Local Preparation and Evaluation of Double-antibody Liquid Phase Radioimmunoassay System for Detection of Human Testosterone

H.M.Shafik¹, Kh.M.Sallam¹, N.H.Ebeid¹, M. S.Mohamed², M.R.Elshaer¹

¹Labeled Compounds Department, Hot Laboratories and Waste Management Center, Atomic Energy Authority, Cairo, Egypt
²Biochemistry Specialty, Chemistry Department, Faculty of Science, Cairo University, Egypt

Received: 15/6/2105 Accepted: 26/11/2015

ABSRRACT

Preparation, evaluation and optimization of testosterone radioimmunoassay (RIA) system using liquid phase double antibody is considered to be the main objective. Three primary components were prepared and characterized to obtain valid and accurate system. These components were polyclonal testosterone antibody, the $^{125}$I-testosterone tracer and set of testosterone standards. The production of polyclonal testosterone antibody was undertaken by immunizing two groups of females white New-Zealand rabbits with testosterone-3-(O-carboxy methyl oxime) : BSA as immunogen through primary immunization and five boosters. Both R₁ and R₄ gave anti-serum has a high immunoreactivity. The preparation of $^{125}$I-testosterone tracer was carried out using three different conjugates (testosterone-3-TME, testosterone-3-histamine and testosterone-3-BSA) by electrophilic substitution mechanism using chloramine-T as oxidizing agent. Tracers were characterized in terms of radiochemical yield %, radiochemical purity %, specific activity and immunoreactivity. A set of testosterone standards were prepared using highly purified testosterone antigen. Optimization and validation tests of the local liquid phase RIA system were carried out. In conclusion, the results showed that, the local testosterone RIA system is sensitive, specific and accurate with significant cost reduction in comparison with commertial kit and extended use of the method for routine investigation of variety of diseases especially hypogonadism and associated male infertility.

Keywords: RIA, Liquid phase, Testosterone, Radioiodination, Polyclonal antibody.

INTRODUCTION

Testosterone hormone is the predominant circulating androgen in men, with roughly 6–7 mg produced per day. Over 95% of testosterone originates from within the testes, where 400 million Leydig cells process cholesterol through the steroidogenic pathway, the adrenal glands contribute the remainder of circulating testosterone (¹). The normal blood level of testosterone in adult males is (2.4 - 9.5 ng/ml) while in adult females is (0.08 – 0.6 ng/ml). The blood level of testosterone is an important indicator of a wide variety of pathological conditions. Elevated concentration of testosterone in a male is characteristic of precocious puberty, congenital 21-hydroxylase deficiency, adrenal hyperplasia (Cushing’s syndrome), testicular tumors. In females, elevated testosterone concentration is associated with ovarian and endometrium tumors, stein-leventhal syndrome, adrenal hyperplasia (cushing's syndrome), hirsutism, polycystic ovarian syndrome and glucocorticoid therapy². Decreased concentration of testosterone in male is associated with Klinefelter's syndrome, hypogonadism, agonadism, anorchism, cryptorchidism, Kallman's syndrome, leydig cell aplasia and defect of the pituitary functions an in females at postmonopause (³).

Radioimmunoassay (RIAs) are valid and attractive micro-analytical techniques for determination of a wide variety of molecules present in complex matrices in view of the high degree specificity of the antigen antibody reaction in order to assess various disease conditions⁴. Three
components constitute the basic reagents of RIA. These reagents include polyclonal antibody, labeled and unlabeled antigens (standards). Development of polyclonal antibody involves determination of the specificity, titre and potential sensitivity of a number of candidate anti-sera. Specificity is assessed by determining the cross reactivity of related compounds, metabolites and endogenous compounds(5).

Testosterone like other steroids are not immunogenic (hapten) because of their low molecular weights but production of testosterone antibody can be achieved by coupling testosterone to carrier proteins of high molecular weights (6). Numerous proteins have been employed as carrier molecules. Those most frequently used include serum globulins and albumins, keyhole limpet-hemocyanin (KLH), gelatin, ovalbumin, casein, hemocyanin, thyroglobulin, fibrinogen, and tetanus, cholera, or diphtheria toxoid. The protein carrier should be immunogenic and have a sufficient number of amino acid residues with reactive side chains for conjugation to the hapten. When used for production of antibodies, conjugates can be injected into any animal except the animal of origin for the carrier protein. In this work testosterone conjugated to bovine serum albumin (BSA) was used to produce anti-testosterone.

The radiotracer is at the heart of every immunoassay. However it should never be considered alone, but always together with which it can be considered a close partner. The closeness and importance of this partnership is particularly apparent when using heterogenous tracers, that are tracers which are structurally different to the analyte which is being measured. This is always the case in enzynemimmunoassays and is also generally true for most of radioimmunoassays using 125I tracers, but not when 3H or 14C tracers are used. With a heterologus tracer, bridge recognition effects can seriously reduce assay sensitivity. This can only be avoided or ameliorated if the structure of both the immunogen and tracer are known and understood(7). A method for the iodiumation of aromatic legends such as histamine or tyrosine methyl ester and its subsequent conjugation to an acid using the mixed anhydride method are described by Nars and Hunter (8). It is necessary, therefore, to activate the acid functional group on the analyte molecules to facilitate reaction. Once again the necessary chemistry is identical to that used in the preparation of immunogens. Tantchou and Slaunwhite(9) recommended the use of the N-hydroxysuccinimide esters of carboxylic acid which are formed using the carbodiimide method. Alternatively the acid can be activated as a mixed anhydride by reaction with isobutyl chloroformate(10).

Development of liquid phase RIA system through locally low cost production and preparation of the basic reagents was the aim of the study. Optimization and validation tests were applied to ensure the validity of local kit under study for the assessment of human testosterone.

MATERIALS AND METHODS

For preparation of RIA system for estimation of testosterone in human sera,a number of reagents were used. This includes highly purified testosterone, testosterone–3–(O–carboxy methyl oxime), testosterone–3–(O–carboxy methyl oxime): BSA immunogen, Histamine, Tyrosine methyl ester (TME), complete and incomplete Freund's adjuvant, chloramine-T, sodium metabisulphite, goat anti-rabbit IgG (whole molecule), polyethylene glycol 8000 (PEG) and ANS (8-anilino-1-Naphtalene sulphonic acid) were purchased from Sigma-Aldrich Co., USA, Carrier and reductant free Na225I (5 mCi, 185 MBq), Institute of IZOTOP, (Baudapest, Hungary). All other chemical reagents were analytical grade obtained from reputed manufacturers.

A-Production of Polyclonal Testosterone Antibodies

Production of polyclonal testosterone antibody was carried out according to the immunization schedule of Vaitukaitis(11) and Szafranska et al(12). This study comprised two groups of healthy female mature white New-Zealand rabbits, 2-3 Kg body weight. The first group contains three rabbits (R1–R3) and the second group contains four rabbits (R4–R7). They were immunized with different doses of testosterone–3–(O–carboxy methyl oxime): BSA (testosterone-3-BSA) immunogen to raise anti-testosterone. The production of testosterone polyclonal antibody was carried out through primary
immunization and five boosters along 18 weeks. The immunogen was prepared by dissolving (10 mg) of the testosterone-3-BSA in (10 ml of 0.05M phosphate buffer, pH 7.4), (1mg /ml).

The first group: for primary immunization, each rabbit was immunized with 300 μg/300 μl of the immunogen, which was emulsified with 300 μl of Freund's adjuvant complete and the emulsion was injected subcutaneously and followed by five booster doses at 3 weeks intervals. Booster immunization doses, each rabbit, received 150 μg/150 μl of the immunogen, which prepared by completing to 300 μl by 0.05M phosphate buffer, pH 7.4, then emulsified with 300 μl of Freund's adjuvant incomplete.

The second group: for primary immunization, each rabbit was immunized with 150 μg/150 μl of the immunogen which prepared by completing to 300 μl by 0.05M phosphate buffer, pH 7.4 and then emulsified with 300 μl of Freund's adjuvant complete and the emulsion was injected subcutaneously followed by five booster doses at 3 weeks intervals. Booster immunization doses, each rabbit, received 75 μg/75μl of the immunogen which prepared by completing to 300 μl by 0.05M phosphate buffer, pH 7.4, then emulsified with 300 μl of Freund's adjuvant incomplete.

Blood samples were harvested three weeks after each injection and characterized in terms of titre, displacement and immunoresponse.

B-Preparation of 125I-Testosterone Tracers

In the present study, preparation of 125I-testosterone tracer was carried out through iodination of three different testosterone conjugates using chloramine-T method as follow: testosterone-3-(O-carboxy methyl oxime) was conjugated to each of TME or histamine by mixed anhydride coupling. The prepared conjugates were labeled according to Hunter and Greenwood (13) and Sallam (14). Also testosterone-3-(O-carboxy methyl oxime): BSA conjugate was radioiodinated as described by Hunter and Greenwood (13) and Jeffcoate et al (15).

1-Preparation of Radioiodinated Testosterone-3-TME and Testosterone-3-Histamine

The preparation of radio-iodinated testosterone-3-TME and testosterone-3-histamine was carried out according to the following steps:

a- Steroid Activation

Synthesis of steroid conjugates were similar to those described by Nordblom et al (16). Testosterone 3-(O-carboxy methyl) oxime-TME or testosterone 3-(O-carboxy methyl) oxime-histamine conjugates were prepared by mixed anhydride coupling. Firstly, 50 μl of testosterone 3-(O-carboxy methyl) oxime (5 mg/ml in dioxan) was activated by the addition of 10 μl of pre-diluted tri-n-butylamine in dioxan (1:10) and 10 μl of pre-diluted isobutyl chloroformate in dioxan (1:5) then incubated at 10°C for 45 minutes.

b- Coupling to TME and Histamine

200 μl of dioxan were added to the previous activated reaction mixture and 50 μl from this mixture were used to conjugate with 50 μl of TME (2.6 mg/5ml of 0.05M phosphate buffer) or histamine (1.5 mg/5 ml of 0.05M phosphate buffer). 20 μl of 0.2M NaOH were added and the mixture was incubated for 1 hour then 10 μl of NaOH was added and the mixture was incubated for another 1 hour. Then the mixture was poured into 1.5 ml distilled water and the product was extracted with 1 ml ethyl acetate three times. The extracts were combined and dried using nitrogen gas. Aliquots of the steroid derivatives were stored in ethanol at -20°C. Prior to iodination the solvent was evaporated to dryness under nitrogen gas and the steroid conjugate was then reconstituted in 20 μl phosphate buffer (0.5 M, pH 7.4).

c- Iodination of Prepared Testosterone Conjugates

Iodination was carried out using chloramine-T method according to Hunter and Greenwood (13), as follows: into a small eppendorf tube, 2.4 μg of testosterone-3-TME or testosterone-3-Histamine conjugate in 20 μl phosphate buffer (0.5 M, pH 7.4) and 5 μl Na125I (0.5 mCi, 18.5 MBq) were added.
Then, 10 μl phosphate buffer (0.05 M, pH 7.4) containing 50 μg Ch-T was added. The mixture was incubated at room temperature for about 2 minutes with continuous vortex; the reaction was quenched by addition of 10 μl phosphate buffer (0.05 M, pH 7.4) containing 150 μg of sodium metabisulphate. The prepared 125I-testosterone tracers were purified using high performance liquid chromatography (HPLC) system.

d - Purification of Radioiodinated Testosterone-3-TME and Testosterone-3-Histamine

The reaction mixture was purified using HPLC column previously equilibrated with 20% CH₃CN + 80% 0.05M sodium acetate buffer for 30 minutes. A gradient elution curve is programmed into the HPLC, starting with 20% CH₃CN + 80% 0.05 M sodium acetate changing to 80% CH₃CN + 20% 0.05M sodium acetate buffer pH 4 at flow rate 1 ml/min. Inject 50 µl product into the column using a Hamilton syringe. 1ml fractions were collected. The required fractions were pooled. 0.5 ml aliquot was diluted with 0.05 M phosphate buffer pH 7.4, containing ANS (0.2%) and NaN₃ (0.1%).

2-Prepartion of Radiolabeled Testosterone-3-BSA Conjugate

Iodination of testosterone-3-BSA was carried out according to Jeffcoate et al (15), Chloramine-T method was used as described by Hunter and Greenwood (13). To eppendorf tube, 5 μg testosterone-3-(O-carboxy methyl oxime): BSA in 5 μl 0.05M phosphate buffer PH 7.4 were dispensed followed by addition of 20 μl of 0.5M phosphate buffer pH 7.4 and 5 μl of Na125I (0.5 mCi, 18.5 MBq). The reaction was started by addition of 50 μg of chloramine -T in 10 μl of double distilled water. The reaction mixture was gently vortex for 1 min at room temperature then the reaction was stopped by the addition of 150 μg of sodium metabisulphite in 10 μl of double distilled water followed by addition of 100 μg KI in 20 μl double distilled water. Gel filtration Sephadex G-25 (PD-10 column) was used in purification of 125I-testosterone tracer at a flow rate 0.5 ml/min and fractions were collected. The required fractions were pooled and diluted using assay buffer containing 0.2% ANS.

3-Immuoreactivity Check

The fresh tracers were tested using liquid phase double antibody RIA system. The 125I-testosterone tracers obtained were characterized in terms of radiochemical yield%, specific activity, immunoreactivity and non-specific binding% (NSB %).

C-Testosterone Standards

A stock standard of the testosterone was prepared by dissolving 1 mg of highly purified testosterone antigen in 1 ml of ethanol. Further, serial dilutions were carried out using steroid assay buffer to prepare set of testosterone standards ranged from (0.1 to 16.1 ng/ml). The validity of the local prepared testosterone standards were assessed using IZOTOP RIA kit (14).

D- Optimization Tests

Optimization of assay reaction parameters including sample volume, incubation time, and incubation temperature and separating agent were carried out using testosterone liquid phase RIA system. Radioimmunoassay of testosterone was carried out as follow: poly propylene tubes were marked in duplicates then 100 μl of standards or unknown samples, 100 μl of the obtained testosterone polyclonal antibody dilution 1/10,000 and 100 μl of 125I- testosterone-histamine tracer were mixed in previous poly propylene tubes. The mixture was then incubated for 3 hrs at room temperature. The separation of bound from free fractions was carried out by adding 100 μl of 2nd antibody (goat anti-rabbit IgG) dilution 1:50, 100 μl normal rabbit serum (NRS) dilution 1:200 and 500 μl polyethylene glycol (PEG-8000) at concentration 12% to all assay tubes. The tubes were allowed to stand for 30 min at room temperature and centrifuged for 20 min at 4000 rpm at 4°C. The supernatant was decanted and the bound fraction was counted using multi-crystal gamma counter.
E- Validation Tests

Some extensive studies were carried out to determine each of sensitivity, precision, accuracy and method comparison to assess the validity of local testosterone RIA system.

RESULTS AND DISCCUSION

A- Characterization of Polyclonal Testosterone Antibodies

The production of polyclonal anti-testosterone was undertaken through the immunization of seven female mature white New Zealand rabbits (which classified into two groups) with testosterone-3-BSA immunogen. Each rabbit in the first group (R₁ – R₃) received 300 µg of testosterone-3-BSA immunogen followed by 150 µg for each booster of the same immunogen, while each rabbit in the second group (R₄ – R₇) received 150 µg of testosterone-3-BSA immunogen followed by 75 µg for each booster of the same immunogen. The produced anti-testosterone was characterized in terms of titre, displacement and immunoresponse. The titre and displacement of an antiserum are estimated by determining the percent binding of different antibody dilutions for the individual bleeding antisera harvested with each of Zero and high testosterone standards (0.0 and 16.1 ng/ml) with fixed quantity of tracer. The percentage binding of each of (Bₒ/TA) and (Bₛ/TA) were calculated for each bleeding for each rabbit to obtain the maximum displacement percent between maximum binding percent (Bₒ%) and minimum binding percent (Bₛ%). Immunoresponse profile was carried out by determination of titre and highest displacement percent for each individual bleeding from each rabbit and plotted against time of immunization.

The results of first group: R₁ gave the highest displacement percent (78.2%) at a dilution titer 1/10000 as indicated in Fig. (1), after 15 weeks from primary immunization as shown in Fig. (2). R₂ gave the highest displacement percent (66.8%) at dilution titer 1/20000 after 15 weeks from primary immunization while R₃ gave the highest displacement percent (70.9%) at dilution titer 1:10000 after 12 weeks from the primary immunization.

The results of second group: R₄ gave the highest displacement percent (62.5%) at a dilution titer 1/5000 as indicated in Fig. (3), after 15 weeks from primary immunization as shown in Fig. (4). R₅ gave the highest displacement percent (56.7%) at dilution titer 1/5000 after 9 weeks from primary immunization, R₆ gave the highest displacement percent (58.2%) at dilution titer 1/20000 after 12 weeks from primary immunization and R₇ gave the highest displacement percent (52.3%) at dilution titer 1/10000 after 9 weeks from primary immunization. From the above results, it can be concluded that, R₁ gave the highest displacement percent (78.2%) in group (1) while R₄ gave the highest displacement percent (62.5%) in group (2).

(R₁) gave the best result for the displacement percent between the zero and high standard of testosterone between all rabbits of both groups (1) and (2). So the antiserum from (R₁) was selected at titer 1/10000 to be used for formulating testosterone liquid phase RIA system to measure testosterone hormone levels in human sera.

The data of the displacement percent and immunoresponse of the present study are in good agreement with previous studies (Vaitukaitis, Bauer, Pillai & Bhandarkar and Szafranska et al. The introduction of an antigen into a host, active immunization occurs. When a foreign antigen is introduced into the body for the first time, a primary antibody response occurs. This is characterized by an initial slow production of antibody. When the same antigen is encountered a second time, the antibody production is much faster, greater in amounts. The same author reported that antibodies are serum proteins (γ-globulins) produced by lymphocytes and plasma cells in response to antigenic stimulation.
The titer of an antibody in serum raised in an immunized animal is an important parameter for optimization of RIA technique. It is an index of the concentration of the antibody molecules in the serum. Generally, the dilution giving high displacement percent is reported as the titer of the antibody.

Fig. (1): Titer and displacement of polyclonal testosterone antibody obtained from rabbit (1)

Fig. (2): Immunoresponse curve of rabbit (1) immunized with testosterone-3-BSA immunogen
Fig. (3): Titer and displacement of polyclonal testosterone antibody from rabbit (4)

Fig. (4): Immunoresponse of rabbit (4) after immunization with testosterone-3-BSA
B-Characterization of $^{125}$I-Testosterone Tracers

Extensive studies were undertaken for the preparations of $^{125}$I-testosterone tracers. The mechanism of these preparations is the electrophilic substitution reaction to establish an ideal iodination procedure for testosterone without losing its biological properties. Characterizations of radiolabeled tracers were carried out in terms of radiochemical yield percent, radiochemical purity percent and immunoreactivity.

1- Radiochemical yield

The iodination reaction mixture of both testosterone-3-TME and testosterone-3-histamine conjugates were applied on HPLC column previously equilibrated with the elution buffer. Thirty fractions were collected on a fraction collector and the radioactivity of each fraction was counted, while testosterone-3-BSA conjugate iodination reaction mixture was applied on PD-10 column and forty-five fractions were collected on a fraction collector and the radioactivity of each fraction was counted. Elution profile was constructed by drawing activity (µCi) against fraction numbers using Ch-T method (Figs. 5-7). As illustrated in Fig. (5), three peaks corresponding to free $^{125}$I (14.3%), $^{125}$I-TME (11.5%) and $^{125}$I-testosterone tracer (71.1%) were observed. Similarly, Fig. (6) shows three peaks corresponding to free $^{125}$I (10.6%), $^{125}$I-histamine (8.3%) and $^{125}$I-testosterone tracer (75.9%) while Fig. (7) shows two peaks corresponding to $^{125}$I-testosterone tracer (53.8%) and free $^{125}$I (26.4%). The results are in agreement with Sallam\cite{14}.

![Fig. (5): Purification of $^{125}$I- testosterone-3-TME tracer using HPLC system](image-url)
Fig. (6): Purification of $^{125}$I-testosterone-3-histamine tracer using HPLC system

Fig. (7): Purification of $^{125}$I-testosterone-3-BSA tracer using PD-10 column
2- Radiochemical Purity

As indicated in Table (1), high radiochemical purity was obtained for iodination of testosterone-3-TME, testosterone-3-histamine and testosterone-3-BSA conjugates, (96%, 96.4% and 94.5%), respectively using paper electrophoresis chromatography. The results obtained are in agreement with Jeffcoate et al (15), Grace et al (18) and Sallam (14) who reported that radiochemical purity obtained was greater than 95% for $^{125}$I-testosterone tracers using Ch-T method.

Table (1): Radiochemical purity of prepared $^{125}$I-testosterone tracers

<table>
<thead>
<tr>
<th>Testosterone-3-conjugate</th>
<th>Free iodide %</th>
<th>Radio-chemical purity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone-3-TME</td>
<td>1.6</td>
<td>96</td>
</tr>
<tr>
<td>Testosterone-3-histamine</td>
<td>1.3</td>
<td>96.4</td>
</tr>
<tr>
<td>Testosterone-3-BSA</td>
<td>2</td>
<td>94.5</td>
</tr>
</tbody>
</table>

3- Specific activity

The specific activity of the prepared tracers through iodination of testosterone-3-BSA, testosterone-3-TME and testosterone-3-histamine was found to be 53.3, 147.1 and 158.1 µCi/µg, respectively the results are in agreement with Jeffcoate et al (15), Grace et al (18) and Sallam (14).

4- Immunoreactivity

The immunoreactivity of the tracers prepared was estimated by examining its binding with specific antiserum at Zero standard (0.0 ng/dl) Bo% and high testosterone standard (16.1 ng/dl) Bs%. The results obtained are presented in Table (2). The obtained results show that, the tracer prepared via iodination of testosterone-3-histamine conjugate gave the highest displacement% and the lowest NSB%.

Table (2): Immunoreactivity check of prepared tracers

<table>
<thead>
<tr>
<th>Testosterone-3- conjugates</th>
<th>Bo %</th>
<th>Bs %</th>
<th>Displacement% (Bo% - Bs% / Bo%)</th>
<th>NSB %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone-3-TME</td>
<td>49.3</td>
<td>16.4</td>
<td>66.9</td>
<td>4.7</td>
</tr>
<tr>
<td>Testosterone-3-histamine</td>
<td>50.1</td>
<td>10.6</td>
<td>78.8</td>
<td>3.1</td>
</tr>
<tr>
<td>Testosterone-3-BSA</td>
<td>59.8</td>
<td>25.2</td>
<td>57.8</td>
<td>5.2</td>
</tr>
</tbody>
</table>

4-Stability of $^{125}$I-Testosterone Tracers

The tracer stability was determined by calculating the maximum binding (Bo %) and non-specific binding (NSB %) by RIA after 1, 4, 8 and 12 weeks when stored at 4°C. The results of this study are shown in table 3 which indicate that the binding Bs % decreased and NSB % increased as the life of the tracers preparations increased. At 4°C, the maximum binding percents (BLo%) of the prepared tracers were 49.4%, 48.5% and 59.5%, and NSB were 3.2%, 1.3% and 4.2% at the first week then Bs % decreased to 19.5%, 35.1% and 10.5% and NSB% increased to 5.2%, 4.1% and 7.3% after 12 weeks for testosterone-3-TME, testosterone-3-histamine and testosterone-3-BSA tracers respectively. So testosterone-3-histamine tracer was valid to be used within two months after preparation. The results are in agreement with the data of Jeffcoate et al (15), Grace et al (18) and Sallam (14).
Table (3): Stability of $^{125}$I-testosterone tracers at 4°C

<table>
<thead>
<tr>
<th>Tracer</th>
<th>Testosterone-3-TME</th>
<th>Testosterone-3-histamine</th>
<th>Testosterone-3-BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shelf life (week)</td>
<td>1</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>Bo%</td>
<td>49.4</td>
<td>47.3</td>
<td>35.6</td>
</tr>
<tr>
<td>NSB %</td>
<td>3.2</td>
<td>3.4</td>
<td>4</td>
</tr>
</tbody>
</table>

From previous results it can be concluded that the $^{125}$I-testosterone tracer prepared via iodination of testosterone-3-histamine conjugate gave the highest radiochemical yield% (75.9%) as indicated in Fig.(6), radiochemical purity% (96.4%) as indicated in Table (1), acceptable specific activity (158.1µCi/µg), and high displacement% (78.2%) as indicated in Table (2) and acceptable stability at 4°C in comparison with the obtained results of the two other conjugates under study. So it has been chosen to formulate testosterone RIA system. These results are in good agreement with Allen & Redshaw(19).

C- Evaluation of Testosterone Standards

The local preparation of testosterone standards for RIA system is conducted using a highly purified testosterone antigen in steroid buffer, the data of local standards was estimated by IZOTOP RIA kit (Budapest, Hungary). The results of local standards showed good recovery ranged from 96.2% to 107.3%.

D-Optimization of Testosterone Liquid Phase RIA System

For achieving reliable assay, the general principles of the assay optimization must be followed. The development of testosterone RIA system was carried out by studying sample volume, incubation time, incubation temperature and separating agents.

1- Effect of sample volume: As illustrated in Table (4), 100 µl of sample gave the highest difference between three different testosterone concentrations and a high displacement % (83.3%). The binding percent starts to decrease with the increase of sample volume (from 200 to 500 µl) inverting the dose response curve which is called a high dose hook effect (17). These results are in accordance to Mehany et al(120).

Table (4) the effect of sample volume on testosterone assay.

<table>
<thead>
<tr>
<th>Testosterone standards</th>
<th>Sample volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25</td>
</tr>
<tr>
<td>0</td>
<td>68.7</td>
</tr>
<tr>
<td>1.25</td>
<td>35.1</td>
</tr>
<tr>
<td>16.1</td>
<td>29.2</td>
</tr>
<tr>
<td>Displacement %</td>
<td>53.7%</td>
</tr>
</tbody>
</table>
2-Effect of incubation time: The effect of incubation time on this system was carried out through 24 hours at room temperature. As illustrated in Table (5), it was found that the optimum incubation time which gave the highest displacement percent was three hours. After more than three hours incubation time, a slight decrease in displacement % was observed. It can be explained the three hours was sufficient to reach the equilibrium between antibody and antigen reaction. This result was in agreement with Mehany et al\(^{(20)}\).

Table (5): Effect of incubation time on testosterone assay

<table>
<thead>
<tr>
<th>Testosterone standard</th>
<th>Incubation time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1h</td>
</tr>
<tr>
<td>0 ng/ml</td>
<td>31.5</td>
</tr>
<tr>
<td>1.25 ng/ml</td>
<td>20.7</td>
</tr>
<tr>
<td>16.1 ng/ml</td>
<td>17.3</td>
</tr>
</tbody>
</table>

| Displacement %        | 45.1%| 68.4%| 83.4%| 79.3%| 78.2% |

3- Effect of incubation temperature: As indicated in Table (6), the results obtained reveal that the highest differences in binding between the three concentrations of testosterone and a high displacement (83.9%) are obtained at 25°C incubation temperature. The previous thermodynamic studies showed that, the reaction between antigen and antibody is endothermic reaction in temperature limits that kept the protein without destruction. So by increasing the incubation temperature from 4 to 37°C, the binding % increased. These results are in agreement with Mehany et al\(^{(21)}\).

Table (6): Effect of Incubation temperature on the liquid-phase RIA system for testosterone

<table>
<thead>
<tr>
<th>Testosterone Standard</th>
<th>Incubation temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4°C</td>
</tr>
<tr>
<td>0 ng/ml</td>
<td>48.9</td>
</tr>
<tr>
<td>1.25 ng/ml</td>
<td>25.6</td>
</tr>
<tr>
<td>16.1 ng/ml</td>
<td>11.4</td>
</tr>
</tbody>
</table>

| Displacement%          | 76.7| 83.9| 80  |

4- Effect of separating agent: Different concentrations of PEG-8000 were studied to reach a highest displacement %. The results were listed in Table (7). These results show a high displacement % and a high binding % were obtained by increasing the concentration of PEG-8000 to 12%. So the concentration of PEG-8000 (12 %) was chosen to be the optimum. The efficiency of separation was increased by increasing the concentration of PEG from 4% to 12%. These results are in good agreement with Mehany et al\(^{(21)}\).

Table (7): Effect of different concentrations of PEG-8000 on testosterone RIA system

<table>
<thead>
<tr>
<th>PEG concentration</th>
<th>2nd antibody(1:50)</th>
<th>NRS(1:200)</th>
<th>Displacement %</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG 4%</td>
<td>37</td>
<td>19.1</td>
<td>48.3</td>
</tr>
<tr>
<td>PEG 8%</td>
<td>47.8</td>
<td>13.4</td>
<td>71.9</td>
</tr>
<tr>
<td>PEG 12%</td>
<td>51</td>
<td>7.9</td>
<td>84.5</td>
</tr>
</tbody>
</table>
The standard curve was constructed using the assay optimized conditions as follow: In duplets, polypropylene tubes were marked and placed in a suitable rack. 100 µl of anti-testosterone antibody dilution 1:10,000 (R₁) and 100 µl of testosterone standards with different concentrations (from 0.1 ng/ml to 16.1 ng/ml) were mixed with 100 µl of ¹²⁵I-testosterone tracer the assay tubes were incubated for 3 hours followed by addition of 100 µl of 2nd Ab dilution 1:50 (Goat ant-rabbit IgG), 100 µl of NRS and 500 µl of PEG-8000 (12%). The tubes were allowed to stand for 30 min at room temperature and centrifuged for 20 min at 4000 rpm at 4°C. The supernatant was decanted and the bound fraction was counted using multi-crystal gamma counter.

![Graph](image.png)

**Fig. (8): Optimized standard curve for testosterone using liquid phase RIA system**

**E-Validation of testosterone liquid phase RIA system**

For a valid and reliable assay, certain criteria should be achieved which include sensitivity, specificity, precision and accuracy.

1- **Sensitivity (Minimal detectable dose):** Sensitivity can be defined as minimal detectable concentration (MDC) of the assay, which can be distinguished statistically from zero standards. For this purpose, the zero standards were set up in 20 replicates along with other standards. The mean and standard deviation (SD) of the counts were calculated. Then the dose corresponding to mean -2 SD was read off the standard curves. This value was taken as the sensitivity of the proposed assay. The sensitivity of the present testosterone RIA system is 0.013 ng/ml as indicated in Fig.(7), the data obtained are in agreement with Nesceu *et al.*

2- **Specificity (cross reactivity):** The specificity is defined as the ability of antibody to recognize an antigen even when the antigen is measured in medium of similar substances. Specificity is an essential test of radioimmunoassay. The cross-reactivity of the locally produced testosterone antiserum has been measured against various related compounds. The percent cross-reactivity is expressed as the ratio of testosterone concentration to the concentration of the reacting compound at 50% binding of the 0 ng/ml testosterone standard. The results are listed in Table 8.
Table (8): Cross reactivity of testosterone anti-sera.

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Cross reactivity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone</td>
<td>100%</td>
</tr>
<tr>
<td>Estradiol</td>
<td>&lt;0.02%</td>
</tr>
<tr>
<td>Cortisol</td>
<td>&lt;0.01%</td>
</tr>
<tr>
<td>Progesterone</td>
<td>0.14%</td>
</tr>
<tr>
<td>17α hydroxy progesterone</td>
<td>0.01%</td>
</tr>
</tbody>
</table>

3-Precision

It is a statistical index of the ability of an assay to yield the same result when the assay is repeated using the same sample\(^{(24)}\). The reliability of the present procedure for testosterone RIA system was assessed by examining its reproducibility on three pooled human serum samples. The intra-assay (Within Run) precision was determined for testosterone from the mean of 20 replicates for each of the three human serum samples and the statistics were calculated for each one. The CVs for intra-assay ranged from (7.7 % to 9.7 %).

The inter-assay (Run to Run) precision was determined for the mean of the average of duplicates from 10 separate runs with the same three pooled human sera. The CVs ranged from (8.9 % to 11.6 %) for inter-assay. The results of these statistics are summarized in Table (9). These results are in good agreement with numerous studies \(\text{Kirkpatrick et al}^{(24)}\), \(\text{Pillai & Bhandarkar}^{(17)}\) and \(\text{El-Kolaly et al}^{(25)}\) which stated that the intra-assay coefficient of variation (CV\%) should be less than 10\%, while in case of inter-assay, they reported that the CV\% of the measured ligand concentration should be less than 15\%.

![Graph showing sensitivity of testosterone liquid phase RIA system](image)
Table (9): Precision profile for testosterone assay

<table>
<thead>
<tr>
<th>Samples</th>
<th>Mean (ng/ml)</th>
<th>SD (ng/ml)</th>
<th>CV%</th>
<th>Mean (ng/ml)</th>
<th>SD (ng/ml)</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.4</td>
<td>0.11</td>
<td>7.9</td>
<td>1.2</td>
<td>0.14</td>
<td>11.6</td>
</tr>
<tr>
<td>2</td>
<td>3.9</td>
<td>0.3</td>
<td>7.7</td>
<td>4.2</td>
<td>0.4</td>
<td>9.5</td>
</tr>
<tr>
<td>3</td>
<td>10.3</td>
<td>0.9</td>
<td>8.7</td>
<td>9.5</td>
<td>0.85</td>
<td>8.9</td>
</tr>
</tbody>
</table>

4- **Accuracy**: the assay accuracy in this study was tested by recovery and dilution tests:

a. **Recovery Test**

Recovery test measures the concentrations in three human serum samples of different diagnostic states before and after adding known amounts of testosterone. The measurements are expressed as a percentage of the added mass. The optimal recovery is 100% according to Edwards (26), but according to Pillai and Bhandarker (17) the recovery of an assay should be 100±15%. As shown from the results listed in Table (10), it can be observed that recovery % ranged from (92.5% to 107.1%). The recovery data of the present study for testosterone are in good agreement with Pillai and Bahandarkar (17).

Table (10): Recovery assessment for testosterone using liquid phase RIA system

<table>
<thead>
<tr>
<th>Sample</th>
<th>Endogenous Testosterone (ng/ml)</th>
<th>Added Testosterone (ng/ml)</th>
<th>Expected (E)</th>
<th>Observed (O)</th>
<th>Recovery % (O/E)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.3</td>
<td>1.25</td>
<td>2.7</td>
<td>1.18</td>
<td>92.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.1</td>
<td>8.7</td>
<td>2.5</td>
<td>92.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16.1</td>
<td></td>
<td>9</td>
<td>103.4</td>
</tr>
<tr>
<td>2</td>
<td>4.1</td>
<td>1.25</td>
<td>2.6</td>
<td>2.7</td>
<td>103.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.1</td>
<td>4.1</td>
<td>3.9</td>
<td>95.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16.1</td>
<td>10.1</td>
<td>9.5</td>
<td>94.1</td>
</tr>
<tr>
<td>3</td>
<td>10.2</td>
<td>1.25</td>
<td>5.6</td>
<td>6</td>
<td>107.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.1</td>
<td>7.05</td>
<td>6.9</td>
<td>97.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16.1</td>
<td>13.15</td>
<td>12.7</td>
<td>96.6</td>
</tr>
</tbody>
</table>

b. **Dilution Test**

The results in Table (11) show the concentrations of three human serum samples at various dilutions in the matrix of the assay (assay buffer) to assess the linearity of the assay. Edwards (26) stated that non-linearity indicates inaccurate calibration or inappropriate matrix or both. The results showed that the procedure of the present study for testosterone maintain good linearity under dilution. As indicated in Table (11), it can be observed that dilution test for testosterone assay ranged from (92.2% to 107.1%).
Table (11): Dilution test for testosterone using liquid phase RIA system

<table>
<thead>
<tr>
<th>Sample</th>
<th>Endogenous Testosterone (ng/ml)</th>
<th>Dilution factor</th>
<th>Expected (E) (ng/ml)</th>
<th>Observed (O) (ng/ml)</th>
<th>Recovery% (O/E) (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.3</td>
<td>1:2</td>
<td>0.62</td>
<td>0.64</td>
<td>103.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:4</td>
<td>0.312</td>
<td>0.3</td>
<td>96.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:8</td>
<td>0.156</td>
<td>0.162</td>
<td>104</td>
</tr>
<tr>
<td>2</td>
<td>4.1</td>
<td>1:2</td>
<td>2.05</td>
<td>1.9</td>
<td>92.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:4</td>
<td>1.02</td>
<td>1.1</td>
<td>107.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:8</td>
<td>0.51</td>
<td>0.5</td>
<td>98</td>
</tr>
<tr>
<td>3</td>
<td>10.2</td>
<td>1:2</td>
<td>5.1</td>
<td>4.7</td>
<td>92.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:4</td>
<td>2.55</td>
<td>2.4</td>
<td>94.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:8</td>
<td>1.27</td>
<td>1.3</td>
<td>102.4</td>
</tr>
</tbody>
</table>

Method Comparison

The statistical analysis were undertaken to compare testosterone results of 20 different human serum samples by commercially available kit (IZOTOP, diagnostic kit, Hungary) to those obtained by the present technique. The results were subjected to correlation coefficient "r" and linear regression analysis.

Fig. (10): Regression line equation and correlation coefficient (r) between testosterone values obtained by IZOTOP method and the present liquid phase RIA system
It could be concluded that the preparation of basic reagents of RIA provides materials for the reliable quantitative measurement of testosterone in human serum. Production of polyclonal antibody in addition to preparation of labeled and unlabeled testosterone antigens (standard) covalent in locally produced testosterone RIA kit with low cost has been achieved. A high sensitivity and accuracy make the local RIA kit valid to fulfill the clinical requirements for diagnosis of disorders associated with abnormal blood testosterone levels.

REFERENCES


