PROJECT REVIEW

"Development of a high throughput reference method for multiplex analysis of biomarkers of growth hormone abuse"

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The detection and quantification of small molecule doping agents has become a relatively simple process with mass spectrometry regarded as the definitive, analytical methodology. The threat from biological agents such as proteins, peptides or genetic manipulation, provides a much greater analytical challenge for most doping control laboratories. As a result of this much research has been expended into the use of endogenous proteins as so called biomarkers of abuse.

Until recently the only effective tool for quantitative analysis of large molecule targets has been enzyme linked immunosorbent assays (ELISA) or similar immunoassay based techniques. These assays are very effective for the analysis of large molecules, particularly where sandwich assays, involving the use of two complimentary antibodies, are employed. While it is likely that immunoassays will remain the standard for routine quantitation of single proteins, great strides are being taken in the development of quantitative methods based upon mass spectrometry, a highly sensitive and selective technique widely applied in the fields of pharmaceutical and sports testing.

More recently, developments in sample handling techniques, chromatographic stationary phases and triple quadrupole instruments have made the quantification of proteins by mass spectrometry a reality which could be transferred into the majority of doping control laboratories. A major advantage of using LC-MS is that it provides an independent means of detecting the target protein compared to immunoassay and is a potential gold standard for validating these methods. This is reinforced by the ability to monitor several peptide fragments from the same protein to increase the confidence in the analytical result and it is also possible to analyse multiple proteins in a single analytical run.

The aim of this project is to develop a generic LC-MS approach to the quantification of a range of growth hormone (GH) biomarkers currently used or proposed for use in sports drug testing. The method will be designed for use in a typically equipped doping control laboratory and will make as little use as possible of specialised equipment.

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Results and Conclusions

The aim of this project was to develop a single methodology based upon LC-MS capable of detecting multiple biomarkers of the administration of growth hormone (GH). The preferred aim was to do so without recourse to antibodies or similar bio-reagents for which continuation of supply could be an issue. Potential applications of the developed method would be as a reference method for the detection of GH biomarkers or possibly for use as a confirmatory procedure for the same.

The approach to the project was broken down into:

- the development LC-MS methods to detect the proteins of interest via proteotypical peptides,
- to obtain stable isotope labelled versions of the targeted peptides,
- to develop a generic extraction protocol capable of isolate all the analytes in a single fraction
- to validate the resultant methodology

The markers chosen were based upon those previously identified as providing the most definitive proof of GH abuse PIIINP, IGF-I and its binding proteins including IGFBP-3. In addition a number of additional potential biomarkers (IGFBP-2, IGF-II LRH) were included in the assay development phase.

In the case of PIIINP, direct determination of potential prototypic peptides using traditional approaches proved impossible given the low levels of standard material available. Significant effort was expended identifying possible approaches to hydrolysis of the protein. By means of computer simulation (in-silico digestion) the most likely enzyme for cleaving PIIINP to produce sufficiently selective proteolytic peptides for use in an assay was

determined. The chosen protease, Glu-C, was utilised but detection of the expected peptide fragments remained elusive. Finally, a custom synthesis of the expected peptide was commissioned and using this standard it proved possible to detect a target peptide for the analysis of PIIINP using LC-MS. While a target peptide is now available, this was accomplished a stage in the experimental work to prevent full development of an assay to be undertaken.

Having developed LC-MS methodologies for the detection of proteotypic peptides of IGF-I, IGFBP-3 and related peptides attention was switched to optimisation of extraction methodologies to isolate the proteins from human plasma. In practice, it was found that isolation of IGF-I was related to the concentration of the IGFBP's present in the sample unless steps were taken to completely disrupt the binding of these proteins. Optimised conditions for extraction of IGF-I could be obtained by acidification of the sample and extraction on a polymeric phase SPE cartridge. This approach gave only low and variable extraction efficiency for the IGFBP's and a second methodology optimised for these analytes was developed.

Analysis of samples for IGF-I in both plasma and serum using this approach proved successful. Reasonable results could also be obtained for the binding proteins with acceptable data obtained for calibration lines and QC's. All indications are that these methods, if applied in the correct manner, would provide the basis for successful analysis of IGF-I and IGFBP-3 in plasma / serum samples.

The developed methodologies for IGF-I, the IGF binding proteins and related biomarkers could be used as the basis of confirmatory methods for these markers in human plasma / serum or as reference methods with which immunoassay based methods can be calibrated or controlled. In order to do so reference matrix with known levels of each marker would prove highly beneficial and aid the introduction of LC-MS methodologies into WADA sports testing laboratories. While it proved impossible to develop a methodology for PIIINP in the time frame available, an appropriate proteotypic peptide has been identified which could form the basis for the development of an LC-MS based assay. Publications are in preparation detailing the methodologies developed and initial investigation of PIIINP and this target peptide.