RESEARCH ARTICLE

Reassessment of the Access Testosterone chemiluminescence assay and comparison with LC-MS method

Ruggero Dittadi¹ | Mara Matteucci² | Elisa Meneghetti¹ | Rudina Ndreu³

¹U.O.C. Laboratorio Analisi, Laboratory Medicine Department, Ospedale dell'Angelo, ULSS 3 Serenissima, Mestre, Italy

²Eureka srl - Lab Division, Chiaravalle (AN), Italy

³CNR Clinical Physiology Institute, Pisa, Italy

Correspondence

Ruggero Dittadi, Laboratory Analysis Unit, Department of Clinical Pathology, Ospedale dell'Angelo, ULSS 3 Serenissima, Mestre (Venice), Italy.

Email: ruggero.dittadi@aulss3.veneto.it

Aim of the study: To reassess the imprecision and Limit of Quantitation, to evaluate the cross-reaction with dehydroepiandrosterone-sulfate (DHEAS), the accuracy toward liquid chromatography-mass spectrometry (LC-MS) and the reference interval of the Access Testosterone method, performed by Dxl immunoassay platform (Beckman Coulter).

Material and Methods: Imprecision was evaluated testing six pool samples assayed in 20 different run using two reagents lots. The cross-reaction with DHEAS was studied both by a displacement curve and by spiking DHEAS standard in two serum samples with known amount of testosterone. The comparison with LC-MS was evaluated by Passing-Bablok analysis in 21 routine serum samples and 19 control samples from an External Quality Assurance (EQA) scheme. The reference interval was verified by an indirect estimation on 2445 male and 2838 female outpatients.

Results: The imprecision study showed a coefficient of variation (CV) between 2.7% and 34.7% for serum pools from 16.3 and 0.27 nmol/L. The value of Limit of Quantitation at 20% CV was 0.53 nmol/L. The DHEAS showed a cross-reaction of 0.0074%. A comparison with LC-MS showed a trend toward a slight underestimation of immunoassay vs LC-MS (Passing-Bablok equations: $Dxl = -0.24 + 0.906 LCMS$ in serum samples and $Dxl = -0.299 + 0.981 LCMS$ in EQA samples). The verification of reference interval showed a 2.5th-97.5th percentile distribution of 6.6-24.3 nmol/L for male over 14 years and <0.5-2.78 nmol/L for female subjects, in accord with the reference intervals reported by the manufacturer.

Conclusions: The Access Testosterone method could be considered an adequately reliable tool for the testosterone measurement.

KEYWORDS

cross-reaction, Dxl platform, immunoassay method, liquid chromatography-mass spectrometry, LoQ, testosterone

1 | INTRODUCTION

Testosterone is the principal androgen hormone, and is produced mainly in the male by the Leydig testis cells. It is also present in the female as intermediate metabolite of the estrogens synthesis and derived by androstenedione by peripheral conversion.¹

The testosterone determination can be used in the delayed puberty, in primary or secondary hypogonadism and in some female endocrine disorders, as hirsutism, virilization and/or in polycystic ovarian syndrome.²

It is worth noting that, so far, no reliable routine methods for direct determination of active testosterone free form are available,³ and it is to date suggested the estimate of bioavailable testosterone through total testosterone determination and the mainly binding protein, the Sex Hormone Binding Globulin.⁴ However, the commonly used immunometric assays for testosterone measurement could show limited sensitivity and accuracy at clinically relevant levels.

One of the reasons of these analytical drawbacks could be interferences from other steroid hormones. In particular,

dehydroepiandrosterone-sulfate (DHEAS), although structurally less similar to testosterone in respect to other steroids, could significantly cross-react with testosterone measurements due to its relatively high circulating concentrations, on average of three orders of magnitude greater than those of the testosterone.⁵

Aim of the study is to re-evaluate the technical characteristics of one automatic immunometric testosterone assays (Access Testosterone from Beckman-Coulter), in particular as for accuracy (in comparison with an LC-MS determination) and DHEAS cross-reaction, reported in Instruction for Use as "0.0%", with an approximation rate that could be misleading.

2 | MATERIALS AND METHODS

2.1 | Testosterone determination

Testosterone levels were assessed by the automated Access Testosterone assay (Beckman Coulter Inc., Fullerton, CA), a competitive binding immunometric assay performed by DxI 800 platform. The sample is added to a reaction vessel along with a Treatment Solution, that release the testosterone from the carrier proteins, mouse monoclonal anti-testosterone antibody, testosterone alkaline phosphatase conjugate, and paramagnetic particles coated with goat anti-mouse polyclonal antibody. Testosterone in the sample competes with the testosterone alkaline phosphatase conjugate for binding to the anti-testosterone monoclonal antibody. The antigen-antibody complexes are then bound to the solid phase by the capture antibody. After incubation, materials unbound to the solid phase are washed away, the chemiluminescent substrate Lumi-Phos[®] 530 is added to the vessel and light generated by the reaction is measured with a luminometer. The light production is inversely proportional to the concentration of testosterone in the sample.

2.2 | Imprecision

Imprecision at different concentration was evaluated testing six pool samples (S1, S2, S3, S4, S5 and S6) stored in aliquots at -20°C for a maximum of 30 days and assayed in 20 different runs using two different reagents lots, according to CLSI EP-5 protocol.⁶ Moreover, the pool from S1 to S5 were used to built an imprecision profile and derive a measure of LoQ at 20% of Coefficient of Variation, according to the CLSI EP17-A2 guideline.⁷

2.3 | Cross-reaction with Dehydroepiandrosterone-sulphate (DHEAS)

The cross-reaction with DHEAS was studied by a displacement curve, assaying with testosterone reagent two standard curves (testosterone and DHEAS), and evaluating the ratio between the concentration of the two analytes that causes the halving of the binding capacity respect to "zero" dose.⁸

An interfering solution was prepared dissolving 82 mg of DHEAS (Sigma ref D5297, MW 390.5) in 100 mL of a 1+1 mixture of ethanol absolute (Carlo Erba Reagents, ref. 414608) and deionized water,

mixed for 30 minutes at room temperature and stored at -20°C . The resulting 820 mg/L (2222 nmol/L) solution of DHEAS was used to prepare a standard curve of DHEAS (six concentrations from 4.48 to 94.8 nmol/L) to be assayed with testosterone assay and compared with a calibration curve of testosterone assayed in the same run.

Moreover, two female sera (T1 and T2), with measured testosterone value of 1.87 nmol/L (T1) and 0.73 nmol/L (T2) and with DHEAS concentrations of 3090 nmol/L and 1970 nmol/L, respectively, were added with the previously prepared solution of DHEAS, in order to prepare four samples each with a constant testosterone and increased DHEAS concentrations (about 4000-20 000 nmol/L). The values of the two hormones were then measured in the five samples.

2.4 | Liquid chromatography-mass spectrometry evaluation

Comparison with LC-MS assay was performed on 21 routine samples (median 3.47 nmol/L, range from 0.67 to 20.4 nmol/L), using Steroid Eureka kit and Agilent UHPLC 1290+Triple Quad 6460 ESI Jet Stream.

Briefly, 400 μL of human serum (calibrators, controls or samples) were crashed with 1000 μL of Deproteinization Reagent containing isotopic labeled internal standards, vortexed and centrifuged 10 minutes at 18600 g. 1200 μL of supernatant were transferred, dried and reconstituted with 100 μL of Reconstitution Reagent. 15 μL are injected into LC-MS/MS. Calibration curve is constructed using seven standards at different concentration, prepared in serum matrix.

Moreover, nineteen EQA Immunocheck samples (median 6.8 nmol/L, range from 0.8 to 23.0 nmol/L) among those distributed by QualiMedLab in the years 2014-2016, were analyzed in cooperation with ProBioQual (Centre Lyonnais d'études pour la Promotion de la Biologie et du controle de Qualité, Lyon, France) with mass spectrometry, and the corresponding values were compared with the results obtained by our laboratory during the EQA cycles.

Control materials were prepared by Randox Laboratories LTD (UK), following QualiMedLab/CNR and ProBioQual specification, from human serum not diluted, not heat-denaturated and not delipidized; no preservative substances were added to the control samples.⁹

2.5 | Indirect reference interval verification

The reference interval was verified by the indirect estimation, as previously reported.¹⁰

8967 records from outpatients testosterone routine determination from February 2009 to June 2016 were selected from the Laboratory Information System and subdivided into males aged over 14 years and females. Outliers, identified by using the iterative 4 SD rule, and data from subjects who had repeated measurements within the observation period were excluded.

2.6 | Statistical analysis

Performed with MedCalc © Software, Version 7.4.2.0 (MedCalc Software, Mariakerke, Belgium).

3 | RESULTS

3.1 | Analytical performances

Six pools results showed a coefficient of variation between 2.7% and 34.7% (Table 1).

From the results of the pools from S1 to S5 we built a precision profile, and by fitting a power function to the data we could derive a LoQ at 20% of imprecision of 0.53 nmol/L (Figure 1).

The evaluation of the interference, with standard curve of Testosterone and DHEAS both expressed as nmol/L, showed a cross-reaction of 0.0074% (Figure 2).

The regression between the expected and measured values of the two samples spiked with increasing concentrations of DHEAS were, respectively: $T1_{Testo}=0.000074 \text{ DHEAS}+1.72$ and $T2_{Testo}=0.000067 \text{ DHEAS}+0.59$, confirming the displacement study result.

In fact, the slope indicates a theoretical increase of 0.074 (T1) and 0.067 (T2) nmol/L of the measured testosterone for an addition of 1000 nmol/L of DHEAS. Therefore, only at DHEAS concentrations over about 7000 nmol/L can a significant increase of measured testosterone (>0.5 nmol/L) be found.

Comparison of the 21 routine samples with LC-MS showed a good correlation between the two methods. The Passing-Bablok equation was: $DxI=-0.24(-0.63/0.18)+0.906(0.805/0.985)$ LCMS, that show a relative underestimation of the immunometric assay (Figure 3A).

Also the comparison of the 19 EQA samples evaluated (Figure 3B) showed the results obtained by our laboratory well correlated but slightly lower than those obtained with LC-MS ($DxI=-0.299+0.981$ LCMS). A similar trend is also observed using the consensus mean of all laboratories using Access DxI method (data not shown).

3.2 | Verification of reference interval

Extracted results were subdivided into females and males over 14 years old. Outliers were removed from each group by using the iterative 4 SD rule. The group of males aged over 14 years showed a bimodal distribution, and the group with the lowest concentration (under 5.2 nmol/L) has been excluded. We found a 2.5-97.5 percentile distribution of 6.6-24.3 nmol/L for male over 14 years ($n=2445$) and <0.5-2.78 nmol/L for female subjects ($n=2838$). The 2.5-97.5 percentile range was calculated according Kairisto and Poola.¹⁰ These results are in accord with the reference intervals reported by the manufacturer (6.07-27.1 nmol/L for males and <2.6 nmol/L for females). The lowest concentration of the upper reference limit for males could be

TABLE 1 Imprecision of Access Testosterone (nmol/L) at different concentrations

	S1	S2	S3	S4	S5	S6
Mean (nmol/L)	0.27	0.70	1.07	3.50	4.87	16.30
SD (nmol/L)	0.09	0.14	0.10	0.14	0.17	0.44
CV %	34.70	20.10	9.24	4.14	3.45	2.71

FIGURE 1 Imprecision profile of the Testosterone method. The relationship between the Testosterone concentrations (on X axis) and the relative CV % values (on Y axis) were interpolated with a power function using 5 serum samples ($y=11.66 x^{-0.859}$, $r^2=0.973$). The dotted lines indicate the interpolation of the 20% CV on the abscissa, to identify the LoQ

FIGURE 2 Cross-reactivity between Testosterone and DHEAS. The standard curves of testosterone (filled squares) and DHEAS prepared in testosterone-free matrix (filled circles) were assayed with testosterone reagent and the results were reported as percentage of Bound/ B_0 . The cross-reactivity was defined as a percentage of the concentration of DHEAS at the point where the reduction in signal reach the 50% of the binding capacity (50% of the signal achieved in the absence of analyte), respect to the concentration of testosterone giving the same fall in signal. The concentrations are indicated by the arrows

in part due to the different age range of the subjects considered in our study (15-80 years, median 52) respect to the range reported by the manufacturer (18-66 years, median 41).

FIGURE 3 A, Passing-Bablok analysis between the Testosterone values measured by LC-MS (X axis) and Access immunoassay (Y axis) methods in 21 serum samples. B, Passing-Bablok analysis between the Testosterone values measured by LC-MS (X axis) and Access immunoassay (Y axis) methods in 19 EQA control samples. The dashed lines represent the confidence interval for the regression line. The dotted lines represent the identity line ($x=y$)

4 | DISCUSSION

Our study confirms adequate analytical performances of Access Testosterone assay, in terms of precision, sensitivity, and accuracy. We confirm the DHEAS interference, which resulted of a similar magnitude to that previously found.⁵ Due to tested cross-reactivity, in typical health adult male the increase of testosterone concentration can be <10% even with very high DHEAS amounts. In prepubescent children or females, conversely, DHEAS presence should be considered, as apparent and clinically relevant testosterone increase could be found in case of extremely high DHEAS levels. However, also given the nonconstant trend of the displacement curve, it can be calculated

that under 7000 nmol/L of DHEAS the interference extension could be negligible for the majority of the clinical needs.

LC-MS method comparison gives overall data well correlated, at least within the limits of our evaluation. This finding is in accord with other recent studies,^{11,12} that, using different immunometric platforms, report both analytically and clinically acceptable correlation with mass-spectrometry methods, although at low concentrations the relationships are weaker and more scattered. However, considering the interferences from other steroid hormones that affect the immunoassays testosterone determination, the relative underestimation found in this paper in comparison with LC-MS is unexpected. The comparison was evaluated also in a subset of samples used in the framework of the Immunocheck EQAS program, and for which a verification with LC-MS was carried out. This comparison confirmed a trend toward the underestimation of the immunometric method vs LC-MS, although more limited. The comparison with the consensus mean achieved during each cycle of Immunocheck EQAS for the Access Testosterone method within 2 years time-span shows that this trend is not related to an inaccuracy of our analytical system, or to a short term bias (eg, due to a particular reagent lot).

The verification of reference interval by the indirect method is particularly useful for the “screening” laboratory tests, for which the health-related samples could be considered the major part of the total distribution. Although testosterone cannot be considered actually a “screening like” analysis, the exclusion of the individuals with repeated tests in a wide span of time and the use only of outpatients could give a reasonable confidence that most of the results should actually have originated from healthy subjects. The results found supported the presently used reference intervals, although the wide span of male population age routinely evaluated in our laboratory could produce the reduction of the upper reference limit in comparison with that reported by the manufacturer.

In conclusion, the Access Testosterone method could be considered an adequately reliable tool for the testosterone measurement. The interference from DHEAS, although it must be taken into account, does not seem to overly influence the determination.

ACKNOWLEDGMENTS

All the immunoassay kits used in this study were kindly supported by Beckman Coulter S.P.A Italy (Cassina de' Pecchi, Milan, Italy).

REFERENCES

1. Ghayee HK, Auchus RJ. Basic concepts and recent developments in human steroid hormones biosynthesis. *Rev Endocr Metab Disord*. 2007;8:289-300.
2. Larsen PR, Kronenberg HM, Melmed S, Polonsky KS (eds). *William Textbook of Endocrinology* 10th edn. Philadelphia: Elsevier Saunders; 2003.
3. Winters SJ, Kelley DE, Goodpaster B. The analog free testosterone assay: are the results in men clinically useful? *Clin Chem*. 1998;44:2178-2182.
4. Vermeulen A, Verdonck L, Kaufman J. A critical evaluation of simple methods for the estimation of free testosterone in serum. *J Clin Endocrinol Metab*. 1999;84:3666-3672.

5. Middle JG. Dehydroepiandrosterone sulphate interferes in many direct immunoassays for testosterone. *Ann Clin Biochem.* 2007;44:173-177.
6. CLSI EP5-A2. Evaluation of precision performance of quantitative measurement methods; approved guideline. 2004, Vol. 24, No. 25. Wayne, Pennsylvania.
7. CLSI EP17-A2. Evaluation of detection capability for clinical laboratory measurement procedures; approved guideline. 2012, Vol. 32, No. 8. Wayne, Pennsylvania.
8. Wild D, ed. *The immunoassay handbook*, 3rd edn. Oxford: Elsevier Ltd; 2005.
9. Giovannini S, Zucchelli GC, Iervasi G, et al. Multicentre comparison of free thyroid hormones immunoassays: the Immunocheck study. *Clin Chem Lab Med.* 2011;49:1669-1676.
10. Kairisto V, Poola A. Software for illustrative presentation of basic clinical characteristics of laboratory tests - GraphROC for Windows. *Scand J Clin Lab Invest.* 1995;55(Suppl. 222):43-60.
11. Bui HN, Sluss PM, Blincko S, Knol DL, Blankenstein MA, Heijboer A. Dynamics of serumtestosterone during the menstrual cycle evaluated by daily measurements with an ID-LC-MS/MS method and a 2nd generation automated immunoassay. *Steroids.* 2013;78:96-101.
12. Knudsen CS, Hoejskov CS, Moller HJ, Nexø E. Testosterone Measured with an Automatic Immunoassay Compares Reasonably Well to Results Obtained by LC-MS/MS. *Clin Lab.* 2016;62:2039-2043.

How to cite this article: Dittadi R, Matteucci M, Meneghetti E, Ndreu R. Reassessment of the Access Testosterone chemiluminescence assay and comparison with LC-MS method. *J Clin Lab Anal.* 2018;32:e22286. <https://doi.org/10.1002/jcla.22286>