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Uninary reference intervals of gonadotrophic hormones in Brazilian athletes using the IMMULITE immunoassay system

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Abstract

Human Chorionic Gonadotrophin (hCG) and Luteinizing Hormone (LH) can be used to obtain anabolic effects. Screening approaches are based on immunoassay methods and the reference intervals are related to the population considered and the assay's specificity. Quantitative validation in urine became necessary, since such methods are originally developed for serum. The objectives were: (i) to validate the IMMULITE 1000 immunoassay for urine, (ii) to evaluate the stability of hCG and LH in different storage conditions, (iii) to determine the reference ranges of urinary excretion for LH and hCG profile from Brazilian athletes. The validation process evaluated specificity, linearity, intra-assay precision, inter-assay precision, LOD, LOQ and stability. The validation results were similar to those obtained by the manufacturer in the serum and confirmed that the urine method quantification meets the criteria established by WADA. The hCG showed stability over 9 cycles of freezing (-20 °C) and thawing. And LH was stable at 4 °C and -20 °C for at least 40 days and for 10 days at room temperature. The LH was unstable only after the second cycle of freezing and thawing. The urinary reference range for hCG (n= 1402) and LH (n = 597) showed a non-normal distribution. For hCG, the reference values was less than 5.0 mIU/mL. Regarding the LH, the upper reference limit was 27.3 mIU/mL. The IMMULITE 1000 system can be used to decide which athletes should undergo follow up investigations for LH and hCG.

Introduction

The hCG and LH are used by athletes aiming to stimulate the secretion of testosterone, maintaining the testosterone/epitestosterone ratio unchanged and to reverse testicular atrophy due to anabolic steroids misuse [1,2]. As a consequence, hCG and LH are listed by WADA as prohibited substances and screening analyses of them are immunoassays based. Since they are endogenous substances, a strategy to allow the discrimination between the hormones naturally produced and the exogenously administered, is necessary to configure an adverse analytical finding. The aim of this project was to evaluate the urinary excretion profile of Brazilian male athletes using the IMMULITE 1000 assay. Since the assay was originally developed for serum analysis, the validation of the assay's performance in urine becomes necessary.

Experimental

IMMULITE 1000 from Siemens with specificity for total hCG [3]; LH-LH β-subunit. The urine samples were analyzed after previous centrifugation (3000 g/5 min).
hCG experimental:
The linearity was evaluated in the range from 2.5 mIU/mL to 40.0 mIU/mL. Intra-assay precision was evaluated by using seven spiked urines and expressed as CV%. Inter-assay precision was investigated using a diluted pregnant urine analyzed 162 times (different days and different operators). Specificity was evaluated comparing similar molecules with the hCG.

Carry over was evaluated alternating analyses of negative and high concentration hCG samples. LOD was estimated by the mean value for chemiluminescence obtained from 145 urines with concentration estimated <1 mIU/mL, added to three times the standard deviation of this average. Stability was evaluated after freezing and thawing cycles (n = 9) (triplicate) considering only one sample pregnant urine and this parameter may vary from one sample to another.

LH experimental:
Intra-assay and inter-assay (3 days) precision were evaluated analyzing seven replicates of three urines. Both CV% expressed. Specificity was evaluated analyzing hCG solution at: 50, 100, 500, 1000, 2500 and 5000 mIU/mL and 3 urines with known LH concentrations (LHuQC, 114 mIU/mL predetermined by IMMULITE 1000 system) spiked with hCG at 5000 mIU/mL. Linearity was established analyzing diluted LHuQC. The range was 0.1 - 50 mIU/mL. Matrix interference was evaluated by the analysis of ten urine samples, whose LH concentrations were measured, then were pooled together two by two (same proportion) and measured again. Carry over was evaluated alternating low LH and high LH concentration samples. Stability was evaluated in 3 different urines. All urines were evaluated in the storage conditions: (-20 °C, 4 °C and RT) during 10, 30 and 40 days. Freezing/thawing cycles were also tested. This experiment used a limited number of samples of urine, and therefore, it must be considered a possible variability of the results according to the content of each sample.

Results and Discussion

hCG results:
The hCG linearity showed homoscedasticity (Cochran test). Intra-assay precision was 3.5% and inter-assay precision was 19.8% (Both are equivalent to the supplier data for serum analysis). LOD was 1.2 mIU/mL, the concentration was estimated after comparing the chemiluminescence value obtained for a calibration urine of 1.1 mIU/mL. Both are equivalent to the supplier for serum analysis.
The LOQ was 2,5 mIU, estimated by the level of concentration in an analytical curve with the lowest intra-assay precision less than 10% (the first point of the curve). The hCG stability of one pregnant women sample is presented in Figure 1 and the variability between individuals must be considered.
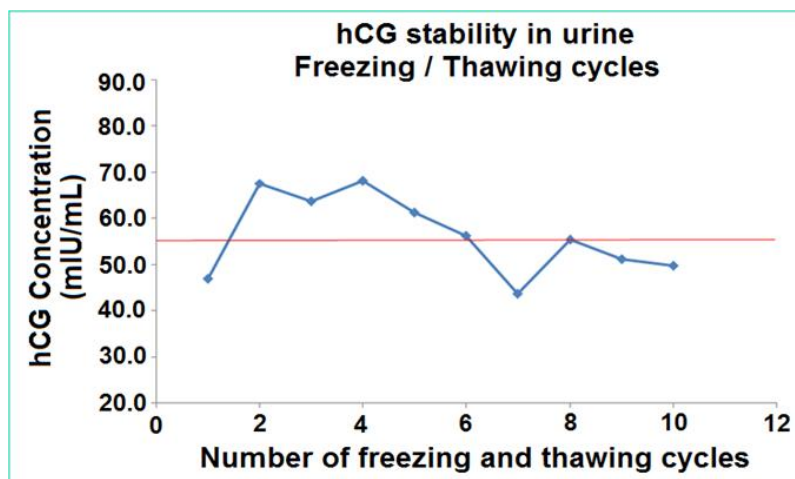

Figure 1: Concentration of hCG in one urine sample after 9 cycles of freezing/thawing. The red line indicates the mean value (56 mIU/mL).
Note: The parameter stability may be different from one sample to another one.

LH results:
The LH linearity showed homoscedasticity. Intra-assay and Inter-assay precision were 2.7% and 5.2%, respectively (lower than the supplier for serum analysis). Cross-reactivity was less than 0.2% and no carry over was observed. LOQ was

established at 0.1 mIU/mL (intra-assay precision < 10%). No matrix interference was observed (Figure 2). For each of the 3 evaluated urines, LH was stable at 4 °C and -20 °C for at least 40 days and for 10 days at RT. LH was unstable after the second cycle of freezing/thawing. The stability may be very different according to the urine samples. Then, the stability is far to be guaranteed in any sample.
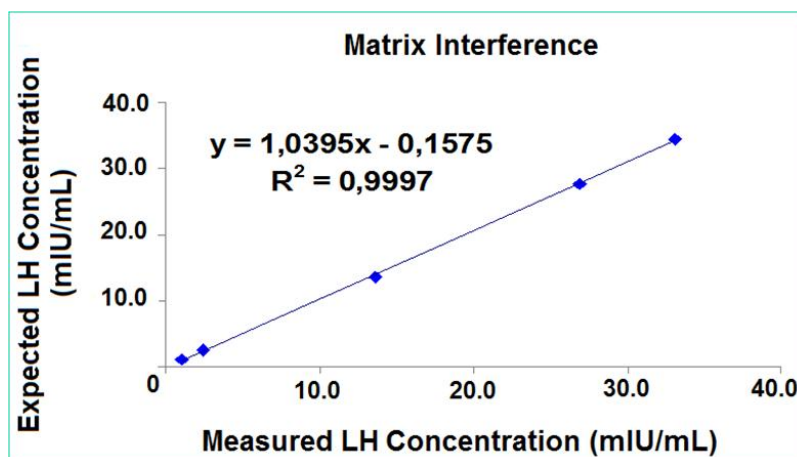

Figure 2: Comparison of urinary LH data obtained with 10 urine samples: the expected LH concentrations (mean LH value obtained for two urines) correlated with those measured after pooled (mean LH value obtained from pooled urine).

Urinary Reference Intervals:

Both populations showed a non-normal distribution according to Kolmogorov-Smirnov test. Following the approach recommended by IFCC (97.5 percentile), the upper reference limit was 3.9 mIU/mL for hCG and 27.3 mIU/mL for LH [4]. These values were confirmed by the far outside value approach: the upper reference limit was 4.3 mIU/mL for hCG and 37.4 mIU/mL for LH [5]. Distribution profile of the total urinary hCG and LH is presented in Figures 3 and 4. Based on this, results above 4.3 mIU/mL and 37.4 mIU/mL could be considered as Presumptive Analytical Findings for hCG and LH, respectively. A second assay should be used to confirm the results.
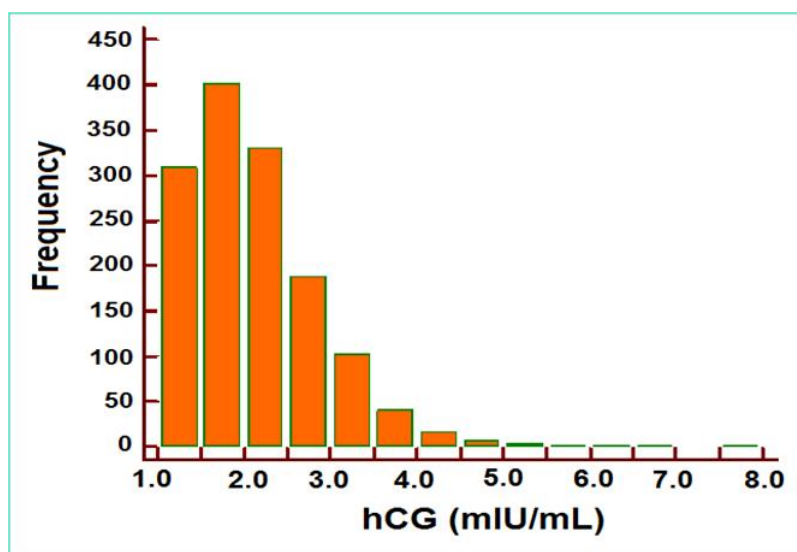

Figure 3: Frequency chart of urinary concentrations of hCG for Brazilian athletes population. The values of hCG samples have not been adjusted for the specific gravity.
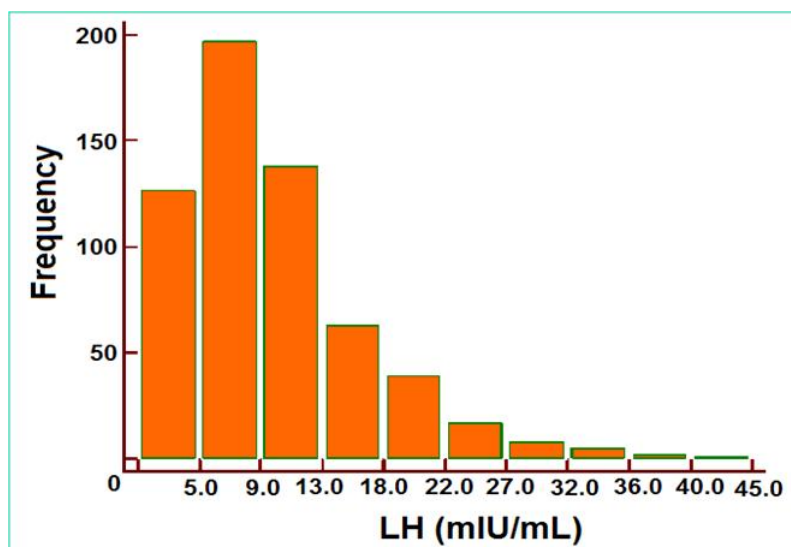
Figure 4: Frequency chart of urinary concentrations of LH for Brazilian athletes population. All values of LH samples have been adjusted for the specific gravity.

Conclusions

The IMMULITE quantitative assay for hCG and LH originally developed for serum matrix was evaluated for urine matrix aiming at the doping control analysis. The validation process in urine demonstrated that the assay is fit for purpose in that matrix. The Brazilian population was evaluated regarding the hCG and LH urinary excretion profiles. Both populations presented a non-normal distribution. Through this evaluation, the Far Out Side Value for hCG was estimated in 4.6 mIU/mL. This value is below the criteria stipulated by WADA (5.0 mIU/mL). Regarding the LH, the Far Out Side Value was established at 37.4 mIU/mL. These results indicated the assay is suitable for application in doping control analysis.

References

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