Clarke E, Goebel C

Identification and Detection of LH in Urine

National Measurement Institute, Australian Sports Drug Testing Laboratory, North Ryde, Sydney, Australia

Abstract

Luteinizing hormone (LH) is a naturally occurring hormone which is secreted by the pituitary gland. The measurement LH is a means of detecting changes in the hypothalamic-pituitary-gonadal axis which may have been induced by doping. Suppression of LH has already been proven as an indicator of doping for anabolic steroids such as testosterone [1-3] while elevated levels can be from gonadotrophin releasing hormone or its analogs [4]. The monitoring of LH can potentially reveal the use of as yet untested for compounds and identify the urine for long term storage and further analysis in the future when new methodologies become available.
Currently WADA laboratories measure LH in urine using a variety of immunoassay techniques which have been primarily designed for the measurement of LH in serum. Previous work which reviewed ten of the currently commercially available immunoassay techniques, many of which are used by WADA laboratories, had shown that two of these detected LH in urine reliably, the Immulite assay (a solid phase, two-site chemiluminescent immunometric assay) and the Delfia assay (a solid phase, two-site flouroimmunometric assay) [5]. A third assay which did not detect LH reliably after long term storage, the DRG Elisa (a solid phase enzyme-linked immunosorbent assay) was used for comparison. The primary objective of this project was to cross validate the Immulite assay to the Delfia assay to determine a correlation factor and to explore different purification and digestion techniques to sequence urinary endogenous LH.

Introduction

The detection of LH in urine is somewhat of a hit and miss endeavour unless it is well understood that LH is actually unstable in urine when it is kept at room temperature or frozen at -20°C. The Immulite assay reliably detects LH even in its degraded form because of the antibody used for that analysis. The Delfia assay has shown similar characteristics to the Immulite kits but a more comprehensive study needs to be completed to prove its reliability and to correlate the results from the two assays. The use of immunoaffinity assays that can yield reproducible and comparable results is critical and it needs to be taken into consideration that urine samples are often transported long distances and not necessarily under ideal conditions [6]. It is for these reasons that the assay used by WADA laboratories need to detect the degrading product in urine as well as the excreted LH directly.
In this project we aimed to conduct stability studies on both assays, validate the Delfia for urinary LH, use the assay to analyse a selection of athlete and excretion study samples, then to statistically determine the correlation factors for the two assays. Finally to purify and concentrate urinary LH in order to gain a greater understanding of the degrading product in urine.

Experimental

LH kits and assays

Testing for LH was completed on the analysers and plates listed below as per manufacturer's instructions.

- Immulite 1000 (Siemens)
- Delfia ELISA LH (Perkin Elmer)
- DRG ELISA LH (Serum, EIA-1289, DRG International)

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LH purification
Purification of urinary endogenous LH was performed using the following technique.

Concentration:
Aliquot 20 mL of urine into a 50 mL falcon tube, add 400 µL complete protease inhibitor and 2 mL of 3.75 M Tris-HCl at pH 7.4. Filter the urine through an Acrodisc Premium syringe filter, 0.45 µm, using a disposable 20 mL syringe. Transfer the filtered urine to a Centricon Plus 20 MWCO 10000. Centrifuge at 4000 rpm fpr 20 min at 20°C. Buffer wash the retentate with 20 mL of 50 mM Tris-HCl, pH 7.4 and 400 µL of complete protease inhibitor. Centrifuge at 4000 rpm fpr 20 min at 20°C. Collect the retentate.

Purification:
Take 30 µL retentate and add 180 µL glycine buffer at pH 8. Transfer into an Immulite LH sample cup and cover the opening with a breathe-easy tube membrane. Incubate overnight at 4°C with gentle rocking. Wash sample cup with 200 µL PBS five times, firmly tapping cup onto tissue between washes. Add 200 µL glycine buffer at pH 2 to the sample cup and incubate for 5 min with gentle shaking. Transfer the sample to a vivaspin 500, 5kDa MWCO filter and centrifuge. Collect the retentate and add 1 M tris HCl to adjust the sample to pH 7.4-8.

Digestion-1:
Dry the retentate using a MiVac rotary evaporator. Add 16 µL of 50 mM ammonium bicarbonate and 2 µL 100 mM DTT and incubate at 60°C for 60 min. Cool and add 2 µL 100 mM iodoacetamide nad incubate in the dark for 30 min. Add 3 µL trypsin at 500 µg/mL and digest overnight at 37°C. Add 2 µL 1% formic acid to stop digestion.

SDS-PAGE:
Load sample onto a Criterion pre-cast gel and run SDS-PAGE using standard conditions of MOPS buffer, setting the voltage to 160 V, current limit at 200 mA/gel and a run time of approximately 90 min. Silver stain the gel to identify the LH bands before digestion

Digestion-2:
Using an OMX-S Protein picker, pick the LH band of the gel, invert and digest using trypsin. For samples that were not run on SDS-PAGE, trypsin spin columns (Sigma) and Hypersep Spin Tips (Thermo) were used for tryptic digestion.

Mass Spectrometry:
Digested samples were run on an Eksigent nanoLC coupled to an LTQ Orbitrap XL (ThermoScientific) using reverse phase conditions of A: 0.1% formic acid on water (BioSolve) and B: 0.1% formic acid on acetonitrile (BioSolve) on a 3u 200A Magic C18AQ 0.2 x 50 column (Michrom Bioresources Inc.)

Results and Discussion

The stability study was conducted simultaneously on the Immulite and Delfia assays as well as on DRG LH Elisa plates. The study included samples from normal subjects with different levels of endogenous LH, three LH suppressed urines spiked with different levels of pituitary LH (WHO 80/552, NIBSC) and three urines spiked at different levels with recombinant LH (WHO 96/602, NIBSC). Urines from recombinant LH and gonadotrophin releasing hormone analogs excretion studies also formed part of the stability study. Urines were kept at different conditions (40°C water bath, room temperature, fridge and freezer) over a period of 36 days. All three assays were run simultaneously to ensure that any changes observed for the Delfia were confirmed by the Immulite assay.
There were a few trends observed from the stability study. As expected the samples kept at 40°C (displayed in red) were inconsistent on the Immulite and Delfia assays (figure 1) from the second measurement at day 4. Interestingly the samples kept at room temperature (displayed in yellow) showed a steady decline from recombinant and pituitary LH on both the Immulite and Delfia assays, with a greater variance from the initial LH amount observed than was seen with the 40°C samples. The data obtained in this stability study reinforced the previous finding that urinary LHis indeed unstable at room temperature. However, in the recombinant and pituitary LH spiked urines, the frozen samples (displayed in dark blue) proved to be stable along with the samples kept in the fridge (displayed in light blue).

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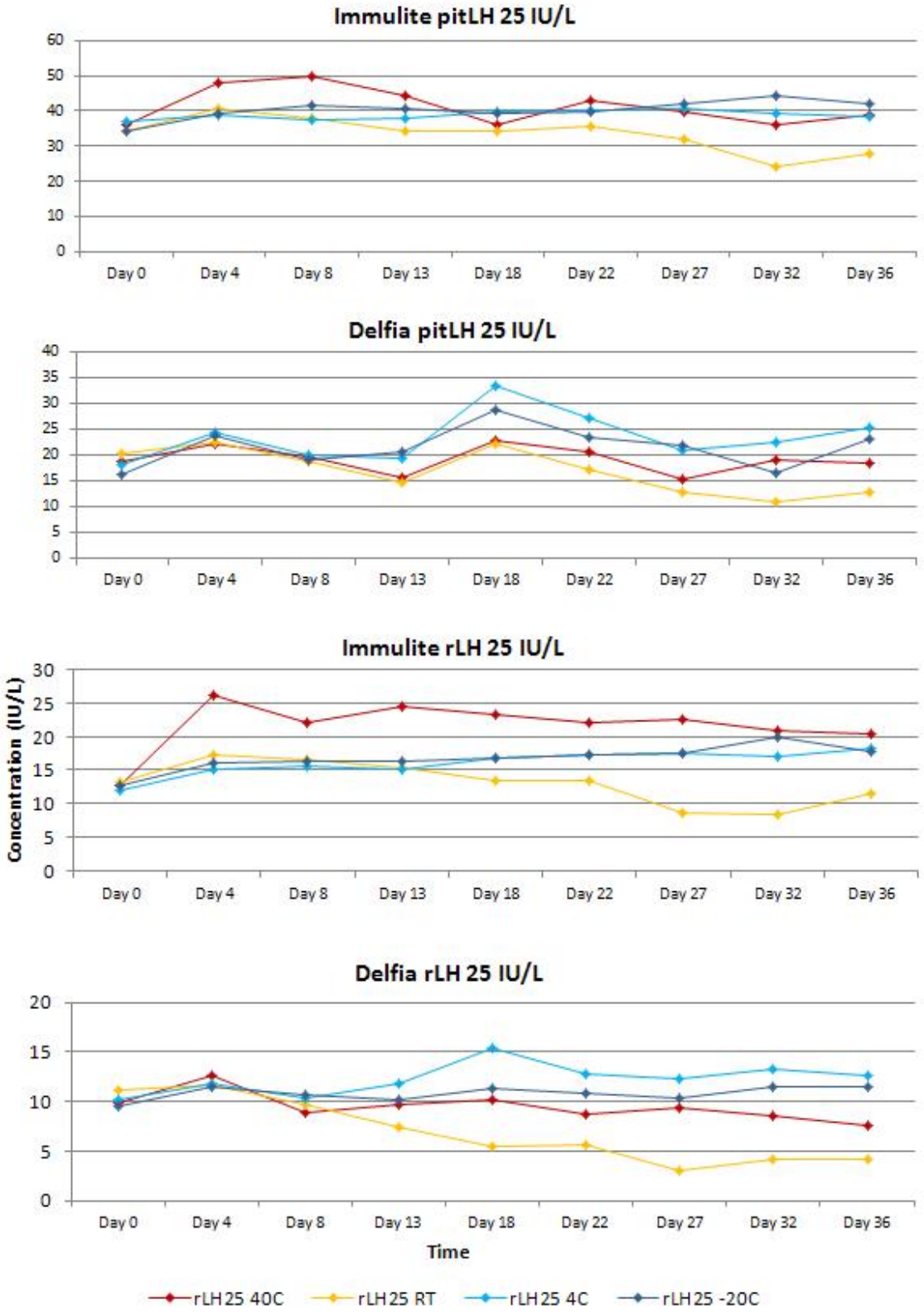

Figure 1. Graphs depicting urine spiked with pituitary LH at 25 IU/L and urine spiked with recombinant LH at 25 IU/L, stored at 40°C (red), 21°C (RT) (yellow), 4°C (light blue) and -20°C (dark blue) over a period of 36 days and measured by the Immulite 1000 and Delfia assay.

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The DRG assay, which was run concurrently with the Delfia and Immulite assays, showed an immediate loss in LH sensitivity and stability after day 4 at both 40°C and room temperature, and a smaller yet still measurable decrease in LH concentration after day 4 at 4°C and -20°C (Figure 2).

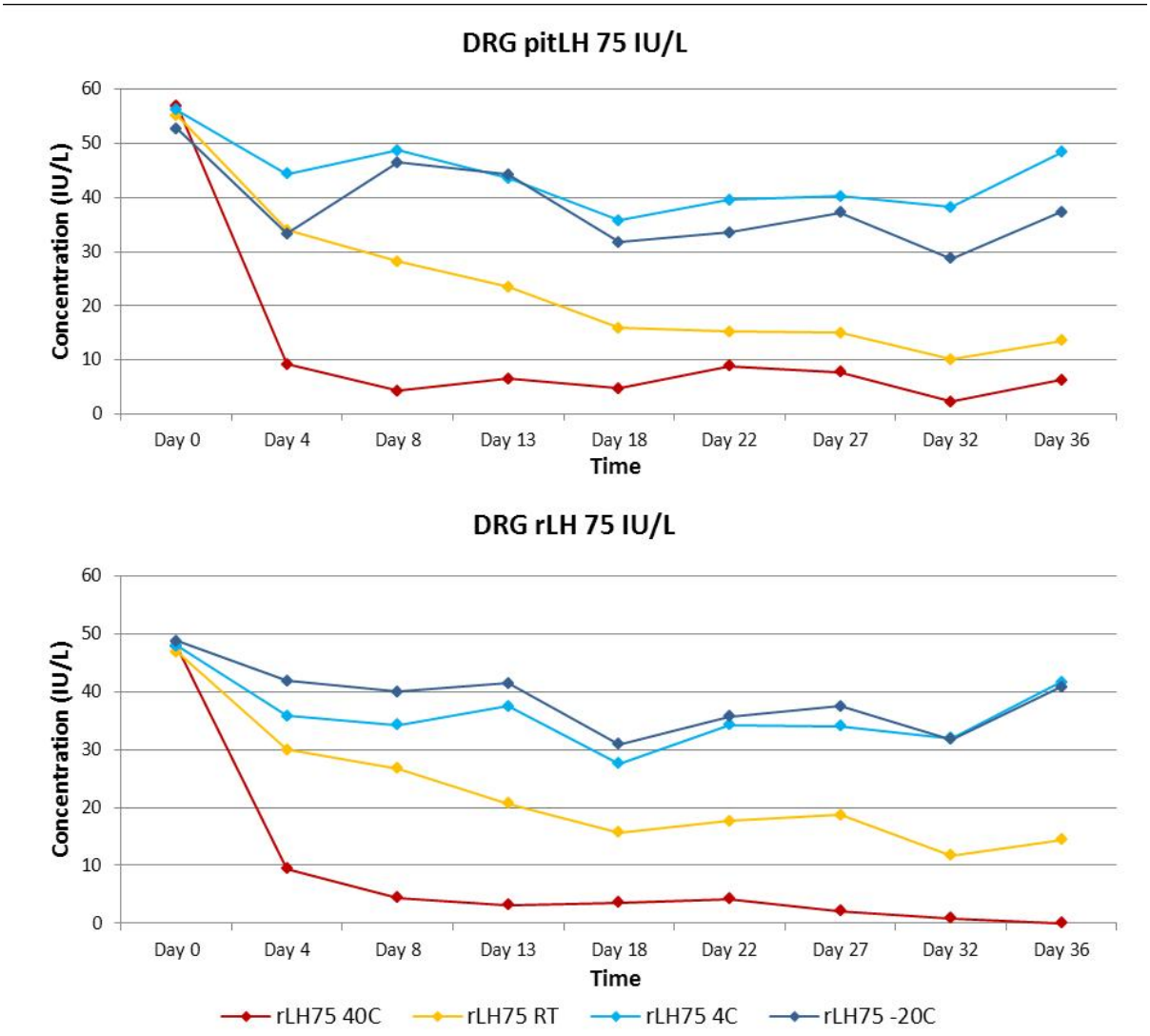

Figure 2. Graph depicting urine spiked with pituitary LH at 75 IU/L and recombinant LH at 75 IU/L, stored at 40°C, 21°C (RT), 4°C and -20°C over a period of 36 days and measured by the DRG Elisa assay.

The potential of multiple freeze thaw cycles was also explored in the stability study. Urines spiked at 75 IU/L with pituitary LH were stored at -20°C and -80°C. All samples were thawed and brought to room temperature on each day of testing before being frozen again. The DRG Elisa (Figure 3) showed a consistent decline in LH concentration (displayed in green). The Delfia assay revealed negligible loss or increase of LH (displayed in red) whilst the Immulite increased LH concentration for the urine kept at -20°C (displayed in dark blue) but stayed more constant for the sample stored at -80°C (diplayed in light blue).

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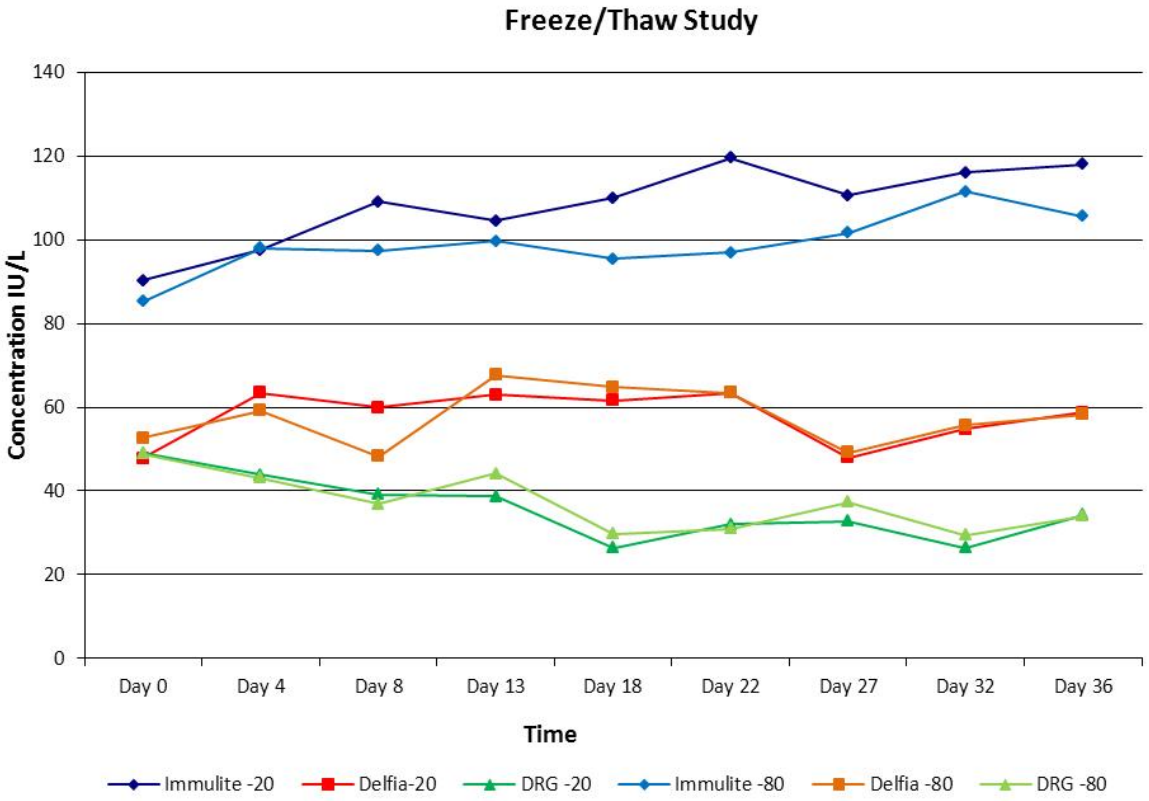

Figure 3. Graph comparing samples stored at -20°C and -80°C over a period of 36 days and allowed to thaw completely on testing days, measured by both the Immulite and Delfia assays.

For the validation of the Delfia assay the parameters of specificity, precision, robustness, ruggedness, limit of quantitation (LOQ) and linearity were performed. A summary of the results can be found in Table 1.

WHO Std	Precision (%)		LOQ	Linearity			Stability at 18°C			
	2 IU/L	15 IU/L	IU/L	Slope	Intercept	R^2	40°C	21°C	4°C	-20°C
	(n=6)	(n=6)					Days	Days	Days	Days
pitLH	16	9	2	0.99166	0.03686	0.99101	<1	<1	36	<8
rLH	14		2	0.84546	0.12886	0.98594	<1	<1	36	<8

Table 1. Delfia assay validation results.

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Two groups of 500 samples were analysed by both assays. The first was used to determine a correlation factor for the Immulite and Delfia assays and the second set was used to validate the determined correlation factors. The correlation factors for the first 500 samples were determined by plotting the Immulite and Delfia assay results against each other with the y-intercept giving each correlation factor. The correlation factor for the Delfia assay was determined at 0.5771 and for the Immulite at 1.5644. A second set of 500 athletes samples were run on both assays to validate the previously determined correlation factors. The correlation factors for the second 500 samples were determined in the same way. The correlation factor for the second set run on the Delfia assay was determined at 0.6193 and for the Immulite at 1.5035.
The correlation factors were then further validated by testing them against previously conducted excretion studies of recombinant LH and gonadotrophin releasing hormone analog. When the samples were adjusted by each correlation factor the concentrations reflected the opposite assay results (Figure 4).

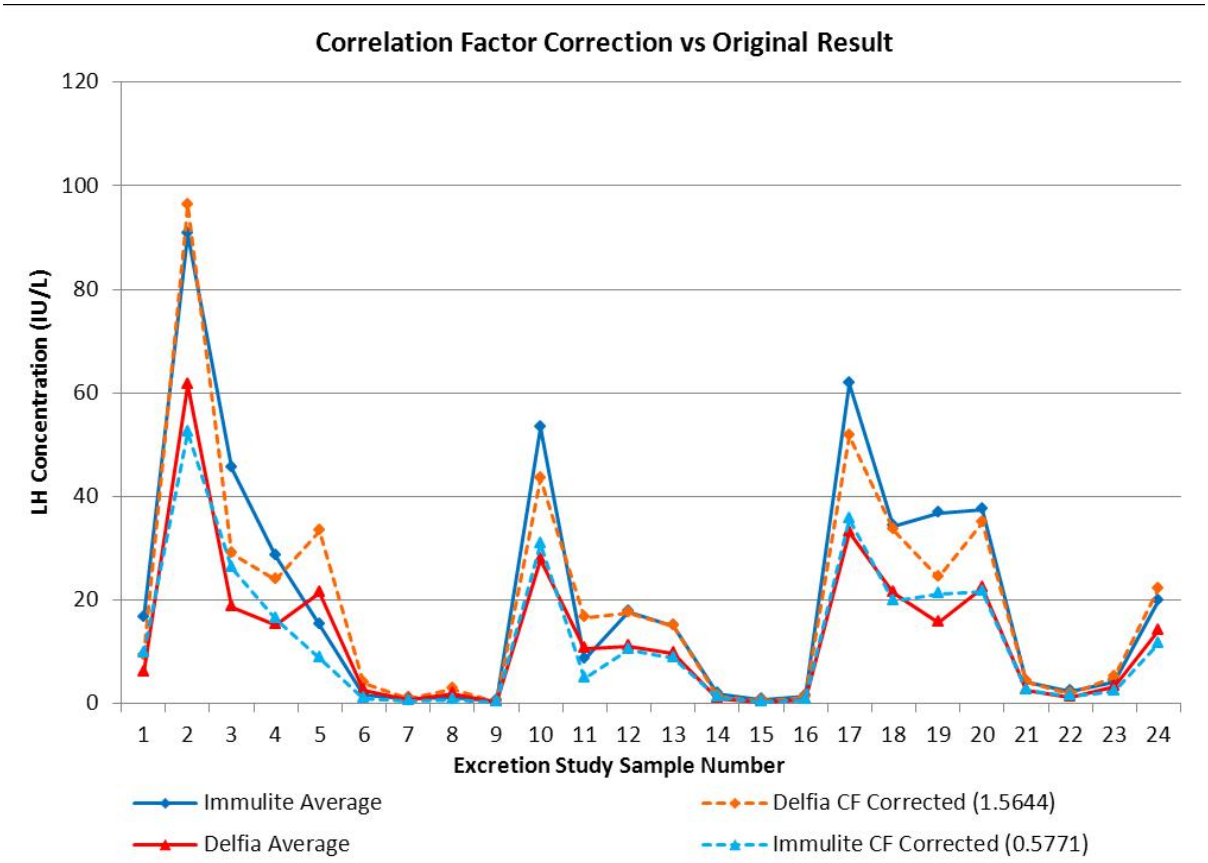

Figure 4. Graph of the correlation factor adjusted concentration against the original measured concentration. The immulite original result (dark blue), the Delfia orginal result (red), Delfia corrected result with Immulite correlation factor 1.5644 (orange dotted line) and the Immulite corrected result with Delfia correlation factor 0.5771 (light blue dotted line).

The purification and sequencing of urinary endogenous LH and excreted recombinant LH was then undertaken. The most effective method for the sequencing of LH in urine is outlined in the methods section under concentration, purification and digestion-1. Sequencing of LH standards and LH extracted from urine had varied results with approximately half coverage of the beta subunit achieved for urinary LH, pituitary LH and recombinant LH (Figure 5).

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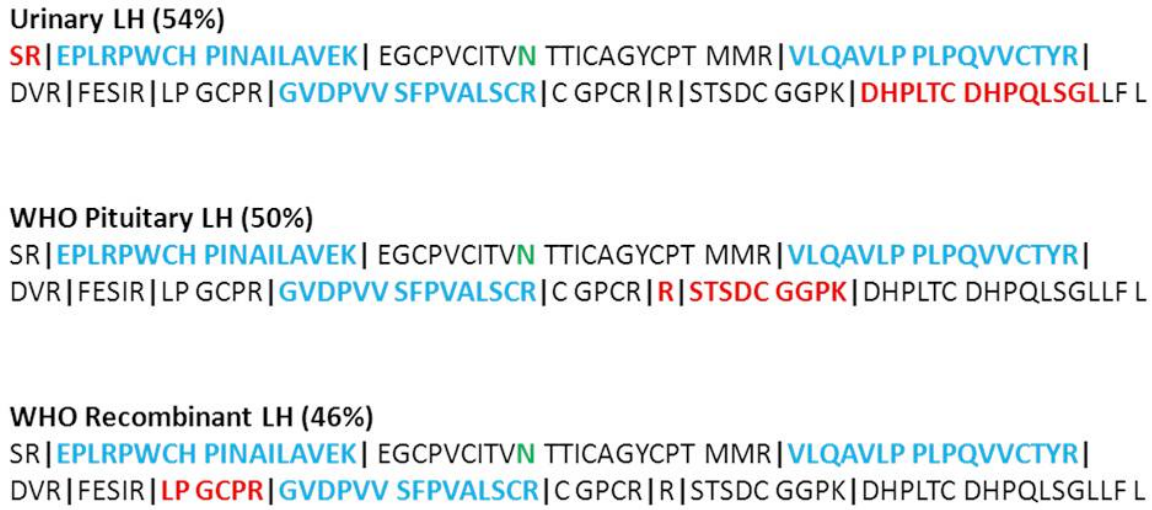

Figure 5. Sequencing achieved on the beta subunit of Urinary LH, WHO pitLH and WHO rLH, with common identified peptides shown in blue and identified yet individual peptides shown in red. The expected cleavage sites for trypsin digestion are shown with vertical black lines and the percentage of protein coverage achieved is shown at the start of each sequence.

Sequencing of the beta subunit of LH revealed the C-terminal and N-terminal of the protein are still largely intact meaning that changes to the protein chain must be occurring within the protein backbone. The tertiary structure of LH may be allowing certain areas to more readily degrade due to turns and disulphide bond placements in the structure as well as the influence of post translational modifications such as the single N-linked glycan on the beta subunit.

Conclusions

The Immulite and Delfia assays are able to detect LH in urine after extended periods of storage at 40°C, 21°C, 4°C and -20°C. The results for the Delfia stability study reflect what was observed for the Immulite stability study, that LH is unstable at room temperature and although it is unstable when frozen, quantifiable amounts are still able to be detected with the Delfia and Immulite assays. Statistical analysis of 1000 athlete samples gave correlation factors for both the Immulite and the Delfia, enabling a more accurate comparison of results between the two assays. This will be particularly useful to the anti-doping community as different laboratories use only one or the other of the assays for detecting LH in urine.

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