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Measurement of LH in urine. A test for doping?

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Abstract
The ANZAC Research Institute and ASDTL have been partners in a WADA funded project to evaluate detection tests for the administration of recombinant human luteinizing hormone (LH). The measurement of LH in urine has been a significant part of this project. This paper will outline some of the problems encountered in the immunoassay detection of administered recombinant LH.

Introduction
The LH concentration in urine has been used as a marker for testosterone (T) and human chorionic gonadotrophin (hCG) abuse through the T/LH ratio by WADA laboratories (Cowan *et al.*, 1991; Perry *et al.*, (1997)). Figure 1 plots the concentration of urinary LH and T measured for an individual given a weekly injection of 250 mg of T from weeks three to eight (Nelson *et al.*, 2006). The urine T concentration was observed to remain relatively consistent during the period of administration while the LH level dropped significantly to consistently low concentrations. The T/LH ratio increased from less than 10 to over 40 nmol/IU during the T administration period remaining elevated fro at least 3 days before returning to baseline a week after cessation of treatments. Thus an elevated T/LH ratio attributable mainly to suppressed LH level is an indication of testosterone abuse.

The recent marketing of recombinant LH from a single manufacturer has lead to the speculation of its possible doping use. There are several possibilities as to why an athlete would use LH, firstly it could be used to mask the use of prohibited substances, such as anabolic agents, and/or to increase the production of endogenous T (Handelsman 2006). Here we aim to outline some of the problems encountered in the detection of endogenous and administered LH in urine samples. The stability of LH in urine under different storage

conditions, the preparation protocols of the urine sample before analysis and a comparison of analysis kits for the detection of endogenous (pituitary) and recombinant LH will be discussed.

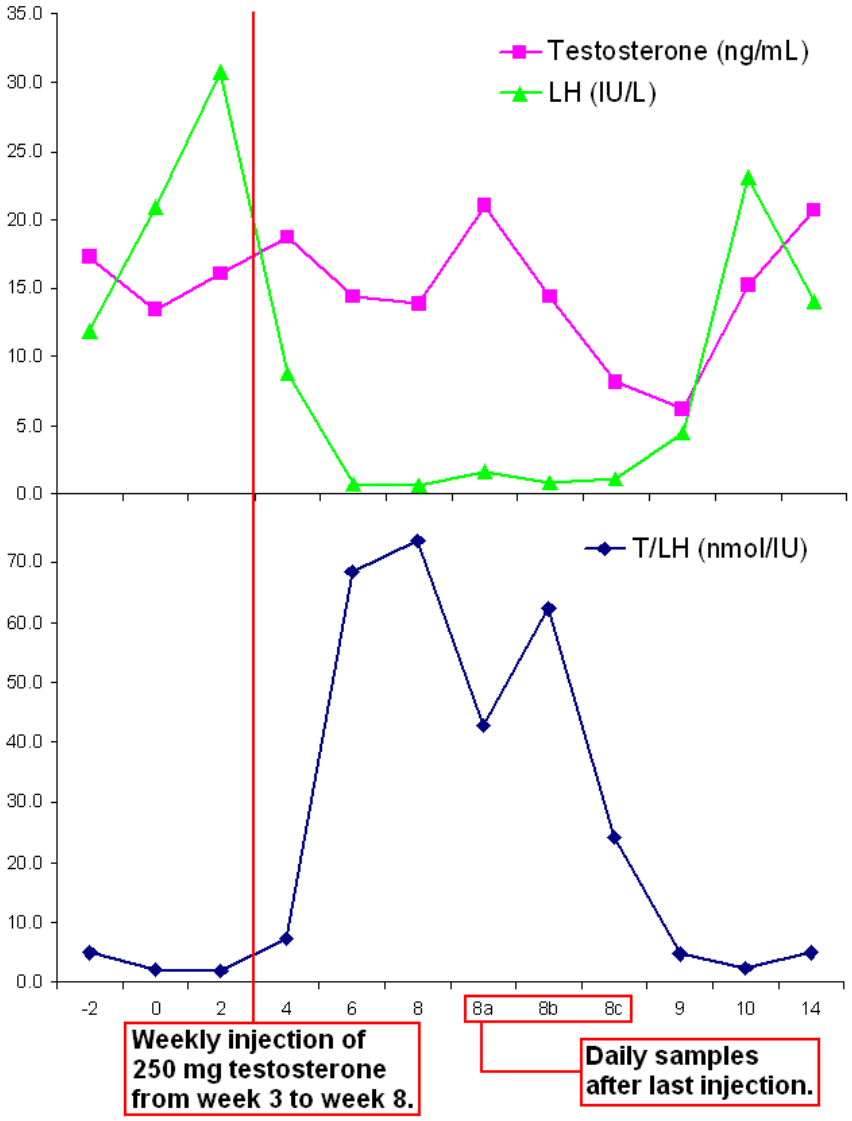

Figure 1 Plot of urine T, LH and T/LH results for an individual given 250 mg of T from weeks 3 to 8. Sample collection was for 16 weeks.

Materials and Methods

Recombinant human LH from Serono Australia Pty Ltd (Luveris, same as injected into study subjects), endogenous LH from human pituitary (WHO International Standard, 80/552) and recombinant human LH (WHO International Standard 96/602).

Standard protocols were followed as per manufacturer's instructions for the LH

measurements by Immulite LH assay (Siemens Australia, NSW) and by Delfia hLH assay (Perkin Elmer, Victoria). The sample storage and preparation protocols are described in the results and discussion section. Samples were assays in a single batch as far as possible and within assay coefficient of variation for all assays were <10%.

Results and Discussion

Sample storage and preparation

Initially storage condition and preparation protocols were compared to determine their effect on the LH concentration in the samples. The different storage conditions examined included room temperature, refrigeration (4°C), freezer (-20 °C) and freeze/thaw cycles, over a time period of two weeks. All samples were equilibrated to room temperature prior to undergoing the preparation protocols. The three sample preparation methods compared were:

- shake sample to mix and disperse any precipitate
- mix sample, aliquot then centrifuge to remove any particulates (5 min, 1000×g) and
- warm sample to redissolve any precipitate.

The samples were then analysed using an Immulite analyser.

The starting measured mean concentration of LH in urine for the sample used in this study was 13.7 IU/L. The urine LH concentration varied over time with the different sample preparation protocols and storage conditions, Figure 2. The samples stored at fridge (4 C) or frozen and redissolved, frozen and shaken, frozen and centrifuged, freeze/thaw cycle and shaken, fridge shaken or centrifuged were within acceptable variation from the starting mean with a final mean of 13.2 IU/L. Some general trends were observed for the change in LH concentration under the different study conditions. The fridge and freezer storage conditions with the redissolved sample preparation protocol showed a trend over two week period to an increasing concentration whereas samples kept in the fridge over two weeks had a decreasing level of LH whether shaken or centrifuged during preparation. The LH concentrations measured in samples that were stored in the freezer prior to either shaking or centrifuging or going through freeze/thaw cycles over the two week period remained similar to the starting mean concentration. One anomalous observation with this sample was an apparently increasing LH concentration when kept at room temperature.

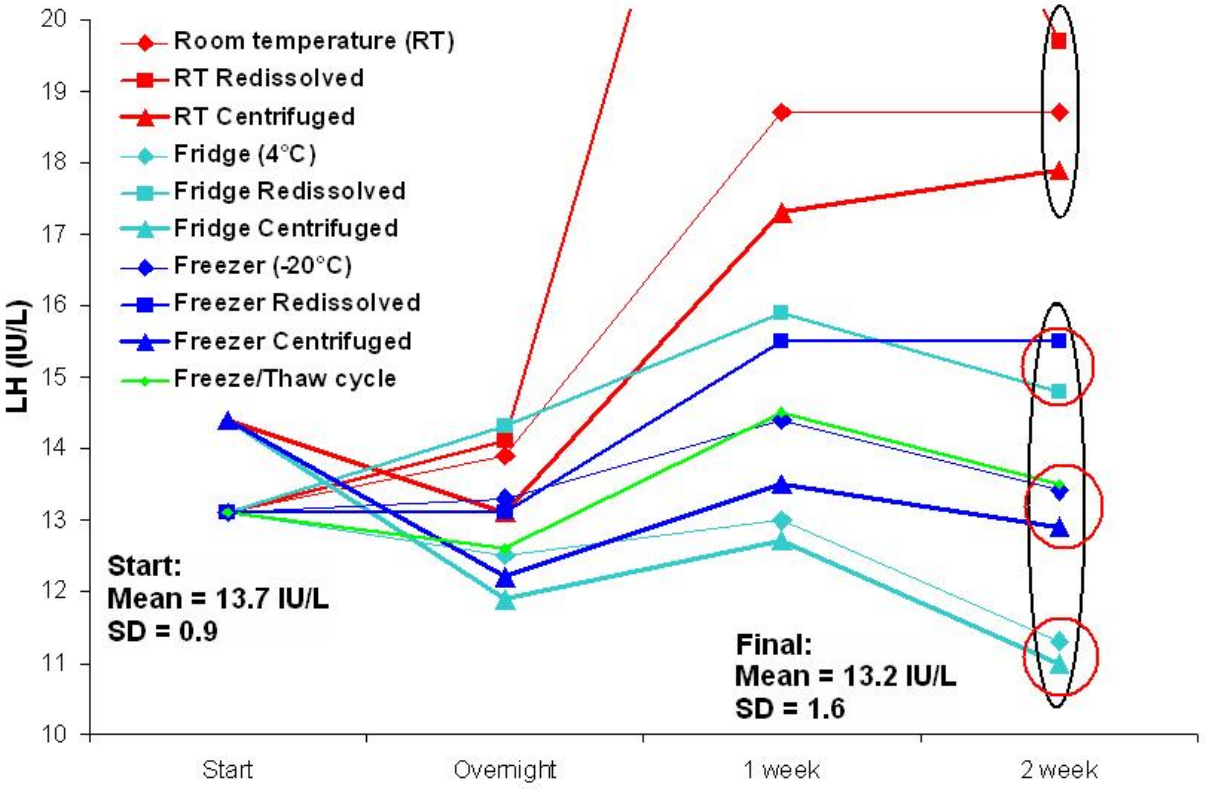

Figure 2 Plot of LH concentrations for different storage conditions and preparation protocols.

The previous experiment was repeated to further examine the apparently increasing LH level when the sample was kept at room temperature. Using a different urine sample, higher temperature storage conditions were included (room temperature, 37°C water bath, 50°C oven and 4°C fridge) and only the shaking protocol was used for sample preparation. The LH concentration did not increase to the same degree as observed previously. Whereas in the previous experiment increases of ~7 IU/L were seen at room temperature, in this experiment the LH concentration in the fridge, room temperature and 37°C samples in the second experiment only changed by ~2 IU/L. Even at 50°C the increase was only 3 IU/L after 2 weeks. We conclude that the previously observed increase of ~7 IU/L may have been due an unexplained anomaly of the individual urine sample used previously.

The sample storage and preparation protocol used for the following experiments was chosen from previous experimental results. The urine was stored in a freezer and equilibrated to room temperature, shaken to mix and disperse any precipitate before sampling for analysis.

Circadian rhythm

Serial samples were collected from six normal males over a 48 hour period to examine for any circadian rhythm, Figure 3. The mean LH concentration for the group of samples was 12.3 IU/L. Higher LH levels were generally observed in the early morning specimens. Some individuals had little variation in LH levels during the day while in others it ranged as high as 27 IU/L during a 24 hour period. The observed differences between individuals did not appear to be age related.

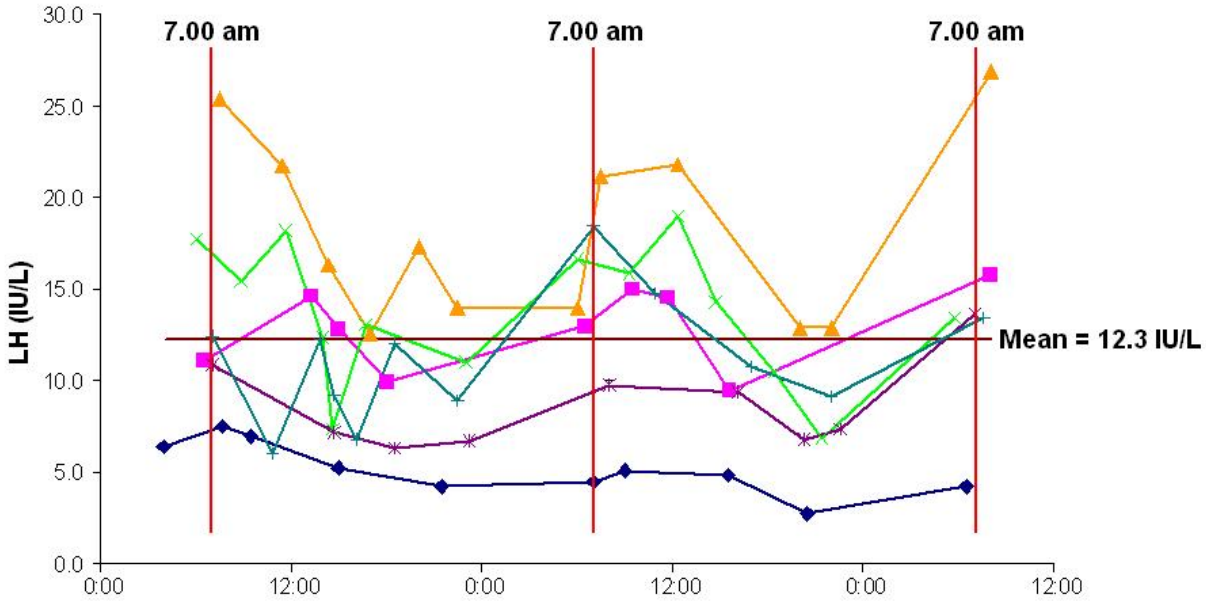

Figure 3 Plot of LH concentrations in 6 normal males over a 48 hour period.

Population distribution

A selection of 165 urine samples from male athlete were analysed to obtain a population distribution for LH concentration. These samples had tested negative in either in or out of competition screening and were analysed using the Immulite LH immunoassay to measure urine LH concentration. The LH concentrations were corrected for specific gravity and the distribution plotted, Figure 4.

The mean corrected urine LH concentration for male athletes was 13.3 IU/L. The distribution for the 6 normal male laboratory control samples compares well with the athlete distribution as does the mean of 12.3 IU/L. It is important to note that the mean LH concentration in urine obtained using the Immulite assay is 3-4 times higher than what has been published by other laboratories (Cowan *et al.*, 1991; Robinson *et al.*, 2007). It was noted that a reading of over 30

IU/L was not unusual in samples from athletes when using the Siemens (DPC) Immulite assay.

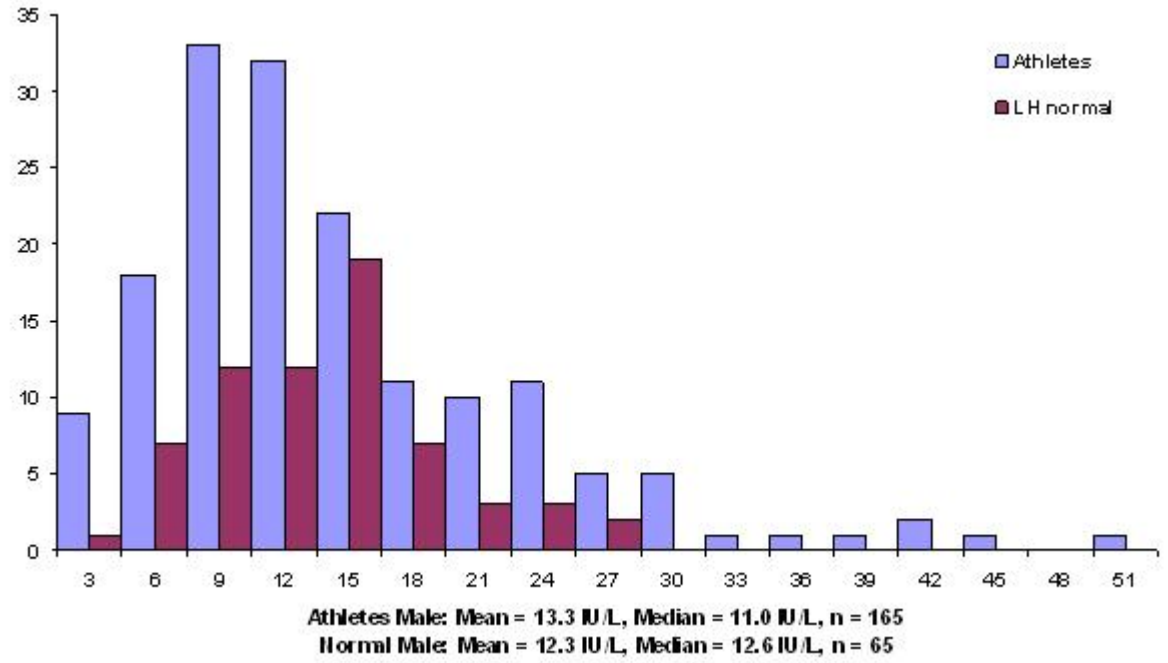

Figure 4 Distribution of corrected urinary LH in male athletes and 6 normal male subjects.

Comparison of immunoassay performance for endogenous pituitary LH and recombinant LH

A comparison between the Immulite and Delfia hLH assays for the detection of both endogenous and recombinant LH was performed. The study involved spiking LH suppressed urine with endogenous and recombinant LH standards. The urinary LH was suppressed by nandrolone decanoate pre-treatment 3 days prior to sample collection. Urine spiked with pituitary LH over a concentration range of 5-250 IU/L gave very similar results for both the Immulite and the Delfia assays, Figure 5. Although urine spiked with recombinant LH gave similar concentrations using the Immulite and the Delfia assays, the measured LH concentration in both assays was about 4 times lower than expected when recombinant LH was spiked into the suppressed LH urine. The Delfia kit calibration standards were analysed using the Immulite assay and were in good agreement. These results clearly indicate that neither the Immulite nor Delfia assay are as responsive to recombinant LH products when compared to endogenous pituitary LH standards for which the commercial immunoassay are calibrated.

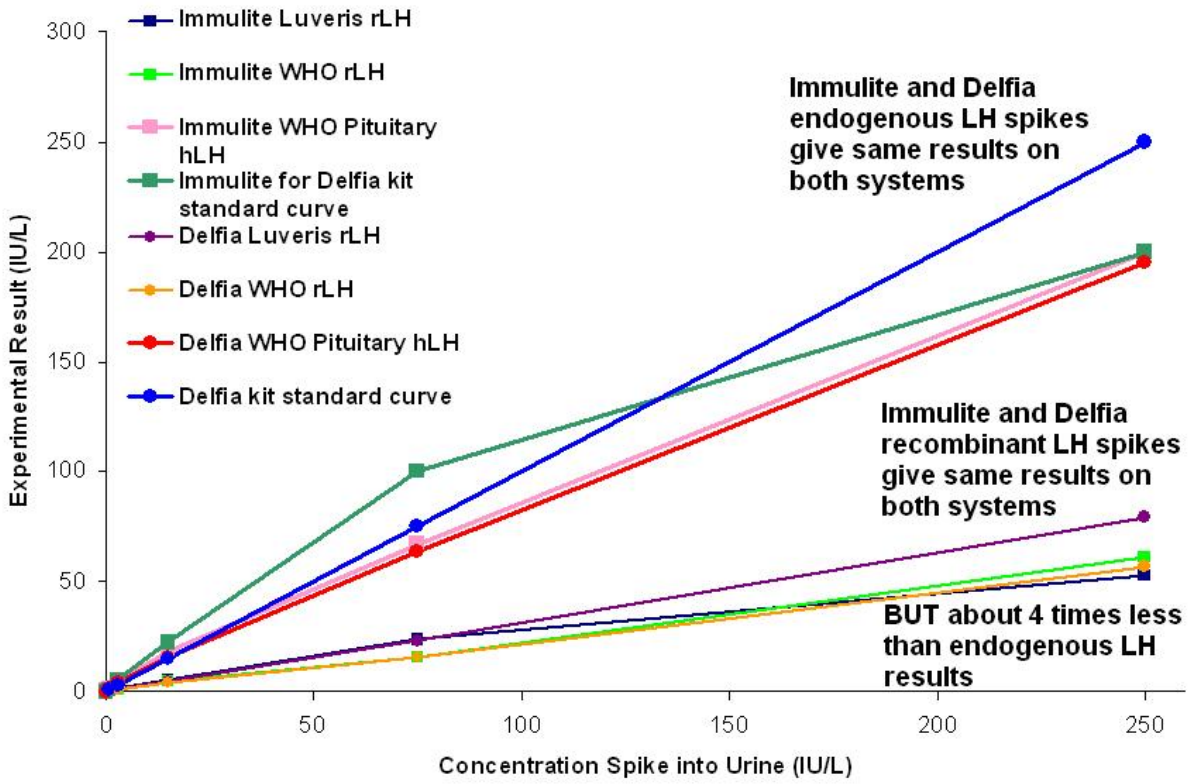

Figure 5 Plot of experimental result versus spiked concentrations for the Immulite and the Delfia assay.

Excretion study

An excretion study which included healthy young men aged 18-45 years was performed to evaluate the detection of recombinant LH in urine. The endogenous LH and testosterone production was either intact or suppressed by administration of nandrolone decanoate. The nandrolone decanoate pre-treatment was 3 days prior to recombinant LH injection. A single subcutaneous dose of recombinant LH at 75 IU or 225 IU was administered. These doses are at or above the recommended range of application for the product.

An example of the excretion study results for a single individual given a 75 IU injection of recombinant LH is shown in Figure 6. The injection of recombinant LH was given immediately after the initial urine collection. A drop in the measured LH concentration was observed for the first 24 hours. Interestingly the Immulite and the Delfia readings do not give the same results as would have been expected given the similar results obtained in the earlier experiment comparing spiked samples. It is assumed this difference is due to the fact that we are measuring the response for excreted (and possibly modified) LH rather than the

unmodified pre-injection standard solutions spiked into urine.

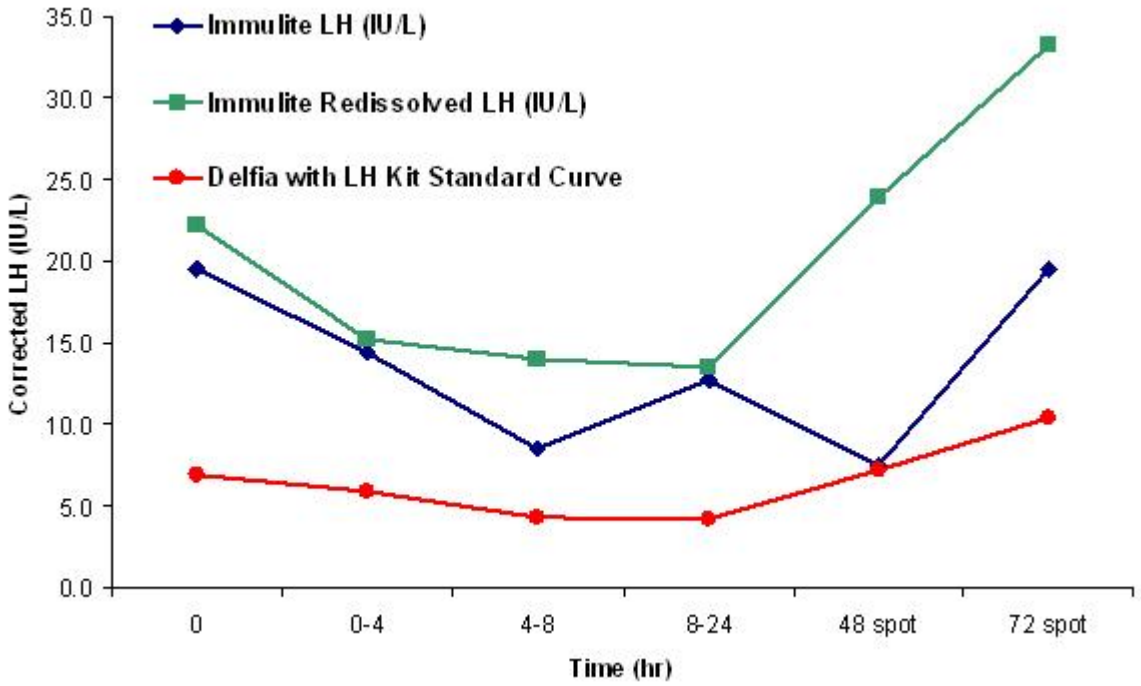

Figure 6 Excretion profile for a male subject given a 75 IU recombinant LH injection over a 72 hour period.

The results used in Figure 6 were calculated using the calibration curves obtained in accordance with the manufacturer's instructions. It is possible to re-examine the same set of data using a recombinant LH calibration curve. Because LH has a relatively short duration of action with a circulating half-life of less than 1 hour, the 0-4 and 4-8 hour collections are the samples most likely to have recombinant LH present. By recalculating the level of LH in these two samples using the recombinant curve we can observe what might actually be happening to the LH level in urine (Figure 7). That is an initial elevation and then a drop back to normal levels.

The concentration of LH in subject samples after either a 75 IU or 225 IU injection were similar (Figure 8) so permission to include a further group of subjects to be given two doses of 375 IU (Total: 750 IU) at time 0 and 4 hours was sought and obtained. No elevation in LH levels was observed in the urine in the expected period of excretion following this high dose.

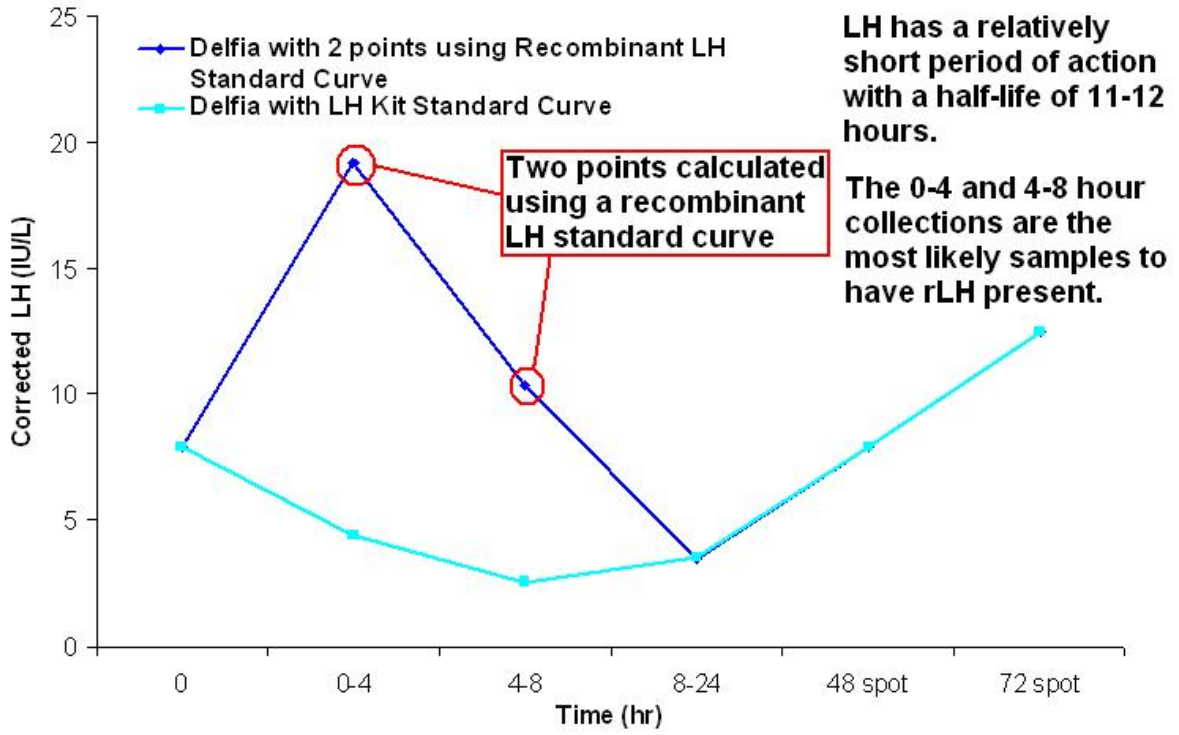

Figure 7 Excretion profile for a male subject given a 75 IU recombinant LH injection over a 72 hour period with two points recalculated using a recombinant LH standard curve.

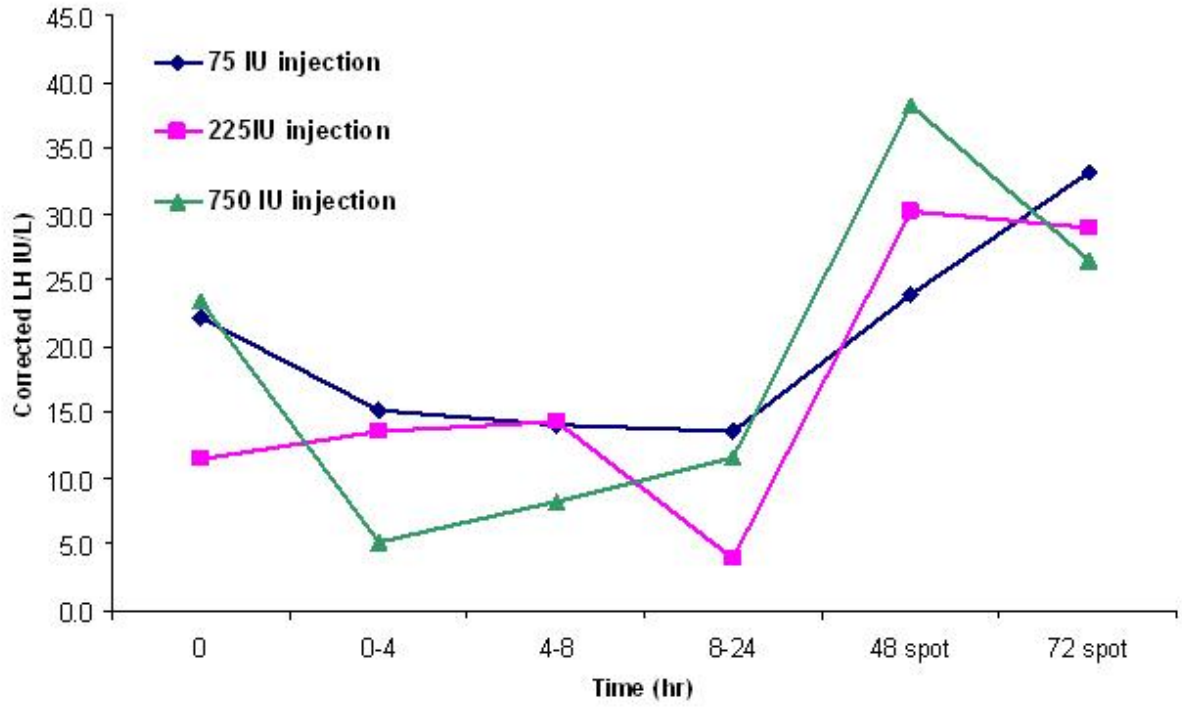

Figure 8 Excretion profile for three male subjects given 75, 225 and 750 IU (2 doses of 325 IU) injections of recombinant LH injection over a 72 hour period

Conclusions

Immunoassay measurement of urine LH concentration using assays developed and optimised for serum measurement is method dependent. Therefore results from each laboratory may not be directly comparable. Laboratories should validate the assays to be used within their own facility and establish working reference ranges. The urine LH concentration measured by commercial immunoassays is dependent on sample pre-treatment therefore a consistent protocol is also essential. Although urine LH remains a useful adjunct test to detect testosterone or hCG doping, we were unable to detect doping with recombinant LH by measuring LH levels in urine. Full discussion and results of the complete LH and hCG study are being published (Handelsman *et al*., submitted).

Acknowledgements

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