. Their scientists have a proven research track record in innovative methods for protein analysis, including protein separation, identification and characterization of their modifications. The company has developed and manufactured several instruments to automate the electrophoretic and mass spectrometric analysis and have produced informatics programs to track samples and data. They have also engineered diagnostic antibody/antigen point-of-care new technology that will facilitate the introduction of any developed antibody based drug screening test. Proteome Systems is thus well positioned to take on the task of solving the varied challenges of detecting the abuse of clinical proteins by athletes who hope that the similarity of these drugs to the human proteins will make their detection difficult.

The close collaboration with the Australian Sports Drug Testing Laboratories and the Penang Doping Control Centre will enable athlete urine and blood samples that they have collected for other tests to be used for method development, as well as providing a highly experienced testing laboratory for validation of the assays developed. As the protocols and instrumentation become available they

will be transferred to these laboratories for accreditation. It is expected that substantial improvements and provision of instrumentation and quality controlled consumables to enable a more reliable and automated EPO test will become available to IOC laboratories by the end of the grant.

Development of Protocols for the Detection of Protein-Based Drugs in Sports

Results and Conclusions

In 2003, the Peltre –Thormann report commissioned by the World Anti-Doping Agency identified specific issues to address in order to improve the one-dimensional isoelectric focusing electrophoresis method currently being used for identifying drug doping with recombinant erythropoietin (rHuEPO).

The normal human form (HuEPO) and the drug (recombinant) form of EPO (rHuEPO) have exactly the same protein component. The reason they can be differentiated in tests is that the number and type of sugar groups attached to the protein, which account for about 40 per cent of the mass of EPO, differ between the two proteins and change the charge and mass of the different forms.

The **First Year** report described a new method for the detection of recombinant drug erythropoietin (EPO) in urine using two dimensional gel electrophoresis (2D Method). The new protocol covered changes to urine sample preparation, 2D electrophoretic separation of the endogenous from the exogenous protein isoforms, single blotting, Western detection and a software algorithm for analysis of the 2D image data.

The 2DE method separates HuEPO and rHuEPO by both iso-electric point and molecular mass.

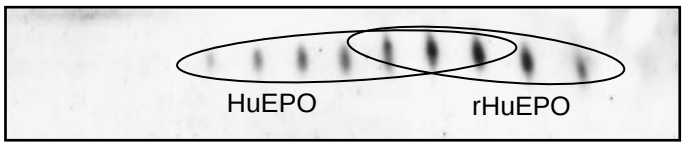

Figure left: separation of normal human EPO (HuEPO) from the recombinant drug form (rHuEPO) in urinary proteins separated by the 2D method

This method was published in *Clinical Chemica Acta* Vol. 238 (2005) p.119-130, and was presented at the Manfred Donike workshop in Cologne in 2004. A favourable comparison of this method with that of the current testing protocol (1D Method) was carried out on spiked samples in conjunction with the WADA accredited doping control laboratory in Sydney, Australia (National Measurement Institute) and Penang, Malaysia (Doping Control Center), and was presented at the Manfred Donike workshop in Cologne in 2005 and published in the proceedings. The 2DE method presented dealt with most of the WADA recommendations for an improved EPO test and provides a sensitive and accurate detection of the EPO drug in urine.

The **Second Year** of the project largely concentrated on optimising this EPO 2D method protocol for transfer to the anti-doping laboratories for validation and for comparison with the current IPG separation method (1D Method). We had identified major urinary proteins, and removed some but not all, non-specific binding by addition of an acidic wash of the blot in the revised protocol. An optimized 2DE method Standard Operating Procedure for EPO testing in urine was developed with the support of some WADA accredited laboratories.

Conclusions: It is our conclusion that once fully validated in an anti-doping environment the 2D Method for EPO testing could become an attractive complementary test of the current 1D Method until such time as it becomes proven in reproducibility and reliability in accredited WADA laboratories. The 2D Method is still laboratory based and requires some skill level and time but likely offers improvements in separation of the isoforms from each other and from other urinary proteins which may react with either the primary and/or secondary antibodies, removes the need for double blotting, and has a horizontal as well as vertical separation of the drug form from the endogenous EPO protein. The two dimensional gel separation in the 2D Method also offers the advantage of being able to add a migration reference standard to the 2D gel electrophoresis for accurate measurement of the migration of the detected EPO, and the opportunity to use image analysis of this result for automated identification of the drug form.

Preliminary studies were also carried out on the feasibility of using two dimensional electrophoresis as a method for the detection of human growth hormone (hGH). There was question as to whether the high turnover and variability of the endogenous hormone make the detection of the actual administered protein a desirable drug doping test method. In addition, the primary differences between the endogenous form and the exogenous drug appears to be low level, variable proteolysis as well as possible single amino acid substitutions, phosphorylation, oxidation and other modifications. These low abundance, small mass and pI differences are difficult to detect by the method of 2D PAGE so this approach is not recommended to pursue further for the development of a hGH drug detection protocol.