

***“Two-dimensional liquid chromatography high resolution mass spectrometry screening method for peptide hormone in urine”***

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**Project Overview:**

The number of peptide hormones, growth hormones and related substances which are required to be tested for are consistently increasing. The main catalyst for this fact is the marketing of products which have had limited data published in medical journals and are then produced and sold via internet websites. This makes them easily accessible and a concern for fair play in sport as well as the health of the athlete as the products may not have therapeutic use approval in humans and can be of poor quality.

In general the analysis of peptide hormones has been addressed one by one with many of the techniques requiring large volumes of urine and complicated extraction protocols. Recently several publications have multiplex methods to include a selection of peptides of interest to antidoping laboratories. These methods have involved either a direct analysis of urine samples for compounds expected at high concentrations or used antibodies for purification to enable lower detection limits.

Particular areas have been identified as playing an important role for the development of a multi-residue analysis technique; extraction/concentration including the use of two dimensional (2D) liquid chromatography, availability of standards and metabolites of peptide hormones. The extraction/concentration of the samples will be examined include solid phase extraction (cartridges/pipette tips), magnetic beads with antibodies, molecular weight cut-off filtration and 2D liquid chromatography. Many of the peptides which are sold over the internet are not available from suppliers of standard materials which makes it more difficult for laboratories to provide testing methodologies. This project includes making available standards suitable for laboratories to develop method with for testing procedures. An in vitro enzyme assay will also be setup with kidney microsomes to be applied to new peptide based drugs to determine the metabolites that are likely to be found in urine.

**Results and Conclusions**

For over a decade the Australian Sports Drug Testing Laboratory (ASDTL) has been analyzing black market seizures for the Australian Border Force. Many different peptide hormone products have been identified and include Gonadotropin Releasing Hormone (GnRH) and analogues (eg. triptorelin), Growth Hormone Releasing Hormone (GHRH) and analogues (eg. CJC-1295), Growth Hormone Secretagogues (GHS) and mimetics (eg. ipamorelin) and Growth Hormone Releasing Peptides (eg. GHRP-6). Many of these substances

continue to be confiscated at the border which means the risk of abuse by athletes in Australia, as well as other in countries around the world remains. The detection in bodily matrices is important, not only for ensuring fairness in sport but also ensuring the health and wellbeing of athletes.

As instrument sensitivity improved, publications were emerging outlining 'dilute and shoot' methods for small molecules. Given the difficulties encountered using SPE with the increasing list of peptides being analysed, a 'dilute and shoot' method was developed and validated where diluted samples were passed through Agilent Captiva ND Lipid plate to remove interferences. This methodology was superseded with the implementation of ionKey/MS systems and SPE sample preparation which can analyse approximately 50 small peptides, metabolites and peptide mimetics. Limits of detection for the majority of sample range between 0.1-0.5 ng/mL with recoveries between 20-99%. The workflow for the small peptide should also allow the addition of conjugated small molecules and the development will be included in future planned work.

The most significant impact of this project has been the production of reference material and the availability of these to WADA Laboratories. Some of the materials are available from commercial providers but the identification and production of new materials in particular metabolites can be considerably more responsive when initiated by a WADA laboratory than a commercial provider. The ASDTL now has available 60 parent and metabolites peptides.