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Performance characteristics of two immunoassays for the measurement of urinary luteinizing hormone

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Abstract

Urine luteinizing hormone (LH) concentration is routinely measured in all anti-doping laboratories to exclude recombinant LH abuse and to test any potential alteration of the hypophyseal-gonadal axis. Before establishing proper reference values among professional top level athletes, an extended validation of two commercial immunoassays for LH measurements was performed.

Elecsys® 1010 and Access® are two automated immunoanalyzers for central laboratories. The limit of detection, the limit of quantification, intra-laboratory, inter-technique correlation, precision, accuracy were determined. Furthermore, reference urinary LH distribution values for male and female top level athletes were determined. Stability studies of LH in urine following freezing and thawing cycles ($n = 3$) as well as storage conditions at room temperature, 4 °C and −20 °C were performed.

Male and female subjects showed important urinary corrected (specific gravity correction) LH distribution differences. Intra-assay precision for the Access® analyzer was less than 8.0% whereas inter-assay was close to 11%. Intra and inter-assay precision for the Elecsys® 1010 analyzer was slightly better. A good inter-technique correlation was obtained ([Elecsys® 1010] = 1.0434[Access®] + 1.146, $R = 0.953$). No urinary LH loss was observed after two freezing and thawing cycles. On the other hand, time and bad storage conditions such as elevated temperature can deteriorate rapidly urinary LH.

In conclusion, both analyzers showed acceptable performances and are suitable for screening anti-doping analyses. Each anti-doping laboratory has to settle its own reference distribution values and then determine when to launch a confirmation procedure. This takes place then depending on the positivity criteria the anti-doping laboratory has established and validated. This study also clearly showed that the time delay between the urine collection and the analysis should be reduced as much as possible and urine samples should be transported in optimal conditions (low temperature and quickly) to decrease urinary LH deterioration.

Keywords: LH; Urine; Doping; Validation; Stabily; Immunoassay

1. Introduction

The luteinizing hormone is a glycoprotein with two subunits (α and β). These subunits are bound together covalently; the α subunit is common to other hormones such as human chorionic gonadotropin (HCG), follicle stimulating hormone (FSH) and thyroid stimulating hormone (TSH) whereas the β subunit is different for each hormone. These differences come from the specific oligosaccharide structure bound on the β subunit. This guarantees the physiological and immunological specificity of each glycoprotein. The approximate weight of LH is 28.5 kDa [7].

Among women, LH has an effect on the hypothalamic-pituitary axis, the ovaries and the regulation and control of the menstrual cycle. LH is released from the gonadotropin cells of the pituitary in a pulsatile way and reaches the ovaries through the blood. In the ovaries, LH stimulates growth and maturation of the follicles as well as the synthesis of oestrogens and progesterone. LH reaches a peak in the middle of the menstrual cycle and induces the ovulation and the formation of the yellow body. Among men, LH stimulates interstitial cells and influences testosterone production by Leydig cells [12].

Usually, LH in combination to FSH is measured to determine the reasons of the dysfunctions of the hypothalamic-pituitary axis. It provides very useful information notably at the time of menstrual cycle exams, fertility and abnormality during puberty, premature ovary insufficiency, menopause, pituitary

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insufficiency and ovulation problems. Deficiency of Leydig cells is also identified by measuring LH concentration.

As mentioned, in women, LH affects also the production of female sex hormones, whereas in males LH causes testosterone to be produced and released. Therefore and since the availability of recombinant LH, sport's authorities decided to include it in the prohibited list [19]. Consequently, it requires the development of a reliable and valid detection test. LH is most likely to be used mainly by athletes who have reduced testicular size and suppressed endogenous testosterone production due to the use of synthetic androgens, but with a low risk of detection. The effects of LH are fairly short making it easier to continue use closer to competition events which are subject to doping tests. Furthermore, LH occurs normally in easily detectable concentrations in urine so it becomes necessary to establish reference range values. In this way, a population analysis could theoretically lead to an estimation of the proportion of athletes abusing of recombinant LH. An alternative would be to distinguish between exogenous recombinant LH from endogenous LH.

In this report, we describe the analytical performances of two immunoassays which were initially designed to measure serum LH concentration, but were used in this specific study to measure urinary LH concentration. This study also pointed out some limitations related to the measurement of LH in urine for anti-doping purposes, because of a lack a stability of LH in urine and the impossibility of establishing reference values specially for female athletes.

2. Material and methods

2.1. Immunological techniques

Two immunoassays were evaluated for LH measurement in urine.

The first immunoassay (Test 1) was the Access® LH from Beckman Coulter (Beckman Coulter International SA, Nyon, Switzerland). This test was a two step ("sandwich") immunoenzymatique assay. Goat anti-mouse antibodies were fixed on magnetic beads. The sample was put together with mouse anti-LH antibodies and a solid phase; a magnetic field kept the complex bead–antibody-LH whereas the non-specific material was eliminated during the washing period. Anti-LH goat antibodies conjugated to alkaline phosphatase were then added and bound to LH previously fixed on the beads. A second washing step got rid of the goat antibodies not bound to LH. The chemiluminescent substrate was added and the light generated by the reaction was measured by a photometer. A volume of 55 μl of urine was required. The Access® analyzer used stored master curves generated by Beckman Coulter for each internal batch of reagents. Each new batch had to be calibrated before its use. The calibration was done by using six adjustor points supplied by the manufacturer (0, 2, 10, 25, 100 and 250 mIU/ml) and was valid over a period of 28 days. Each point was analyzed in duplicate. Quality controls (QC) were supplied by an external manufacturer, Bio-RAD Laboratories (Lyphochek Immunoassay Plus Control, trilevel).

The second immunoassay (Test 2) was the Elecsys® 1010 LH from Roche (Roche Diagnostics, Rotkreuz, Switzerland). This test was also a "sandwich" type immunoenzymatique assay. During the first incubation, the sample was put together with a monoclonal anti-LH antibody specially marked with biotine and a second antibody marked with ruthenium. Micro particles covered with streptavidine were added to the reaction chamber. The immunological complex got bound to the solid phase via the streptavidine-biotine bond. A washing step got rid of the free fraction whereas the micro particles were kept in the measuring chamber thanks to a magnetic field. On the electrode, a tension was applied which then generated some light measurable by a photometer. A volume of 20 μl of urine was required. The Elecsys® 1010 analyzer used stored master curves generated by Roche Diagnostics for each internal batch of reagents. Each new batch had to be calibrated before its use and was valid over a period of 7 days. The calibration was done by using two adjustor points supplied by the manufacturer; a linear regression was then plotted while the intercept was set at zero. Each point was analyzed in duplicate. Two internal controls (Elecsys® PreciControl Universal 1 and 2) were used on a regular basis as well external quality controls (QC). These latter were supplied by Bio-RAD Laboratories (Lyphochek Immunoassay Plus Control, trilevel).

According to the literature and to common practice in anti-doping laboratories, urinary LH concentrations were corrected in case the specific gravity was >1.020. This was necessary to fit a log normal distribution. The determination of the specific gravity and the correction factor have been published elsewhere [5,13,16].

2.2. Urine samples

Urine samples coming from regular in and out competition anti-doping tests were used for this study. The gender was known whereas the age of the athlete was unknown. All samples were collected, transported and analyzed within less than six days except for those analyzed for stability studies and for freezing and thawing cycles. Before analysis, all urine samples were centrifuged for 5 min at $1000 \times g$.

2.3. Validation assays

The validation assays were all conducted in the Swiss Laboratory for Doping Analyses, Institut Universitaire de Médecine Légale de Lausanne. Inter-technique analyses were all performed on the same days.

2.4. Intra-laboratory and inter-technique validation

Intra-laboratory validation was performed following a 4-day validation protocol. The best fit between signal and concentration were calculated automatically by the analyzers themselves.

Five replicates of three QC samples were analyzed for the determination of intra-assay precision and accuracy, while the inter-assay precision and accuracy were determined for all values with all independent experimental assays of QC samples.

Precision was expressed as the percent relative standard deviation (R.S.D.%) of the measurements performed. Accuracy was evaluated as "correct" or "incorrect" if the concentration obtained was inside or outside the acceptance concentration range defined by the manufacturer of the QC samples. According to previous publications, the theoretical limits of detection (LOD) and quantification (LOQ) were calculated by measuring the blank calibration sample (absence of analyte) five times in the same run and in the same week. The standard deviation of the values obtained was taken as the measure of the noise. Theoretical LOD was defined as the mean value obtained for the blank sample plus three times the estimated value of noise. Theoretical LOQ was defined as the mean value obtained for the blank sample plus 10 times the estimated value of noise. In order to determine the real limit of quantification (LOQ_R) of both tests in urine, one urine sample was measured and then diluted up to nine times (geometrical dilution) with a urine sample considered as negative (urine sample measured with both analyzers and with a mean LH concentration below the detection limit). The limit of quantification (LOQ_R) was reached when the daughter urine did not show half the concentration of the previous mother urine.

Inter-technique validation was realized by analyzing the same set of human urine samples with both analyzers (Test 1 and Test 2). The evaluation of the dispersion of the results obtained between different techniques was obtained with Bland–Altman analyses [3]. The mean values of concentrations were represented versus the differences between concentrations. The 95% limits of agreement was calculated (mean difference ± 1.96 standard deviation of differences).

2.5. Stability studies

Stability studies were performed using external QC controls (QC1, QC2, QC3). These test were performed with both analyzers.

2.6. Freeze/thaw cycles

LH stability in human urine samples was tested via regular freezing and thawing cycles. Three urine samples (3 ml each), low, medium and high concentrations were analyzed three times consecutively (F/T0), then frozen and kept at −20 °C for 48 h. After that, they were unfrozen at room temperature for 90 min, were homogenized and re-analyzed three times consecutively (F/T1). Afterwards, all three samples were put back into the freezer. Two more cycles freezing/thawing (−20 °C for 48 h, unfreezing 90 min) were repeated (F/T2 and F/T3). Stability was evaluated by monitoring the percentage of changes regarding initial values (F/T0). All analyses were performed with both analyzers.

2.7. Storage conditions

LH stability in human urine samples was tested while storage conditions were different. All samples were analyzed three times consecutively. They were then aliquoted into different tubes. All tubes were either kept at room temperature (RT), at 4 °C or at −20 °C. After 5, 10 and 90 days, one of each tubes was analyzed three times consecutively to determine the stability of urinary LH according to time and storage conditions. Stability was evaluated by monitoring the percentage of changes regarding initial values (T0). All analyses were performed with both analyzers.

2.8. Matrix effect

The matrix effect was evaluated. Urine samples were measured once, then they were pooled together two by two and measured once again. In this way, it was possible to find out whether differences could appear between expected values (mean values between two urine samples) and measured values (values of two samples pooled together).

2.9. Statistics

All statistical analyses were performed on Matlab® Version 6.1.0 with Statistics Toolbox Version 3.0. For distribution testing, we employed a Bera-Jarque test of normality. For other hypothesis tests, we used a two-sample *T*-test. A significant level of $p < 0.05$ and $p < 0.01$ were considered for all tests.

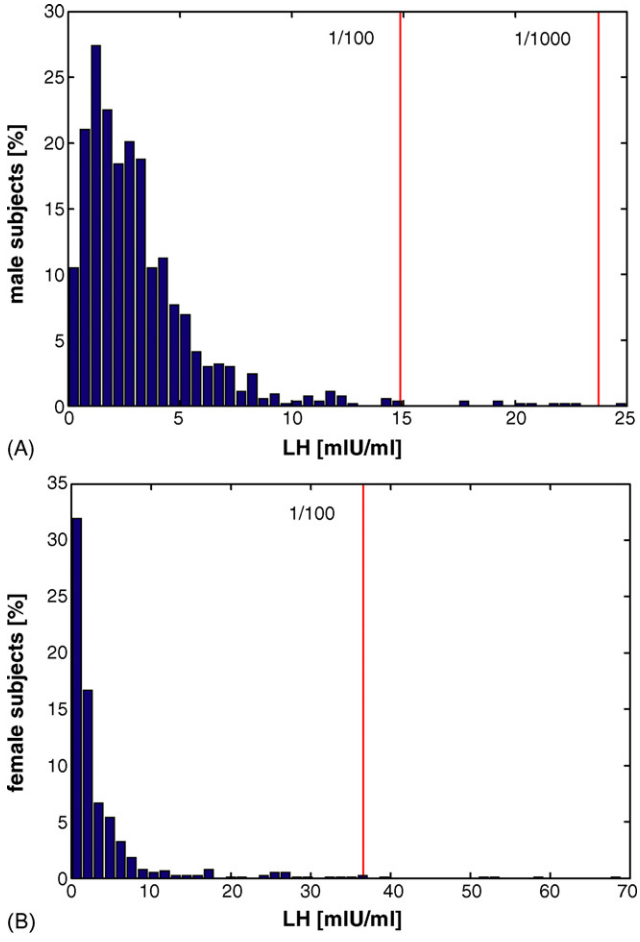

Fig. 1. Distribution of corrected urinary LH concentrations of male (A; $n = 1066$) and female (B; $n = 552$) top level athletes measured with the Elecsys® 1010 instrument. Vertical lines represent the probability of observing a urine sample with a corrected urinary LH concentration above 1/100 and 1/1000.

3. Results

3.1. Urinary LH concentrations observed among top level male and female athletes

Fig. 1 represents the corrected distribution of urinary LH observed among male (Fig. 1A, $n = 1066$) and female (Fig. 1B, $n = 552$) sportsmen. The probability of observing a urine sample with a urinary LH concentration was estimated at 1/100 and 1/1000 levels in men and at 1/100 in women (see vertical lines). Both distributions are well represented by a lognormal distribution.

3.2. Detection (LOD) and quantification (LOQ) limits

Theoretical LOD and LOQ were calculated and determined with both analyzers. The calculation of the standard deviation (S.D.) of the blank sample for Test 1 was excellent. LOD was equal to 0.03 mIU/ml and LOQ was equal to 0.10 mIU/ml. For Test 2, it was impossible to calculate LOD and LOQ, because the instrument did not return LH results below 0.10 mIU/ml. These results did not take into account the possible matrix effect of urine. The determination of LOQ_R (real LOQ in urine) showed it was higher for Test 1 than for Test 2, because the instrument reached a plateau around 0.30 mIU/ml (see Table 1). This experience showed a good linearity and precision in urine for both tests in their respective quantification domain.

3.3. Detection (LOD) and quantification (LOQ) limits

Intra and inter-assay results are presented in Table 2. Intra-assay precision for Test 1 was acceptable (≤8.0%) and within expected values provided by the manufacturer (≤10.0%). On the other hand, inter-assay for the lowest control level (QC1) was above 10%, respectively, 10.8%. Intra-assay precision was better for Test 2 than for Test 1. Inter-assay confirmed these results. Acceptable accuracies were obtained with both tests even though Test 1 showed on two occasions to have an accuracy slightly below reference range (see QC 1, T1 and T3) (see Table 1).

3.4. Inter-technique comparisons

In Fig. 2A, urinary LH concentrations observed among 152 samples were plotted. Different concentrations were measured according to the test. Mean values with Test 1 were equal to 3.18 mIU/ml (min. = 0.04; max. = 26.62) and equal to 5.03 mIU/ml (min. = 0.00; max. = 35.01) with Test 2. Bland–Altman representation showed the underestimation of urinary LH determination of Test 1 in comparison to Test 2. Considering a 95% limit of agreement for the 152 samples measured, only 7 values were out of the defined range; this is a non-significant result (see Fig. 2B).

3.5. Stability tests

Freezing and thawing altered human urinary LH concentrations after three cycles. This was noticeable when measuring three different urine samples three times consecutively with both instruments. All results were expressed in percentage changes. Differences between samples having gone through three cycles or not did not exceed 10% (see Fig. 3). Storage conditions, on the other hand, showed that urinary LH concentrations changed rapidly while samples were kept at room temperature (less than 5

Table 1
Evaluation of the limit of quantification of one urine sample following multiple dilutions ($n = 9$) with a negative urine sample (urinary LH < 0.02 mIU/ml)

Dilution	n	Mean (mIU/ml)	S.D. (mIU/ml)	Precision R.S.D. (%)	Theoretical value (mIU/ml)
Test 1					
1	3	23.80	0.93	3.9	23.80
1/2	3	10.98	0.28	2.5	11.90
1/4	3	4.99	0.05	0.9	5.95
1/8	3	2.37	0.07	3.0	2.98
1/16	3	1.26	0.02	1.6	1.49
1/32	3	0.71	0.02	2.1	0.74
1/64	3	0.50	0.03	6.0	0.37
1/128	3	0.36	0.02	4.2	0.19
1/256	3	0.30	0.03	8.3	0.09
1/512	3	0.30	0.02	5.1	0.05
Test 2					
1	3	31.07	0.86	2.8	31.07
1/2	3	14.22	0.42	3.0	15.54
1/4	3	6.26	0.06	1.0	7.77
1/8	3	2.98	0.03	1.1	3.88
1/16	3	1.48	0.10	6.8	1.94
1/32	3	0.80	0.05	5.8	0.97
1/64	3	0.41	0.02	5.6	0.49
1/128	3	0.22	0.02	9.6	0.24
1/256	3	0.13	0.02	17.2	0.12
1/512	3	<0.100	NA	NA	0.06

Table 2
Validation parameters of Test 1 (Access®) and Test 2 (Elecsys® 1010) obtained in our laboratory

	QC range (mIU/ml)	Assay	Intra-assay					Inter-assay				
			n	Mean (mIU/ml)	S.D. (mIU/ml)	Precision R.S.D. (%)	Accuracy error (%)	*n*	Mean (mIU/ml)	S.D. (mIU/ml)	Precision R.S.D. (%)	Accuracy error (%)
Test 1												
QC1	1.4–2.3	T1	5	1.21	0.08	7.0	Incorrect	13	1.35	0.15	10.8	Correct
		T2	5	1.49	0.05	3.4	Correct					
		T3	3	1.33	0.10	7.7	Incorrect					
QC2	12.0–20.0	T1	5	12.80	0.19	1.5	Correct	15	13.84	0.81	5.8	Correct
		T2	5	14.19	0.24	1.7	Correct					
		T3	5	14.55	0.27	1.9	Correct					
QC3	34.0–56.0	T1	5	36.01	1.95	5.4	Correct	15	38.16	2.15	5.6	Correct
		T2	5	38.24	0.76	2.0	Correct					
		T3	5	40.23	0.80	2.0	Correct					
Blank		T1	5	0.00	0.00	NA	Correct	14	0.01	0.02	151.9	Correct
		T2	5	0.04	0.02	62.8	Correct					
		T3	4	0.01	0.01	200.0	Correct					
Test 2												
QC1	1.4–2.3	T1	5	1.93	0.04	1.8	Correct	15	1.92	0.04	2.2	Correct
		T2	5	1.94	0.05	2.5	Correct					
		T3	3	1.90	0.04	1.9	Correct					
QC2	12.0–20.0	T1	5	17.21	0.07	0.4	Correct	15	17.08	0.22	1.3	Correct
		T2	5	17.15	0.16	0.9	Correct					
		T3	5	16.89	0.25	1.5	Correct					
QC3	34.0–56.0	T1	5	48.10	0.46	1.0	Correct	15	48.00	0.43	0.9	Correct
		T2	5	48.19	0.40	0.8	Correct					
		T3	5	47.71	0.34	0.7	Correct					
Blank		T1	5	<0.10	NA	NA	Correct	15	NA	NA	NA	Correct
		T2	5	<0.10	NA	NA	Correct					
		T3	5	<0.10	NA	NA	Correct					

QC range: acceptance concentration range according to the manufacturer; S.D.: standard deviation; R.S.D.: relative standard deviation; accuracy error: inside or outside the acceptance range defined by the manufacturer; LOD: Test 1: 0.03 mIU/ml; LOQ: Test 1: 0.10 mIU/ml.

days). At 4 °C and −20 °C, alteration process was slowed down but was nevertheless significant after 10 days (see Fig. 4).

3.6. Cross-reactivity

Under our experimental conditions, urinary matrix did not affect significantly the results as depicted in Fig. 5. For both analyzers the expected urinary LH concentrations (mean LH value obtained with two urine samples) correlated extremely well with those measured (LH concentration obtained from two urine samples pooled together). A linear regression gave the following results for Test 1: [Expected LH concentration] = 0.9605[Measured LH concentration] + 0.0622, $R = 0.991$ whereas for Test 2 we obtained: [Expected LH concentration] = 0.985[Measured LH concentration] + 0.4612, $R = 0.985$.

4. Discussion

In this study, two different immunoassays were evaluated for the measurement of urinary LH. Both tests were simple to perform, entirely automatic and did not require much sample preparation except a short centrifugation before analysis to avoid any clogging which could potentially be caused by cell or bacteria fragments and protein precipitation/agglutination. Once the calibration procedure and the controls were run, the turnaround time for results was less than one hour. Both immunoassays gave good diagnostic results, but in order to reach forensic standards, some harmonization still needs to be done.

This study clearly demonstrated that the establishment of a cut off limit for urinary LH concentration for male and female athletes is subject to discussion. According to the immunoassay, the time delay between the urine collection and the analyses or the transport conditions (pre-analytical conditions), the result can be strongly different [9,14]. Each anti-doping laboratory has to validate its immunoassay in place in its facilities, but the validation cannot take into account the possible interferences due to bad pre-analytical conditions. Furthermore, and according to the International Standard for Laboratories (ISL), on receipt of urine anti-doping samples, B-samples – sample to be used in case of counter analysis – have to be frozen. Our results as well as previous studies clearly showed that LH deteriorates over time even if kept at −20 °C. Consequently, results can be confirmed with great difficulty in case of counter analysis unless very strict precautions are taken. Nowadays, anti-doping laboratories have

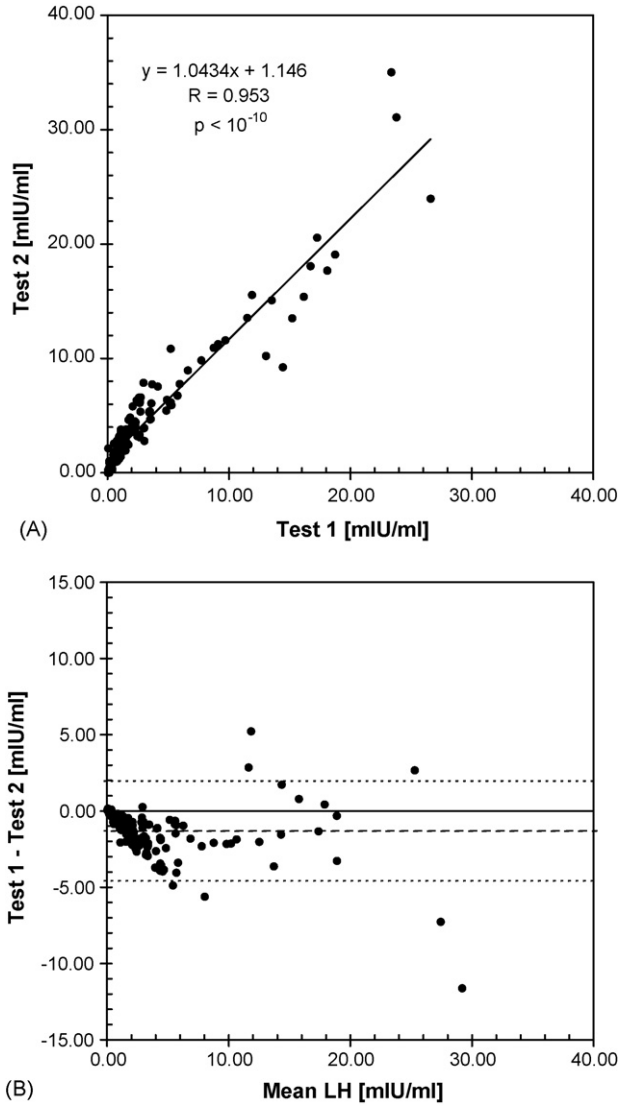

Fig. 2. (A) Inter-technique comparison of LH immunoassays ($n = 150$). (B) Bland–Altman representation is shown in 'B' with the dotted lines representing the 95% confidence limits and the mean difference between both tests.

the right to communicate results within 10 working days. This time delay is too long to guarantee optimal urinary LH results, specially when the time delay between the urine collection and the delivery to the anti-doping laboratory is long. So, it would be strongly recommended to perform LH measurements on receipt of urine samples and return the results in a short delay. In this way, the counter analyses could theoretically take place before LH deteriorates in urine.

These results also showed the lack of harmonization between both commercial immunoassays used for this validation. As mentioned, this suggests that both tests can be used for screening purposes, but in order that any athlete tested in any anti-doping laboratory around the World have the same chances of being tested negative/positive to LH in urine, a unique confirmation test should be performed (confirmation performed with a second antibody specific to an other epitope). As most anti-doping laboratories are spread out all over the planet, it is not conceivable that each laboratory gets equipped with a unique automatic

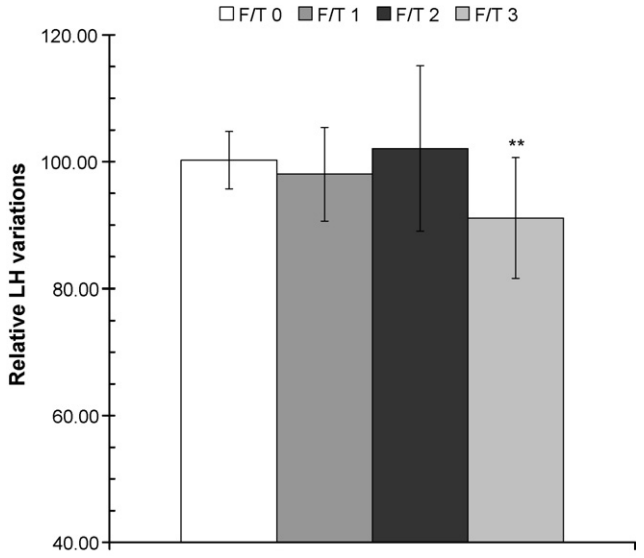

Fig. 3. Relative urinary LH variations following 1 (F/T1), 2 (F/T2) or 3 (F/T3) freezing and thawing cycles (mean ± S.D.). All urine samples were measured three times consecutively with both analyzers. Variations were established according to initial data (F/T0). A significant level of $^{**}p < 0.01$ was considered.

analyzer. Therefore, the probable solution would be to use for confirmation purposes a unique test such as an ELISA test which can be easily implemented in any anti-doping laboratory.

Urinary LH is also commonly measured together with other steroid markers (epitestosterone, etiocholanolone, androsterone . . .) to determine whether sportsmen are abusing of exogenous testosterone [1,2,4,10]. Indeed, the ratio of testosterone glucuronide to LH (T/LH ratio) in urine is a valuable criterion in detecting the administration of testosterone. It is well known that serum LH concentration will be increased upon testosterone

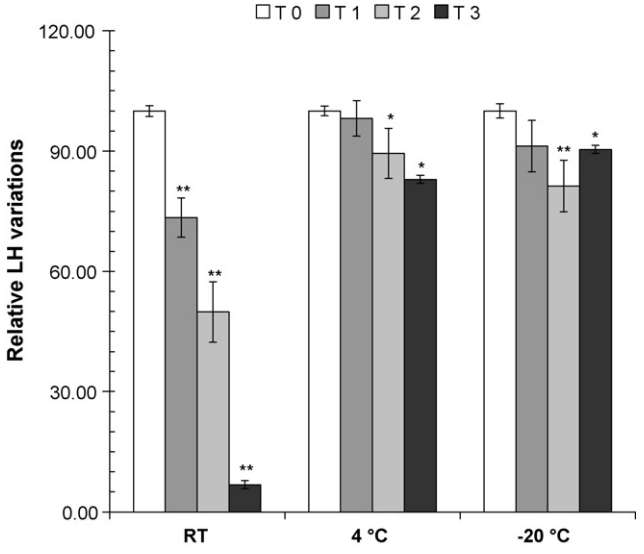

Fig. 4. Relative urinary LH variations over time (T1 = 5 days, T2 = 10 days, T3 = 90 days) according to temperature storage conditions (RT = room temperature; 4 °C = 4 degrees Celsius; −20 °C = −20 degrees Celsius) (mean ± S.D.). All urine samples were measured three times consecutively with both analyzers. Variations were established according to initial data (T0). A significant level of $^{*}p < 0.05$ and $^{**}p < 0.01$ were considered.

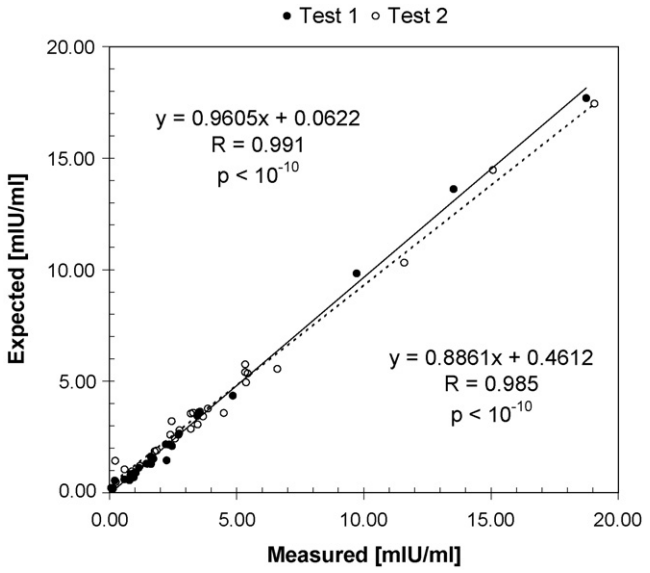

Fig. 5. Comparison of urinary LH data obtained with 30 urine samples pooled two by two (measured; same volume for each sample) and with the mean value of both samples measured individually (expected). All urine samples were selected on a random basis. Correlations for both tests were excellent (see coefficient of determination).

administration and may lead to an increase in urinary T/LH ratio [1]. Consequently, the determination of urinary LH has to be precise, exact and reproducible within different anti-doping laboratories. This is specially necessary in case of a longitudinal follow up (analyses performed in different laboratories) to find out whether a sportsmen has naturally an elevated urinary testosterone concentration [16]. In case of wrong urinary LH determination, an athlete could be suspected of manipulation while the origin of the abnormal concentration could be due to degradation of LH [6,8] or possibly a poor correlation between different immunoassays [18].

The 2005 World Anti-Doping Agency's (WADA) prohibited list included abnormal urinary LH concentration for male and female athletes [19]. The establishment a reference values for female athletes is extremely difficult as urinary LH concentration changes significantly during the time of ovulation. Previous publications clearly showed that urinary LH concentration can increase up to five times on the peak excretion day [15]. For that reason, most of the anti-doping laboratories probably did not declare adverse analytical findings for female urine samples, because the result were not relevant in front of a court. Elevated urinary LH could come from a potential abuse of recombinant LH but also from a disease or a possible cross-reaction with other hormones (e.g. HCG, TSH) in the immunoassay [11,14,17]. For that reason and according to our data during this evaluation, urinary LH should be measured only among male urine samples. Our validated method and our results clearly showed it is possible to save money by pooling urines samples two by two and nevertheless exclude a recent recombinant LH injection.

In conclusion, both tests evaluated during this study can be used for urinary LH determination and for longitudinal profiling of the T/LH ratio in urine samples of athletes provided that all analyses are conducted with either one or the other analyzer. On the other hand, due to the necessity of harmonization in the anti-doping field, counter analyses should preferably be conducted with the same test in the anti-doping laboratories so that all athletes are treated equally. This study also clearly showed that urinary LH is most probably under estimated in quite a few occasions due to bad pre-analytical conditions which leads to a degradation of LH in urine. For that reason, we strongly recommend to standardize transport conditions (monitor the temperature during transportation with a temperature below 10 °C, reduce the time delay between the urine collection and the delivery of the samples to the laboratory). In that way and by following usual guidelines of good laboratory practice, urine LH concentration should be determined properly.

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