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## The use of isotopically labelled internal standards for hCG analysis

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### Introduction

Human chorionic gonadotrophin is a peptide hormone that is responsible for the stimulation of sex hormone secretion. Intact hCG is comprised of two noncovalently bound subunits,  $\alpha$  and  $\beta$ , each are glycosylated i.e. have carbohydrates attached at specific amino acid residues, and are highly cross linked internally by disulfide bridges. The  $\alpha$  subunit contains 92 amino acids and has a molecular weight of 14.5 kDa. The  $\beta$  subunit contains 145 amino acids and has a molecular weight of 22.2 kDa (Vankrieken et al., 2000). The hormone occurs naturally in humans in the case of pregnancy, trophoblastic diseases, testicular cancers and other tumours (Vankrieken et al., 2000). hCG is of interest in the area of sports drug testing as it can be misused by male athletes to induce the increased production of endogenous testosterone without affecting the T/E ratio (Gam et al., 2003)

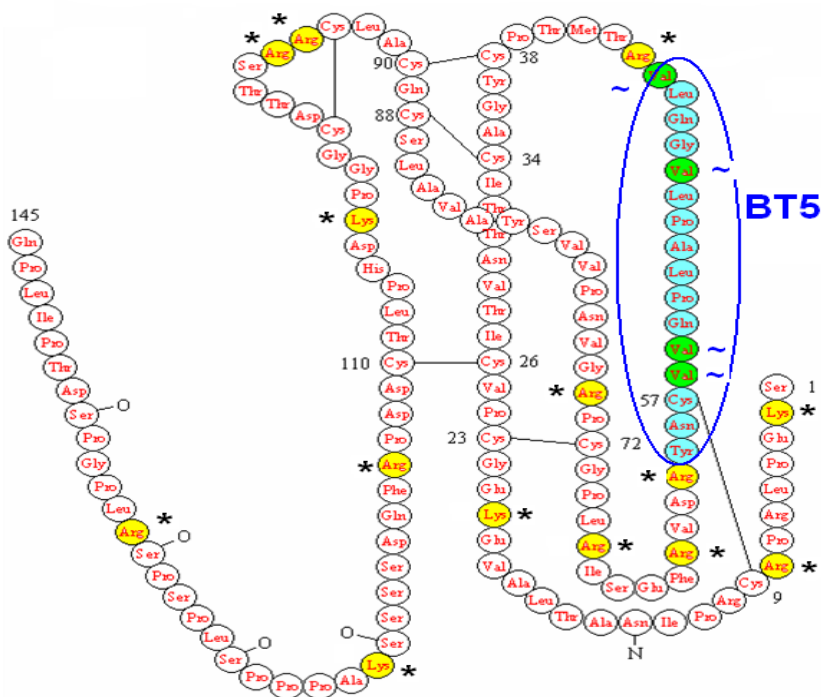


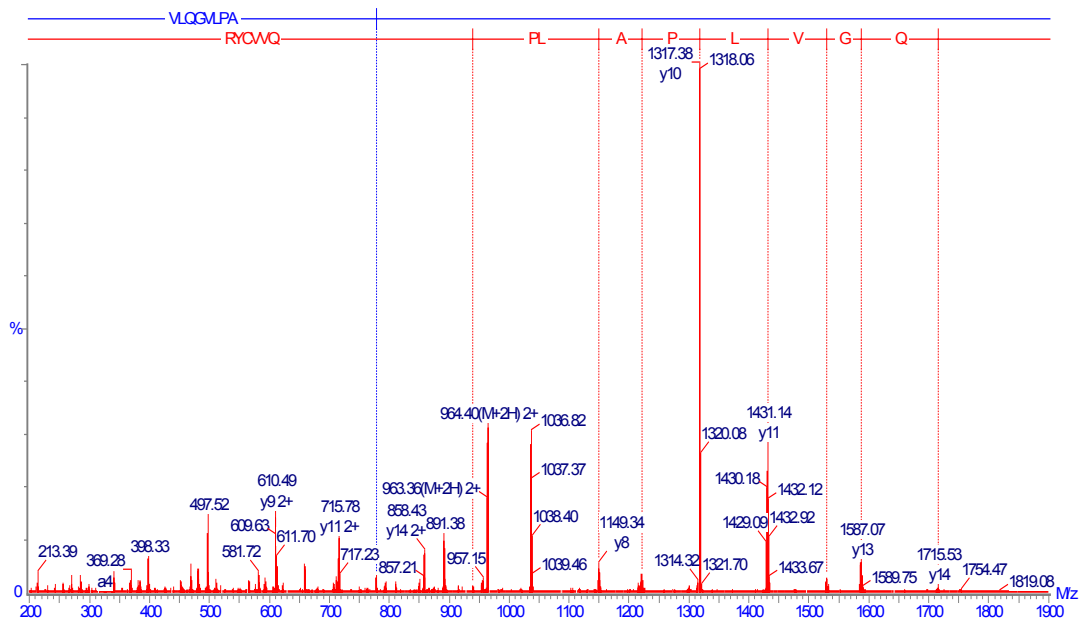
Figure 1: Representation of the structure of  $\beta$  subunit of hCG with the highlighted amino acids representing the cleavage sites for the tryptic digestion (\*),  $\beta$ -hCG-T5 segment (circled region) and isotope labelled valines (~).

## Quantification

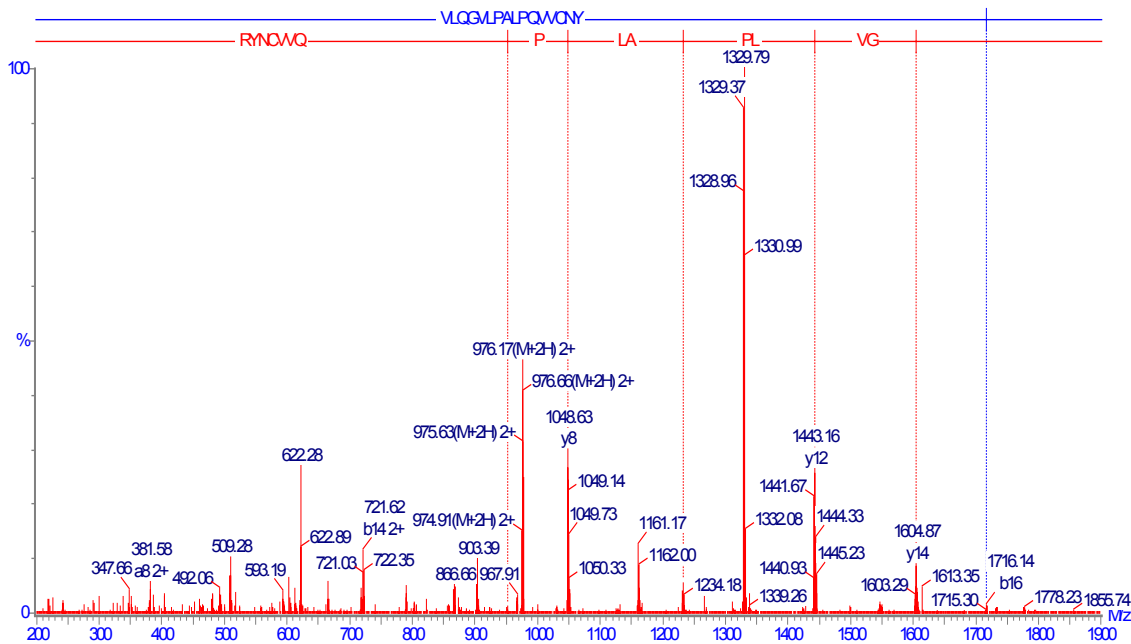
Figure 1 illustrates the  $\beta$  subunit of the hCG molecule. Highlighted also is the region corresponding to the tryptic fragment hCG- $\beta$ T5. This fragment has been identified as the section of the molecule that interacts with the hCG receptor in the testis thereby producing testosterone (Gam et al., 2003). The tryptic digest fragment  $\beta$ T5, when reduced and alkylated (using dithiothreitol and iodoacetic acid) is characteristic of the  $\beta$  subunit of hCG, and detection of this peptide has formed the focus of the continuing work in the development of an Liquid Chromatography tandem Mass Spectrometry (LC/MS/MS) method for the quantitative detection of hCG.

Quantitative detection of peptides in biological samples such as urine, by Electrospray ionisation (ESI) MS, is complicated by the effects of the matrix components. Ion suppression occurs, as the numerous components of the urine matrix compete for ionisation with the analyte in the ion source of the MS (Stokvis et al, 2005). This interference does not affect the validity of the mass spectral identification but has been referred to as the Achilles heel of quantitation using ESIMS (Taylor, 2005). For doping control purposes quantification is likely to be important and so techniques such as isotope dilution are being incorporated in the methods to facilitate precision at femtomole levels (Bronstrup, 2004).

As quantitative measurements are to be made, both a pure standard of  $\beta$ -hCG-T5 and a suitable internal standard were required. There are two options available: structural analogues or isotopically labelled analogues. Structural differences between the analyte and the internal standard result in varied ion suppression experienced by the compounds, therefore it is important that the analyte and the internal standard are as similar in chemical structure as possible. The ideal internal standards for LC/MS are isotopically labelled analogues of the compound of interest as they co-elute from the LC into the MS ion source they experience the same amount of matrix effect (Stokvis et al, 2005). Cambridge Peptides (UK) produced both the natural  $\beta$ -hCG-T5 and a labelled analogue with each of the four valines in the molecule having  $^{13}\text{C}$  rather than  $^{12}\text{C}$  and  $^{15}\text{N}$  rather than  $^{14}\text{N}$  atoms. This results in a total mass difference of 24 Da for  $\beta$ -hCG-T5. Figure 2 and 3 shows the MS/MS comparison of the unlabelled peptide and the isotopically labelled variant produced by Cambridge Peptides (these were reduced with dithiothreitol and alkylated with iodoacetic acid) which are in agreement with the expected sequence of  $\beta$ -hCG-T5. These analyses were carried out on the Micromass Quattro Micro MS.



**Figure 2. MS/MS spectrum of natural  $\beta$ -hCG-T5 peptide generated from the 964 precursor ion.**



**Figure 3: MS/MS spectrum of  $^{13}\text{C}_5$   $^{15}\text{N}_1$  isotope  $\beta$ -hCG-T5 peptide generated from the 976 precursor ion.**

An initial experiment was carried out without using an isotopically labelled internal standard. A series of five urine spikes were prepared at intact hCG concentrations ranging from 8 to 5000 IU/L. These 10ml spiked samples were concentrated to 200ul using the Centricon 10 kDa ultrafilters (Millipore), each sample then reduced and alkylated. Samples were desalted

prior to tryptic digestion and analysed on the Micromass Quattro Micro MS. The results showed a linear response over the full range of concentrations ( $R^2=0.998$ ).

Another experiment was conducted to determine the efficacy of using the labelled internal standard during the LC/MS/MS analysis. Solutions containing concentrations of the synthesised  $\beta$ -hCG-T5 ranging from 13 to 10,000 ng/mL with a constant concentration of the labelled  $\beta$ -hCG-T5 (10,000 ng/mL) were prepared and reduced and alkylated as above. However these peptide standards were desalted prior to analysis on the ABI Q trap 4000 using Vivaspin C18 microcentrifuge filters, as the 10 kDa ultrafilter devices were not suitable for the peptide. The results demonstrated linearity for the quantification over the wide concentration range when correction for the internal standard is made ( $R^2>0.999$ ). The addition of a high concentration peptide internal standard also reduces the potential losses of the analyte due to adsorption.

### **Conclusion**

These preliminary results show the potential of the method using a labelled internal standard and a high sensitivity LC/MS/MS to not only detect and confirm the presence of hCG at low concentrations but also to precisely measure its concentration. The lowest concentration standard used corresponds to an hCG level of approximately 5 IU/L in urine assuming a concentration factor of 50 during extraction and purification.

### **References**

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