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Article

Urinary Excretion Profile of Luteinizing Hormone in Brazilian Athletes

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Luteinizing hormone (LH) can be used by athletes as an alternative way to take illicit advantage of androgenic effects, since it stimulates the secretion of testosterone by the Leydig cells. As consequence, LH use is illegal for male athletes and the hormone is included in the World Anti-Doping Agency (WADA) prohibited list. Since LH is an endogenous substance, the strategy used to allow the detection of LH abuse was the establishment of a reference value. All analyses were developed by IMMULITE®, which was submitted to a complete validation performance with urine. The hormone showed to be stable at 4 °C and −20 °C for at least 40 days and for 10 days at room temperature and unstable after the second cycle of freezing and thawing. The reference population of male Brazilian athletes showed a cut-off value of 37.4 mIU mL^{-1}. The results indicate the assay is suitable for application in doping control analysis.

Keywords: luteinizing hormone, doping control, chemometrics/statistics, validation, endogenous profile, immunoassay

Introduction

Luteinizing hormone (LH) is a glycoprotein synthesized and secreted in the anterior pituitary gland, by the gonadotropic cells, playing a crucial role in the production of estrogens and androgens in gonadal function and gametogenesis.[1]

The glycoprotein hormones are heterodimers consisting of an α-subunit and a β-subunit, non-covalently linked together.[2]

LH is primarily responsible for regulating the synthesis of gonadal steroid hormones. In males, LH stimulates the interstitial Leydig cells to produce androgens, and in females, LH acts with follicle-stimulating hormone (FSH) to stimulate follicular development. LH also acts on the mature follicle inducing ovulation and stimulates the corpus luteum during the luteal phase of the menstrual cycle, producing estrogens and progesterone.[3]

Anabolic androgenic steroids (AAS) remain the most effective ergogenic aid in sports requiring strength and power. However, the prohibition of the use of AAS in sport encourages the use of androgens with structures identical to endogenous but industrially synthesized. An alternative way used for obtaining illicit androgenic effects are indirect doping of androgens, that is, the use of hormones that increase the levels of endogenous steroids as testosterone.[4]

Therefore, LH is included on the World Anti-Doping Agency (WADA) prohibited list in section S.2 as a peptide hormone whose use is illegal only for male athletes.[5] LH can be used by male athletes aiming to increase testosterone production, in order to improve the physical performance.[6] LH stimulates the production of endogenous steroids without affecting the testosterone/epitestosterone (T/E) ratio, a known biomarker to assess alterations of endogenous steroids profile. Besides, athletes can also use LH in order to normalize the production of endogenous testosterone suppressed during and after prolonged use of synthetic androgens.[7]

After the commercial availability of recombinant human LH in 2000 and the consequent entry of LH on the WADA prohibited list in 2005,[8] there is a need for doping control laboratories to validate and establish a quantitative method for detection and to determine a typical range of urinary LH concentrations to allow the inference of a possible misuse by athletes. Since LH is an endogenous substance, the knowledge of the pattern of excretion in different populations

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is part of the strategy to generate objective criteria for interpretation of results, especially in the screening step.

In clinical analysis, the reference values of LH detection in blood serum are well established. Since LH is related to gonadal dysfunction and failure of the pituitary gland or hypothalamus, the hormone indicates whether its low levels are of a primary origin or a result of weak stimulation by pituitary hormones. LH levels are also used in the evaluation of infertility in men and women.[9] The detection of urinary LH levels may also be important for clinical applications, complementing the already established LH blood analysis.

Virtually all commercial LH assays are designed for analysis of serum blood samples, whereas doping control analyses are generally performed in urine. Consequently, the application of LH detection in urine by immunoassays requires a rigorous validation for a valid interpretation.

The aim of this study is the statistical evaluation of the excretion urinary profile of LH in Brazilian male athletes through the analysis by IMMULITE® assay. Since the assay was originally developed for serum analysis, the validation/verification of the assay's performance in urine in the light of the WADA regulations became necessary. Also, in order to evaluate the immunoreactivity of LH with time and temperature, the stability of the hormone in urine was carefully studied.

Experimental

Information about the samples

The 598 urine samples from the reference population were collected from Brazilian male athletes of several sport modalities (high-risk sports not included), coming from regular in and out competition anti-doping tests. None of the samples presented an adverse analytical finding (AAF) for any classes of substances from the normal set evaluated for doping control purposes.

There is no information available on the personal data of the athletes regarding to age, weight, ethnicity or conditions of the sample collection, such as time of day, storage or transport. These limitations are inherent in the activity of doping control, which ensures the protection of the athlete's identity for ethical reasons.

The samples from the validation analysis were collected from healthy male and female volunteers. All volunteers signed a term of consent according to the Ethical Committee rules.

Analysis

The urinary LH from all urine samples was quantified by IMMULITE®, an automated immunoassay system (Siemens Healthcare Diagnostics Products Ltd., Los Angeles, USA). The IMMULITE® system is a solid-phase, two-site chemiluminescent immunometric assay. The solid phase consists of a polystyrene bead that is coated with a monoclonal antibody directed against LH, which is sealed into a test unit. The urine sample and the polyclonal antibody conjugated to alkaline phosphatase are added, and after an incubation of 30 min at 37 ºC, LH binds to the monoclonal antibody that coats the bead and to the polyclonal antibody conjugated to the enzyme in the form of a sandwich complex. The bead is washed, removing the unbound antibody. LH is quantified by the addition of a chemiluminescent substrate (ester of adamantyl dioxetane phosphate) through the luminescence produced.

The IMMULITE® LH assay specifically binds to the β-subunit. Therefore, the assay detects the intact LH molecule as well as the free β-subunit.

The analyses were accomplished after a periodical maintenance and calibration according to specifications of the manufacturer.

The quality of the analysis was controlled by means of a blank sample and a multivalent control module by Siemens (Los Angeles, USA), composed of three LH plasma concentration levels (low, medium and high).

In order to compare the LH concentrations between different urine samples, they were normalized by means of the urinary density according to the Levine-Fahy equation.[10] The concentrations *per* sample were corrected to a standard specific gravity of 1.020.[11]

Pretreatment of the samples

Eight urine samples from different volunteers were treated concurrently in two different ways to evaluate the appropriate pretreatment before analysis by IMMULITE®. The first one involved centrifuging the samples at 3000 rpm for 5 min at room temperature, and the second involved heating the samples at 37 °C for 15 min. Analyses were done in triplicate.

Validation assays

The method was validated in urine matrix through the evaluation of the specificity, intra and inter-assay precision, linearity, limit of detection (LOD), limit of quantification (LOQ), matrix interference, recovery and carryover contemplating the WADA requirements described in the International Standard for Laboratories (ISL).[12]

Specificity

Specificity was evaluated by the analysis of 1% m/v bovine serum albumin (BSA) in phosphate-buffered saline

(PBS) solutions (endogenous LH absent) spiked with recombinant human chorionic gonadotropin (hCG) solution in the following concentrations: 50, 100, 500, 1000, 2500 and 5000 mIU mL^{-1}. The apparent LH concentration of each sample was determined by the IMMULITE® system. The analyses were performed in duplicate.

Intra and inter-assay precision

The intra-assay and inter-assay precision were evaluated by the analysis of seven replicates of three different urine samples on the same day, and on three different days by different analysts and different lots of LH immunoassay kits, respectively. Both were expressed as coefficient of variation (%).

Linearity

The linearity was established by serially diluting a urine sample with a measured LH of 114 mIU mL^{-1} with a PBS/BSA 1% solution. The linearity was evaluated in the concentration range from 0.1 to 50 mIU mL^{-1}, with 3 replicates for each concentration level.

Limit of detection and limit of quantification

The limit of detection was estimated by the mean value obtained by the analysis of 10 blank samples plus three times its standard deviation. PBS/BSA 1% solutions were used as the negative samples. The limit of quantification was estimated as the lowest concentration of the calibration curve obtained by the linearity whose coefficient of variation was less than 10%.

Matrix interference

The matrix interference was evaluated using an experiment described by Robinson *et al.*[13] and it was evaluated by the analysis of ten different urine samples, whose LH concentrations were measured, and then they were pooled together two by two (same proportion) and measured again in order to evaluate the differences between the expected and the measured values. The analyses were performed in triplicate.

Recovery

Recovery was assessed by the spiking and analysis of five urine samples (with known original LH concentrations) with additional 67 mIU of pituitary LH (International Standard for Human Pituitary LH, NIBSC Code 80/552) before the centrifugation step.

Carryover

The carryover was evaluated observing the analytical signal of an urine sample with suppressed LH analyzed sequentially after an urine sample with high LH concentration (119 mIU mL^{-1}). Analyses were performed in triplicate.

Stability

The stability of urinary LH in different storage conditions (–20 °C, 4 °C and room temperature) was evaluated after 10, 30 and 40 days after the collection and analysis of 3 different urine samples. Urinary LH stability was also tested through 3 regular freezing and thawing cycles of 3 different urine samples. The samples were thawed at room temperature.

Reference interval

In order to evaluate the urinary excretion profile of LH, urine samples of 598 professional male athletes of several sport modalities were analyzed by IMMULITE®. The urinary LH concentrations were normalized by their specific gravity. The reference population was determined from the urinary LH concentrations, for subsequent establishment of the reference range through statistical analysis. The distribution of the LH profile parameter sets was tested using the Kolmogorov-Smirnov test. Outliers were searched with the Tukey test, based on the box plot diagram type.[14,15] The reference value was obtained by two different approaches: the percentile estimation method, recommended by the International Federation of Clinical Chemistry (IFCC) and Laboratory Medicine (percentile of 97.5%),[16] and the inference of a far outside value: percentile of 75% + (3 × interquartile interval).[17,18]

Statistical analysis

All statistical calculations were performed using MedCalc© software, version 12.3.0, and graphical representations were performed using Microsoft Excel software (p-values < 0.05 were considered statistically significant).

Results and Discussion

Pretreatment of the samples

In order to evaluate the proper pretreatment of the urine samples prior to the immunoassay analysis, two different procedures were compared: warming or centrifugation of the samples. The comparison between urinary LH concentrations measured from pre-assay warmed samples and pre-assay centrifuged samples is shown in Figure 1. The linear regression gave the following results: [LH concentration (pre-assay warming)] = 0.9606 × [LH concentration (pre-assay centrifugation)] + 0.6727;

$R^2 = 0.9997$, $R = 0.9998$. The coefficient of determination shows the good correlation between the LH concentrations measured from pre-assay warmed samples and from pre-assay centrifuged samples.

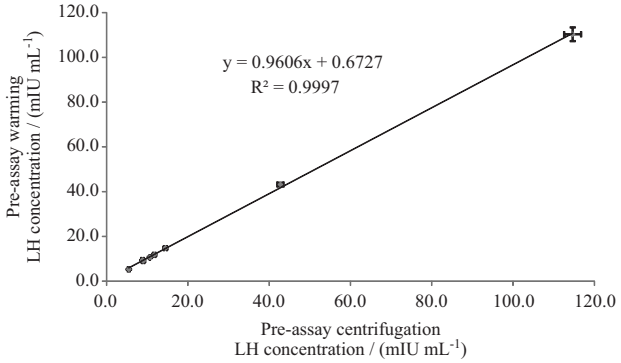

Figure 1. Comparison of urinary LH concentrations measured from pre-assay warmed samples and pre-assay centrifuged samples.

Specificity

Table 1 shows the values of each hCG concentration in the solutions of PBS/BSA 1% with no endogenous LH, as well as, their apparent LH concentrations obtained by the IMMULITE® and the cross-reactivity obtained for each level of spiked hCG.

The apparent LH can be attributed to the cross-reactivity of the immunoassay with hCG (measured by the ratio between the apparent LH and the spiked hCG), which was less than 0.2%, equivalent to that obtained by the supplier for serum analysis (0.15%).[19]

Intra and inter-assay precision

As shown in Table 2 and Table 3, respectively, the intra-assay precision was estimated at 2.7% and the inter-assay precision was estimated at 5.2% (both lower than the reported by the supplier for serum analysis: 4.8% and 10.6%, respectively).[19]

Table 1. Specificity of the analytical method for LH in solutions free of the hormone but fortified with hCG

hCG / (mIU mL^{-1})	Apparent LH / (mIU mL^{-1})	Cross reactivity / %
50	< 0.1	–
100	0.15	0.15
500	0.89	0.18
1000	0.68	0.068
2500	1.95	0.078
5000	4.3	0.086

Linearity

Figure 2 presents the linearity data obtained in urine matrix with LH concentration range from 0.1 to 50 mIU mL^{-1}. A homoscedastic model was observed by the Cochran test (variance test). The linear regression gave the following result: [CPS] = 371550 × [LH concentration] + 175384; $R^2 = 0.9999$, $R = 0.9999$.

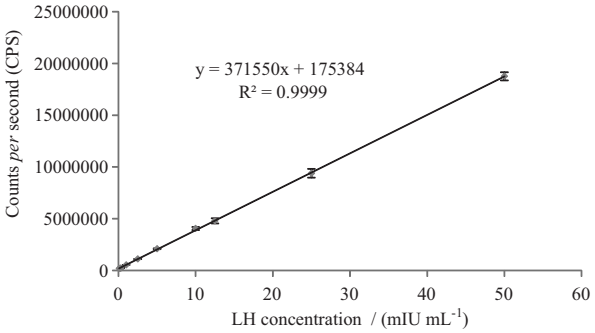

Figure 2. Linearity verification in urine matrix. LH concentration range from 0.1 mIU mL^{-1} to 50 mIU mL^{-1}. Each concentration point evaluated in triplicate (n = 3).

Table 2. Intra-assay precision obtained from different urine samples with different concentrations

Sample	n	Mean LH / (mIU mL^{-1})	Standard deviation / (mIU mL^{-1})	Coefficient of variation / %
A	7	4.3	0.11	2.6
B	7	8.5	0.19	2.3
C	7	11.5	0.31	2.7

Table 3. Inter-assay precision obtained from different urine samples with different concentrations

Sample	n	Mean LH / (mIU mL^{-1})	Standard deviation / (mIU mL^{-1})	Coefficient of variation / %
A	21	4.5	0.23	5.2
B	21	8.0	0.38	4.8
C	21	11.5	0.31	2.7

Limit of detection and limit of quantification

The analytical signal that statistically distinguishes from the noise has a mean chemiluminescence value corresponding to 137239 units. According to the calibration curve obtained in the linearity test, this signal corresponds to a concentration value lower than 0.1 mIU mL^{-1}, which is the lowest detectable concentration of LH by the IMMULITE®. Therefore, we can infer that the limit of detection is less than 0.1 mIU mL^{-1}, but it is not possible to estimate an exact value because the system does not allow such operation.

The lowest concentration of the calibration curve showed a coefficient of variation of 2.54%, which is satisfactory once is lower than 10%. Therefore, the limit of quantification was established as 0.1 mIU mL^{-1}.

Matrix interference

The comparison of the experimental data obtained for 10 urine samples pooled together two by two (measured LH concentration) with the mean value of both samples measured separately (expected LH concentration) is shown in Figure 3. The linear regression gave the following result: [measured LH concentration] = 0.9617 × [expected LH concentration] + 0.1563; R^2 = 0.9997, R = 0.9998. The coefficient of determination shows the good correlation between the expected and the measured concentrations, indicating that there was no significant matrix interference.

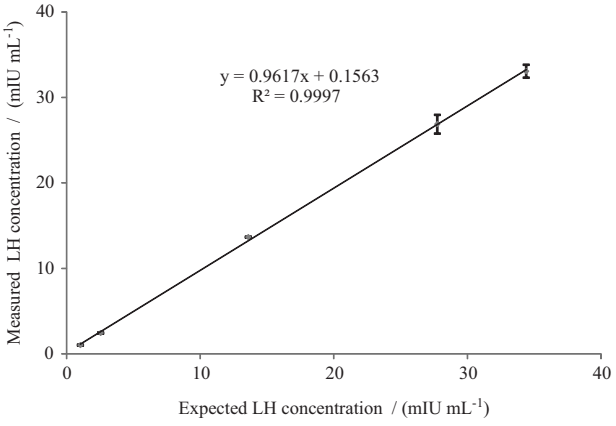

Figure 3. Comparison of the measured and the expected urinary LH of 10 samples pooled two by two.

Recovery

The recovery was calculated from the ratio of the experimentally measured concentration of LH and its expected concentration (ratio between the total amount of LH and the final urine volume). As shown in Table 4, the method obtained a range of recovery from 93.6% to 99.8%, equivalent to the obtained by the equipment supplier (92% to 101%).[19]

Carryover

No carryover was observed after alternated analysis of a sample urine with high LH concentration and a urine sample with low LH concentration.

Stability

Figure 4 shows the stability of LH in urine samples stored at −20 °C, 4 °C and room temperature for 40 days and after regular freezing and thawing cycles. Urinary LH remained stable for at least 40 days when the samples were stored at 4 ° C and −20 ° C and up to 10 days when stored at room temperature. LH was unstable after the second cycle of freezing/thawing. Since the composition of the urine samples may vary significantly, the stability of urinary LH may be very different according to the urine samples. Then, the stability is far to be guaranteed in any sample.

Determination of the reference value

The Kolmogorov-Smirnov test results revealed a non-normal distribution, which can also be confirmed by visual inspection of the histogram shown in Figure 5.

In order to detect outliers by the Tukey test, the data was logarithmically transformed in order to a normal

Table 4. Recovery obtained from different urine samples

Sample	Measured LH / (mIU mL^{-1})	Standard deviation / (mIU mL^{-1})	Expected LH / (mIU mL^{-1})	Recovery / %
A	46.1	2.51	49.3	93.6
B	53.2	1.49	56.0	95.0
C	53.0	1.59	53.2	99.8
D	53.0	2.14	54.1	97.9
E	53.9	2.53	54.8	98.2

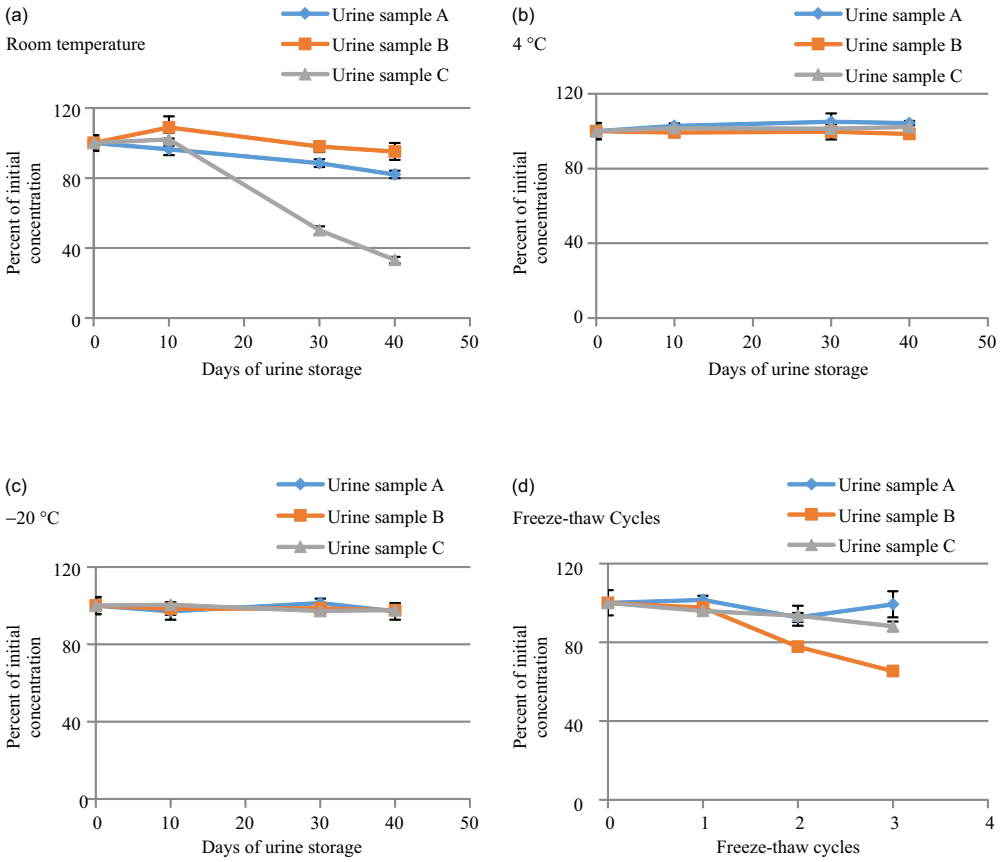

Figure 4. Stability of LH in urine samples stored at room temperature (a); 4 °C (b); –20 °C (c) for 40 days and after regular freezing and thawing cycles (d).

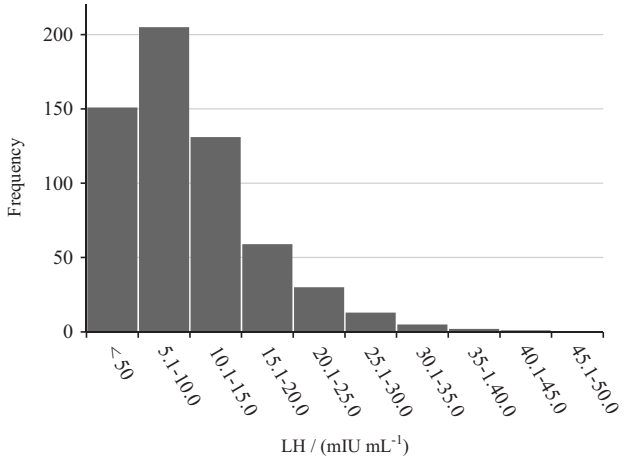

Figure 5. Frequency chart of urinary concentrations of LH for Brazilian athletes' population.

distribution be analyzed. Applying the outlier test, one value was detected and excluded from the distribution.

Because of the non-normal distribution of the data, non-parametric approaches were adopted to the evaluation of the results. Following the IFCC approach, the upper reference limit was determined at 27.3 mIU mL^{-1} with a 90% confidence interval of 25.0-28.6 mIU mL^{-1}. Following the far outside value approach ([percentile 75% + 3 × interquartile interval]),[17,18] the reference value was estimated at 37.4 mIU mL^{-1}.

In analysis by immunoassays, particulate matter present in the urine samples may interfere in the results. Therefore, for the analysis of the analog of LH, hCG, WADA recommends centrifugation of the urine samples prior to analysis in the immunoassay.[20] However, there is no recommendation yet for the determination of LH.

In order to circumvent the problem of the presence of sediments in urine, there are two classical approaches well described: centrifugation of the samples prior to the immunoassay analysis and heating the samples for complete solubilization of the sediments.[21] There is a concern that the removal of the sediments by centrifugation may reduce the original concentration of LH, since it may remove part of the LH that is adsorbed to the sediments, whereas heating the samples would allow the entire content of LH to be preserved. Since both pretreatments tested at this study showed to be equivalent, with no apparent loss of LH, the pretreatment chosen for this study was the centrifugation of the samples of urine, following the protocol previously established for the analysis of hCG.[20]

Once the equipment IMMULITE® was originally developed and validated for analysis of blood serum, a

complete validation protocol was performed in order to evaluate its performance for the analysis in urine. The validation process confirmed that the method used for quantification of LH in urine matrix by IMMULITE® system meets the criteria established by WADA, ensuring the reliability of the results.

As it is not always possible to maintain optimal storage conditions during the transport of the samples to the analytical laboratory, it is necessary to simulate the conditions in which the urine samples can be stored before being analyzed by the immunoassay.

Considering the lack of a standardized way of storing the samples during transport to the place of analysis, the stability of LH in urine for at least 10 days at room temperature ensures that its quantitative assessment seems not to be diminished due to a possible degradation hormone. Thus, analyses with range of up to ten days after the time of collection seem to be feasible if the samples are stored during the transportation at any of the three conditions analyzed in this study.

According to the ISL, "A" samples (athletes samples analyzed in routine), and "B" samples (athletes samples analyzed in case of confirmation of routine findings) must be stored frozen (–20 ° C).[12] Thus, from the results obtained in this study, urine storage at –20 ° C does not compromise the analysis of LH for at least 40 days, as long as the samples are submitted to at most one freezing-thawing cycle. Therefore, it would be recommended to storage all the urine samples at 4 ºC, in which they would not be submitted to any freezing/thawing cycle.

Other studies showed a lower stability of urinary LH over time.[13] However, it depends on a few factors, such as the exposure to high urinary concentrations of urea or microorganisms, for example, and the immunoassay epitope specificity.[22-24] Since IMMULITE® is specific for the β-subunit of LH, it detects both the intact hormone and the β-subunit dissociated, and therefore, the estimate of the LH concentration present in the urine samples is not compromised in the case of a dissociation of the hormone in its subunits.[25] Immunoassays specific for only the intact form will inevitably have the hormone immunoreactivity compromised over time. In fact, the Access® LH immunoassay from Beckman Coulter, used by Robinson *et al*.,[13] has the specificity for the intact LH only and does not recognize LHβ, which is consistent with the lower LH immunoreactivity over time. While IMMULITE®, by having specificity by both intact LH and LHβ, is expected to display a prolonged stability over time.[24]

The establishment of reference ranges of urinary concentrations of different hormones from reference populations is a consecrated strategy in doping control, especially in the diagnosis of abuse of endogenous doping agents.[26,27] The information originated from reference populations are extremely useful in screening methods.[27] Results that show deviations from the population can, *a priori*, be classified as atypical.

Up to now, WADA has not established, clearly, any positivity criterion or cut-off value to characterize a presumptive LH result in doping control. Robinson *et al*.,[13] have discussed the real difficulty on inferring a cut-off value, as well as the current situation of general LH analysis on doping control. Still, because of the absence of a universal cut-off value for LH, each doping control laboratory has to determine its own reference range of urinary LH in order to infer a possible misuse by athletes.

For this study, the urinary excretion of endogenous LH of a reference population composed of 597 Brazilian male elite athletes was evaluated.

The descriptive statistics for reference distribution is presented in Table 5, showing the mean, median, first (Q1) and third (Q3) quartiles, and minimum and maximum values for urinary luteinizing hormone.

Table 5. Descriptive statistics obtained for a population of 597 Brazilian male athletes

Parameter	LH / (mIU mL^{-1})
Minimun value	1.2
25% quartile (Q1)	5.0
Median (intequartile range)	8.1
Mean	9.9
75% quartile (Q3)	13.1
Maximun value	43.1

Following the IFCC approach, the reference value correspondent to the percentile of 97.5% was determined at 27.3 mIU mL^{-1} with a 90% confidence interval of 25.0-28.6 mIU mL^{-1}. Following the far outside value approach, the cut-off value was estimated at 37.4 mIU mL^{-1}. Both are reliable and relevant approaches to the purpose of determination of a cut-off value at doping control. However, due to the absence of a universal reference value, the highest value will be used in our laboratory as the LH cut-off value for further analyses. Based on these findings, any result above of 37.4 mIU mL^{-1} by the analysis of LH in urine through IMMULITE® system can be considered as a presumptive analytical finding. A second assay with a different epitope or a mass spectrometry based method must be used to confirm the result. Therefore, it would be recommended to reanalyze the urine sample with the maximum value of 43.1 mIU mL^{-1} of LH, shown in Table 5,

for example, in a second assay in order to confirm a possible abuse of the hormone.

The anti-doping laboratory of China also determined a cut-off value for LH from 1443 male athletes' urine samples. Using an enzyme immunoassay (EIA), the upper reference limit obtained, following the recommendations of the IFCC and ICSH (International Committee for Standardization in Hematology), was 26.77 mIU mL^{-1} with a 90% confidence interval of 24.40 mIU mL^{-1} to 28.03 mIU mL^{-1}.[28]

Due to the different LH specificities of each immunoassay, the reference range established by each laboratory becomes test-specific, considering the different antibodies used in each one. This means that, in order to try a standardized LH reference range in doping control laboratories, all of them would have to use the same immunoassay.

Besides, reference ranges are also dependent on the population to be tested, and therefore, it cannot be automatically transferred between laboratories.[29] At most, it can serve as the basis for interpreting laboratory results, while studies on the local population have not yet been developed.

Conclusion

The IMMULITE® LH assay, originally developed for serum matrix, was evaluated in urine matrix aiming the doping control analysis in Brazil. The validation process in urine demonstrated that the assay is fit-to-purpose in that matrix. The Brazilian population was evaluated regarding the LH urinary excretion profile and presented a non-normal distribution. The reference population of 597 male Brazilian athletes showed a cut-off value of 37.4 mIU mL^{-1}, following the far outside value approach. These results indicated the assay is suitable for application in doping control analysis. Therefore, Brazilian male athletes' samples with urinary LH concentrations higher than 37.4 mIU mL^{-1}, determined by IMMULITE® system, should be interpreted as a warning for further investigation about a possible abuse of LH. The results obtained in this study are of great importance to doping control and various clinical applications, considering the few works in the literature devoted to this subject and also the need to implement the monitoring of LH in the Brazilian doping control.

Acknowledgments

This work was supported by Fundação Universitária José Bonifácio-FUJB, CNPq, FAPERJ and CBF. The technical assistance of Viviane Goulart Freire de Araújo was greatly appreciated. In the Cover Image of the issue there are the following pictures: Syringe illustration - Copyright© Andrei Alexandru Florian; Athletes' icons - Copyright© Vaclav Kostal; Researcher icon - Copyright© Leremy; Immunoassay reaction - Copyright© Extender01. The pictures were obtained from Dreamstime.com.

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