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Homologous ELISA for detection of 17 α -methyltestosterone in serum

Divya Verma

Department of Reproductive Biomedicine, National Institute of Health and Family Welfare, Munirka, New Delhi, India | Centre for Interdisciplinary research in basic sciences, Jamia Millia Islamia, Jamia Nagar, New Delhi, India

T. G. Shrivastav

Department of Reproductive Biomedicine, National Institute of Health and Family Welfare, Munirka, New Delhi, India

*Corresponding author email: drtg.srivastav@gmail.com

Sonu Chand Thakur

Centre for Interdisciplinary research in basic sciences, Jamia Millia Islamia, Jamia Nagar, New Delhi, India

*Corresponding author email: sthakur@jmi.ac.in

Abstract--17 α Methyl testosterone is a synthetic steroid hormone having androgenic properties. It is used in aquaculture, medical treatments, in sports as doping drugs, in improving food efficacy but its residue in meat and their products can cause risk to the human, animals and environment. In this article, we have focused on developing homologous ELISA using 17 α Methyl testosterone-3-Carboxymethyloxime-Bovine serum albumin antiserum and 17 α Methyl testosterone-3-Carboxymethyloxime-Horseradish peroxidase enzyme conjugate. Immunogen and enzyme conjugate both were prepared by N-hydroxysuccinimide mediated carbodiimide reaction method. The sensitivity, affinity and ED₅₀ of the assay were found to be 0.11 ng/ml, 0.02 \times 10⁻⁸ L/mol and 5.78 ng/ml respectively. The cross reactivity for this assay combination was seen with eight steroids out of 59 structurally related steroids. The assay was then studied for analytical variables like recovery (94.8%-111.6%), precision (Inter and Intra-assay coefficient of variation less than 10%), correlation (R²= 0.96) by comparing with commercially available kit and further validated by measuring levels of 17 α methyltestosterone in rat serum after injecting rats intramuscularly.

Keywords--ELISA, 17 α -methyltestosterone, homologous assay, Bovine serum albumin, Horse radish peroxidase.

Introduction

Anabolic steroids having androgenic properties are the primary male sex hormones. These are known to promote muscle growth and develop male sexual characters (Khalil et al., 2011). One such androgenic steroid is 17 α -methyl testosterone which was synthesized in 1935. Its IUPAC name is *17 α -methylandrosta-4-en-17 β -ol-3-one*, Molecular weight is 302.45 g/mol and chemical formula is C₂₀H₃₀O₂. The methyl group at 17 α position decreases its systemic metabolism, extending its half-life, prolonged stability and making it orally active (Nieschlag et al., 2004). The permissible limit for 17 α -methyl testosterone in muscles is 1 μ g/kg by European commission (European Commission, 2003). The half-life of the steroid is approximately 3 hours and duration of action of the drug is 1-3 days. 17 α -methyltestosterone is commonly used by aqua-culturists because it is capable for masculinizing fries of various fish species and culturing ornamental fishes that are in more demand (Mousavi-Sabet & Ghasemnezhad, 2013; Orose & Vincent-Akpu, 2016). It is used in three main areas- as veterinary medicine (clinical therapy in farm animals), in sports (physique and performance-enhancing purposes by competitive athletes) and medical purposes like treating hypogonadism, delayed puberty and menopausal symptoms (Rivero-Wendt et al., 2014). The drug is also used in women to treat breast cancer, breast pain in non-breastfeeding females, excessive lactation, migraine, dysmenorrhea and osteoporosis. However, anabolic steroid residues in meat and their products cause risk to the human health such as heart palpitations, endocrine disorders, chromosomal aberrations and reproductive toxicity (Khincha et al., 2014). High doses of 17 α -methyltestosterone can cause increase in blood pressure, hepatotoxicity, polycythemia, prostate cancer, gynaecomastia, behavioral changes like aggression, forgetfulness, depression, even suicidal tendencies and delusions. 17 α -MT can cause damaging biological effects even at low concentration i.e. 1-100 ng/L (Zheng et al., 2014). So, there is a need for sensitive and rapid analytical technique to validate the presence of 17 α -methyltestosterone in biological fluids, tissues of animals and as doping drug in sports.

Several important methods and techniques are currently used for the detection of 17 α -methyltestosterone in biological fluids and tissues. Physical methods presently available are High resolution orbitrap MS (Vanhaecke et al., 2013), LC-MS (Gonzalo-Lumbreras & Izquierdo-Hornillos, 2003), LC-MS/MS (Chu et al., 2006; Kaklamanos et al., 2007) LC-MS combined with online turbulent flow extraction (Du Shin et al., 2017), LC-HRMS (Tudela et al., 2015); HPLC (Cravedi & Delous, 1991; Han et al., 2012; Marwah et al., 2005; Shi et al., 2008), HPLC-MS/MS (Regal et al., 2010), Ultra-HPLC with Orbitrap-high resolution MS (López-García et al., 2018); HPLC combined with RIA (Daeseleire et al., 1991), HPLC-UV (Li et al., 2020), GC-MS (Bi & Massé, 1992; Shinohara et al., 1985; Zakhari et al., 1991; Zeng et al., 2010), GC-MS/MS (Wong et al., 2017), GCMS-MIPFs-OCDD-LVI (Zhong et al., 2013), Gas chromatography microchip APPI-MS/MS (Hintikka et al., 2013), hollow fiber solvent-stir bar micro extraction (HF-SSBME) device (Liu et al., 2012); SFE-SPE combined with GC-MS (Stolker et al., 1999); New mixed surfactant MEKC method (Zhang et al., 2009); Fluorescence modulation method (Haynes & Levine, 2020), Arrayed based sensing (Gill et al., 2019) and Thin layer chromatography (Musharraf et al., 2017) for the analysis of 17 α -methyltestosterone. Immunological methods such as RIA (Hampl et al., 1978),

CLIA (Van Peteghem et al., 1989); (Jansen et al., 1985; Xie et al., 2005), ELISA (H. Gao et al., 2021; Huml et al., 2020; Kong et al., 2015; Lu et al., 2006; Risto et al., 2013) and LFIA (Holubová et al., 2021; Kong et al., 2015) are presently used to detect the permissible limit of the steroid. Electrochemical method like Biosensors (Conneely et al., 2007) and Capillary Electrophoresis (Qi et al., 2008) are also used to evaluate the risks associated with high dose of 17 α -methyltestosterone. Due to extensive process, they cannot be used as a screening method for the detection of hormone. Therefore, in this study, a specific, sensitive, reliable, accurate and low cost ELISA has been developed to determine the level of 17 α -methyltestosterone in food, biological fluids, and animal tissues and as doping drug in sports.

Materials and Methods

Institutional Animal Ethics Committee (IAEC) of The NIHFV, New Delhi and CPCSEA approval and guidelines were followed for carrying out the protocols.

Materials

- **Chemicals, salts and solvents** of pure analytical grade have been used for this study were purchased from Sigma chemical company, Missouri, United States and Merck (India) Pvt. Ltd. All steroids used for conjugation, preparation of immunogen, preparation of enzyme conjugate and for cross-reaction were procured from Steraloids Inc., Newport, USA.

- **Instruments**

Thermo ScientificTM Evolution 220 Double beam UV-Visible Spectrophotometer (Thermo Fisher Scientific, USA), Harrison lyophilizer (Harrison Scientific Instruments Co., New Delhi, India), Tecan Infinite M200 Pro ELISA plate reader (Tecan Group Ltd., Mannedorf, Switzerland), Sigma[®] 3-30 KS Centrifuge (Sigma Labor zentrifugen GmbH, Germany).

Methods

Buffers

The buffers used in this study are: 10 mM Coating Buffer (PBS), Enzyme Conjugate dilution buffer, Antibody dilution buffer, Blocking buffer, Washing buffer (PBS), 50 mM TMB Buffer, 1N HCl (Stop solution).

Preparation and characterization of immunogen (17 α -methyltestosterone-3-CMO-BSA)

17 α MT-3-CMO was attached to BSA by N-hydroxy succinimide ester method with the modifications previously described by D. Kumar (Kumar et al., 2018). 17 α MT-3-CMO (10 mg) was dissolved in 400 μ l of dioxan and 400 μ l of dimethyl formamide. To this solution, addition of 0.1 ml water, 20mg NHS and 40 mg EDAC was done. Then, it was vortexed and kept overnight for activation at 4 ° C. The activated mixture was supplemented slowly to the BSA solution (1mg/0.3ml), mixed and kept overnight at 4 ° C. Conjugated 17 α MT-3-CMO-BSA was then subjected to dialysis with de-ionized water and its characterization was done by

UV spectrophotometer. After that, lyophilization was done and stored at 4 ° C in small fractions (1 mg) for immunizing rabbits.

Characterization of immunogen 17 α MT-3-CMO-BSA by Ultraviolet (UV)-visible Spectrophotometry

0.25 mg of 17 α MT-3-CMO and 17 α MT were dissolved separately in 1 ml of ethanol, 1 mg/ml BSA and 17 α MT-3-CMO-BSA was prepared. Then, the absorption spectra from 235 nm to 295 nm of 17 α MT, 17 α MT-3-CMO, BSA and 17 α MT-3-CMO-BSA were taken by using UV-Visible spectrophotometer (Evolution 220).

Measurement of hapten number by ELISA method in 17 α MT-3-CMO-BSA immunogen

10 μ L of 17 α MT-3-CMO-BSA immunogen was dispensed in duplicate to 17 α MT-3-CMO-BSA Ab coated wells along with 17 α MT standards (0-40 ng/ml). Subsequently, 50 μ L of enzyme conjugate 17 α MT-3-CMO-HRP was added in every well and incubation was given at 37 °C for 1 hr. After washing, 100 μ L of the substrate (TMB/H₂O₂) was added in all the wells and incubated for 15 min at RT in dark and 100 μ L HCl (1 N) was added to each well to stop the reaction. The absorbance was taken at 450 nm with ELISA plate reader.

Immunization with immunogen (17 α MT-3-CMO-BSA) in New Zealand white rabbits

An emulsion was prepared freshly using 500 μ L of Freund's complete adjuvant in 500 μ L of saline having 1 mg of 17 α MT-3-CMO-BSA immunogen. New Zealand white rabbit was injected intramuscularly (limbs) with 250 μ L of the prepared emulsion. This procedure of immunization with 17 α MT-3-CMO-BSA was repeated on day 7, 14, 21 and 28. After these five primary injections, the booster was given in Freund's incomplete adjuvant every 30 days for the whole study duration i.e. two years.

Collection of antiserum, Serum from non-immunized rabbits and preparation of NRS

After booster injection, blood was collected on a regular basis between 10th to 14th days counting from the 1st booster injection. New Zealand white rabbits that were non-immunized were used for NRS collection. Secondary antibody i.e. Anti-rabbit gamma globulin was generated by immunization in the goat using purified Rabbit-IgG (as immunogen) following the method reported by Shrivastav (Shrivastav, 2004). Samples were centrifuged at 5000 rpm; 10 minutes and kept in small fractions at -30 °C.

Preparation of enzyme conjugate (17 α MT-3-CMO-HRP)

17 α MT-3-CMO was attached to HRP by NHS ester method with modifications in previously reported method (Dutta et al., 2018). Addition of 0.2 ml each of dioxan and DMF to 5 mg of steroid was done. To the above solution, 100 μ L water, 10 mg

NHS and 20 mg EDAC were added. Activated steroid solution was added to 1 mg/ml HRP solution and stored at 4 °C for 24 hours. After that, resultant mixture was loaded and passed through already equilibrated 'G-25 column' with 10 mM PBS containing 0.1 % thimerosal. The brown colored solution was then preserved by adding an equal volume of ethylene glycol and 1% of sucrose, ammonium sulfate and BSA according to the final volume. Then, HRP conjugate was stored at -30° C in small tubes for further use.

Checkerboard Assay

The Checkerboard for homologous assay was done to determine the optimum dilution using 17 α MT-3-CMO-BSA antiserum and 17 α MT-3-CMO-HRP enzyme conjugate with least NSB for further analysis like displacement assay and validations like sensitivity, affinity and recovery, precision. Coating was done using immunobridge immobilization technique as described by Shrivastav and colleagues (Shrivastav et al., 2003).

Checkerboard assay procedure for determination of optimal loading

In order to determine the immunoreactivity, 100 μ l of 17 α MT-3-CMO-HRP diluted serially in enzyme conjugate buffer i.e., 1:500, 1:1000, 1:2000 and 1:4000 in wells (one dilution in duplicate vertically) was dispensed and incubation was given at 25°C for 1 hour. After incubation, the content was decanted and wells were washed with washing buffer 15-20 times. Then, 100 μ l of substrate solution "TMB/H₂O₂" was added to the wells for measuring bound enzyme activity and incubated for 15-20 minutes at RT. 100 μ l of 1 N HCl (stop solution) was added to stop the reaction and absorbance was taken at 450 nm.

Preparation of standard dilutions of 17 α -methyltestosterone in buffer and assay protocol for displacement assay

To carry out standard displacement assay, working standards with different concentrations of 17 α -methyl testosterone (0, 0.625, 1.25, 2.5, 5, 10, 20 and 40 ng/ml) were prepared in tris base buffer-pH 7.4 having 0.5 % glycerol as stabilizer and 10% charcoal stripped serum. First, 100 μ L standards of 17 α MT (0-40 ng/ml) along with rat serum samples (in duplicate) were added in the wells of microtitre plate coated with 17 α MT-3-CMO-BSA Ab. Then, 50 μ L of 17 α MT-3-CMO-HRP was added in every well. Rest of the steps was repeated same as of checkerboard assay. The illustrative picture of the developed assay in this study is shown in Figure 1 below.

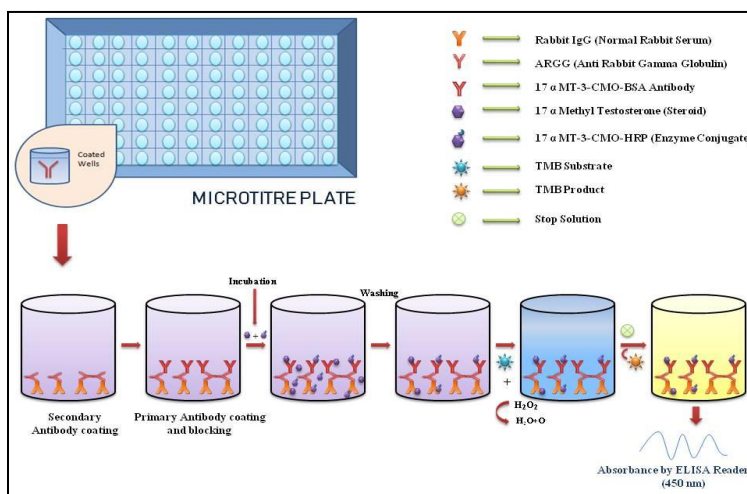


Figure 1. Diagrammatic Illustration of competitive ELISA step by step

Preparation of working standards/pools of 17 α -methyltestosterone in serum for recovery and precision

Five working standards of 17 α -MT in charcoal treated stripped serum were prepared for recovery with concentrations of 0, 0.625, 1.25, 2.5 and 5 ng/ml. The serum samples with similar 17 α -MT concentrations were pooled and stored in different containers of each concentration for repeating the assay number of times.

Preparation of working standards and samples of developed kit and available kit for Correlation coefficient

Five working standards of 17 α -MT in charcoal treated stripped serum were prepared with concentrations of 0, 0.625, 1.25, 2.5, 5, 10, 20, 40 and 80 ng/ml for estimating the 17 α -MT level by our developed ELISA. The working standards were prepared as per given instructions on the leaflet of commercially available kit by *Sincere Biotech Co. Ltd. Beijing, China*. The stored rat serum samples were thawed and labeled before use. The correlation coefficient for 17 α MT values were calculated by developed ELISA with an already available ELISA kit in 80 serum samples and regression graph was plotted.

Dosing of 17 α -methyl testosterone in rats and sample collection

Adult rats have been given different doses of 17 α MT (injectable form) intramuscularly once according to the method published by P. Dutta et al. Six groups having vehicle control and 5 different doses of 17 α MT variants (0.1 mg, 0.2 mg, 0.4 mg, 0.8 mg and 1.6 mg) according to their body weight (mg/Kg). Then, 250 μ L of blood samples from tail vein will be collected at regular intervals i.e. 0 hr (pre-injection), 6 hrs, 24 hrs, 6th day, then after 1 week and at every 15th day till the study period (60 Days). The sample was centrifuged; serum was separated and stored at -30°C. Dosing in rats were done to check the levels of the

steroid 'in-house' by the developed kit at different time intervals and to check the peak time/level of steroid in the body.

Measurement of serum level of 17 α MT by the developed ELISA

Concentration of 17 α -MT in rat serum was measured by the developed ELISA using the developed 'IMMUNOCAL' computer program in the lab.

Analysis of Data

Dose Response Curve for homologous assay of 17 α -methyltestosterone

The composite dose response curve was prepared for homologous assay of 17 α -methyltestosterone using Microsoft Excel.

Standardization and Validation of homologous assay of 17 α -methyltestosterone

The assay standardization and analytical validation of homologous assay was done by calculating sensitivity, affinity, ED₅₀ and specificity in terms of cross reaction (Abraham, 1969; Rodbard, 1974). Concentrations of unknown samples in recovery and precision were calculated by in house developed 'IMMUNOCAL' QBASIC language program.

Statistical Analysis

Statistical analysis of the data which include average (mean), standard deviation, Coefficient of variation, correlation coefficient and level measurement of 17 α -methyltestosterone in serum were determined through 'MS Excel' and graphs were plotted with the help of software *Graph pad prism 8*.

Results

Characterization of Steroid-BSA conjugate by UV spectroscopy

The absorbance spectrum of BSA, steroid, steroid derivative and Steroid-BSA conjugate was taken from 235-295 nm to establish that 17 α MT-3-CMO was coupled to BSA (Figure 2 shown below). The characteristic absorbance spectra of each sample are shown in the above graph. The absorbance maxima of BSA was at 280 nm, steroid (17 α MT) was 243 nm, steroid derivative (17 α MT-3-CMO) was at 249 nm and Immunogen (17 α MT-3-CMO-BSA) was at 253 nm and confirmed that BSA has been conjugated with steroid and its derivative.

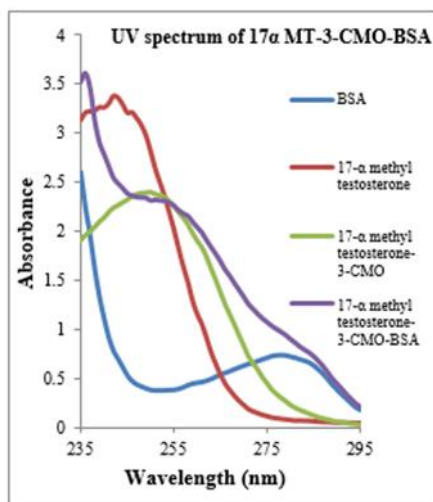


Figure 2. Absorbance spectra of 17 α MT-3-CMO-BSA against BSA, steroid and steroid derivative

Hapten analysis in immunogen 17 α MT-3-CMO-BSA by ELISA method

This is also a method which is used for confirming that steroid and its derivative has been conjugated to the BSA (protein). The amount of 17 α MT conjugated to the immunogen 17 α MT-3-CMO-BSA was estimated to be 15.3 μ g of 17 α MT per mg of immunogen.

Interpretation of checkerboard assay

The optimum dilution of antiserum and enzyme conjugate with highest binding and least NSB (non-specific binding) has been selected for analysis and assay validations. It was found that 1:500 dilution of 17 α MT-3-CMO-BSA antiserum showed appreciable binding and negligible NSB with 1:4000 enzyme conjugate 17 α MT-3-CMO-HRP dilution.

Dose Response Curve for homologous assay of 17 α -methyltestosterone

Fig. 3 gives Composite dose-response curve of the homologous ELISA of 17 α MT using 17 α MT-3-CMO-BSA antiserum and enzyme conjugate 17 α MT-3-CMO-HRP. The increasing concentrations of 17 α MT (ng/ml) were plotted on X-axis as their log values against A/A₀ ratio of standards on the Y-axis using Microsoft Excel. The CV% for A/A₀ ratio of each standard for the homologous assay was ranged from 1.10% to 4.49%.

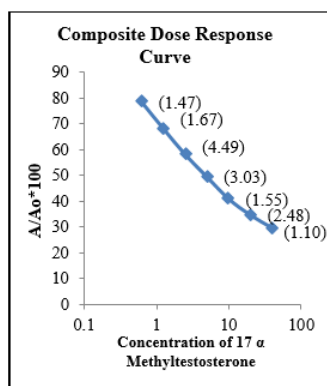


Figure 3. Composite dose-response curve of homologous ELISA of 17α Methyl Testosterone using 17α MT-3-CMO-BSA antibody with 17α MT-3-CMO-HRP enzyme conjugate. Each value is a mean ± SD of 8 assays (In duplicate). The coefficient of variation at each concentration is shown in parentheses

Table 1
Slope, Intercept, Analytical Sensitivity, Affinity and ED₅₀ of homologous assay

Assay Combination	Optimum Dilutions	Slope and Intercept		Sensitivity (ng/ml)	ED ₅₀ (ng/ml)	Affinity (L/mol)
		m	c			
17 α MT-3-CMO-BSA 17 α MT-3-CMO-HRP	Ab.- 1:500 HRP- 1:4000	-1.19	0.90	0.11	5.78	0.02x10 ⁻⁸

Specificity

The specificity of the anti-17 α MT-3-CMO-BSA antiserum in terms of cross reaction % was checked with 59 commercially obtained analogous steroids which belong to C18, C19, C20, C21, and C27 group mentioned in Table 2. The formula for calculating the percentage cross-reaction is:

$$\% \text{Cross - reactivity} = \frac{\text{Concentration of standard at } 50\% \frac{A}{A_0} (S)}{\text{Concentration of cross - reactant at } 50\% \frac{A}{A_0} (C)} \times 100$$

Table 2

Percentage cross reaction of homologous assay combination of 17 α MT-3-CMO-BSA and enzyme conjugate 17 α MT-3-CMO-HRP shown only with 8 analogous steroids out of commercially available 59 steroids

Assay combinations	Steroids	% Cross Reaction
17 α MT-3-CMO-BSA with 17 α MT-3-CMO-HRP	Danazol	25.14%
	Testosterone	38.3%
	Progesterone	6.38%
	Androstenedione	3.52%
	Androstenediol	19.16%
	6-hydrotestosterone	43.75%
	Nandrolone	19%

Validation of homologous assay

The validation of developed immunoassay for 17 α MT was done by measuring its analytical recovery, precision and correlation coefficient.

Recovery

Recovery is the capability to measure accuracy of the assay developed. The recovery percentage formula is as follows:

$$\text{Recovery\%} = \frac{(C-B)}{A} \times 100$$

Where, A- known concentration, **B-** basal **C-** observed total concentration

Recovery for homologous assay of 17 α -methyl testosterone using antibody against 17 α MT-3-CMO-BSA and 17 α MT-3-CMO-HRP enzyme conjugate of 17 α MT standards in spiked serum pools [shown in Table 3 (a)].

Table 3

(a): Recovery of 17 α MT from spiked serum pools using 17 α MT-3-CMO-BSA Ab and 17 α MT-3-CMO-HRP enzyme conjugate (Homologous assay)

Serum pools	Added conc. of 17 α MT (ng/ml)	Observed conc. of 17 α MT (ng/ml)	Expected conc. of 17 α MT (ng/ml)	Recovery percentage
Pool 1 (Basal)	-	1.19	-	-
Pool 2	0.625	2.02	1.81	111.6
Pool 3	1.25	2.37	2.44	97.13
Pool 4	2.5	3.68	3.69	99.72
Pool 5	5	5.87	6.19	94.83

Precision

Precision can be regarded as reproducibility. The coefficient of variation % is

calculated using the following formula [Refer Table 3 (b)] –

$$CV\% = S. D. / \text{mean} \times 100$$

Table 3

(b): Inter and intra-assay coefficient of variation for the measurement of 17 α MT in Serum pools using 17 α MT-3-CMO-BSA Ab and 17 α MT-3-CMO-HRP enzyme conjugate

Variation	Mean \pm Standard deviation	CV (Coefficient of variation) %	Variation	Mean \pm Standard deviation	CV (Coefficient of variation) %
Intra-assay $n = 8$	1.19 \pm 0.10	8.60	Inter-assay $N= 8$	0.66 \pm 0.03	5.90
	2.02 \pm 0.11	5.83		1.33 \pm 0.04	3.03
	2.37 \pm 0.22	9.44		2.07 \pm 0.04	2.18
	3.68 \pm 0.35	9.63		3.1 \pm 0.02	0.68
	5.87 \pm 0.36	6.18		5.11 \pm 0.01	0.24

n = No. of times same sample tested within the assay; N = No. of times whole assay repeated.

Correlation coefficient and Regression Graph of 17 α MT homologous assay

A recently developed method required to produce the same results when compared with other available methods that are clinically validated and reliable. A graph was plotted for both methods opposite to one another by similar axes, accompanied by the intersecting line which measures correlation between the two methods. The correlation coefficient (R^2) generally estimates linear regression. The correlation coefficient for 17 α MT values calculated by developed ELISA with an already available ELISA kit by *Sincere Biotech Co. Ltd. Beijing, China* in serum samples ($n=80$) was found to be $R^2 = 0.96$ (Figure 4 given below).

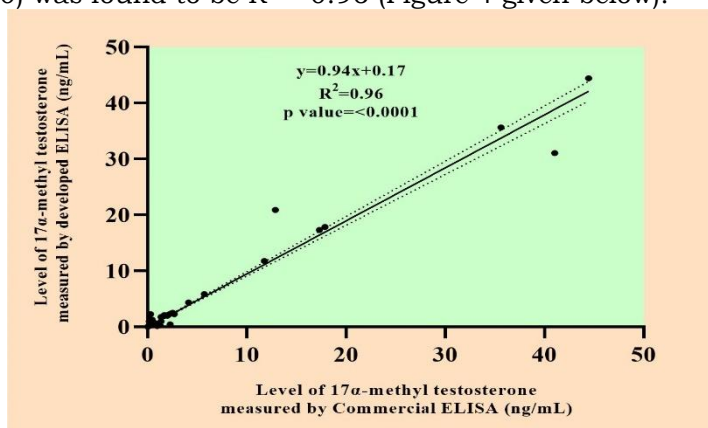


Figure 4. Regression curve of correlation for estimated 17 α MT concentrations in serum using the developed ELISA and available commercially ELISA kit

Determination of 17 α MT levels in the rat serum measured by in-house developed ELISA

The levels of 17 α -MT in the collected rat serum samples were measured by the developed ELISA using 'IMMUNOCAL' software program. The graph was plotted against concentration of 17 α -methyl testosterone (ng/ml) vs. time (in hours and days) shown in Figure 5.

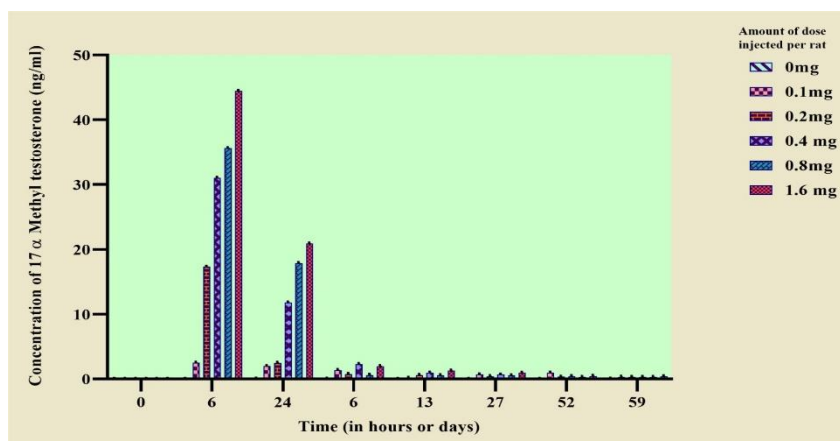


Figure 5. 17 α MT levels in rat serum measured by the developed homologous ELISA at different durations (prior to injections at 0 hr and after injection at 6 hr, 24 hr, 6th day, 13th day, 27th day, 52nd day and 59th day)

Discussion

Enzyme immunoassay is very well known technique in the clinical applications as it is simple, compact, gives fast and accurate results. Enzyme-linked immunosorbent assay (ELISAs) and enzyme immunoassays (EIA) have been the standard diagnostic tool in medical areas, biotechnology and for the study of biomolecules. In ELISAs, antigen-antibody interactions are seen and the analyte is detected by an enzyme system (Chaudhari et al., 2014). There are several techniques currently available for the detection of 17 α -methyl testosterone. LC-MS, GC-MS and HPLC need efforts, time taking, sample processing, extraction in various steps, requires more sample (1-2 ml), costly instruments and trained staff but less throughput. RIA is very sensitive technique but requires license, hazardous to health due to use of radioactive materials and needs proper disposal of materials after use. CLIA, LFIA needs extensive sample processing, costly raw materials, only gives information qualitatively and is time taking. Biosensors also require costly materials.

In this work, direct competitive ELISA of 17 α -methyl testosterone using antibody against 17 α MT-3-CMO-BSA immunogen and 17 α MT-3-CMO-HRP enzyme conjugate has been developed to quantify 17 α MT in the serum directly. In this, absence/lesser color indicate that more the antigen is present in the sample. Competitive ELISA is highly sensitive technique because presence of even small amounts of antibody can be detected (Chaudhari et al., 2014). There has been no ELISA development reported for the detection of 17 α -methyltestosterone in India

till date. However, antigen based indirect competitive ELISAs have been developed in Ireland, China and Czech Republic (Prague) (H. Gao et al., 2021; Huml et al., 2020; Kong et al., 2015; Lu et al., 2006) but they have some limitations like every time antibody will be required to generate for the assay which is time consuming and variations are also likely to occur in binding of the enzyme conjugate. The sensitivity (least detectable dose) and ED₅₀ of our developed assay were calculated as 0.11 ng/ml and 5.78 ng/ml respectively. Affinity of anti-17 α MT antiserum to 17 α MT was 0.02x10⁻⁸ L/mol.

The assay showed cross reaction with only eight commercially available analogous steroids (Testosterone- 38.3%, Danazol- 25.14%, Androstenedione- 3.52%, Androstenediol- 19.16%, Nandrolone- 19%, 17α-dimethyltestosterone- 2%, Metandienone- 5% and 6-hydrotestosterone -43.75%). In a homologous immunoassay for the steroid detection, protein conjugated with steroid to produce the antibody and enzyme-conjugated steroid derivative uses the same hapten. The antibody has better affinity for enzyme conjugate as compared to steroid alone. The conjugation chemistry implicated in the preparation of immunogen and its structure, antigen-antibody bounded affinity interactions determines the specificity and sensitivity of an immunoassay. The shape and complementarities between the structurally related steroids and antibody-binding segment gives the cross reaction pattern (Nara et al., 2008). The recovery percentage calculated by adding known amount of 17 α MT to the serum pools along with working standards were in the range from 94.83% to 111.6%. Inter and intra assay CV% (n=8) were less than 10. The correlation coefficient of the assay when compared with available commercial kit of 17 α MT was 0.96.

Our developed ELISA technique was also validated by estimating the level of 17 α MT in the rat serum after giving doses (0.1, 0.2, 0.4, 0.8 and 1.6 mg/kg) to the rats. The level of 17 α MT increases after 6 hours for different doses i.e. 2.52 ng/ml- 44.45 ng/ml and then started decreasing after 24 hours i.e. in the range of 1.98 ng/ml -20.88 ng/ml. Then, the levels declines slowly till the study period of 60 days. On 59th day, the minimum level was found between 0.16 and 0.38 ng/ml. The above mentioned developed homologous ELISA requires only 1 hour 20 minutes completing the assay and serum sample can be used without any processing, however tissue samples and food samples need to have some initial processing to precede the test.

Conclusions

A specific, sensitive, reliable, accurate and low cost indigenous ELISA based on direct competitive inhibition has been developed. It can be used in future to determine the level of 17α-methyltestosterone in food, biological fluids, tissues of animals and also as doping drug in sports.

Competing Interests

The authors declare that they have no competing interests.

Data Availability

The data and details that support and justify this study can be available on request from the corresponding authors.

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