Enzyme Immunoassay Technique for the Determination of Hormones in Human Serum

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Received 20/10/2008 accepted 12/1/2009

Abstract

Enzyme Linked Immunosorbant Assay (ELISA) is two immunological steps sandwich type assay. In the first step the hormone is captured by a monoclonal antibody bound to the wells of a micrometer plate. In the second step a biotinylated monoclonal antibody is added with streptavidin -peroxidase conjugate. The biotinylated antibody binds to the solid phase antibody complex and, in turn, binds the conjugate. After incubation, the wells are washed and the antigen complex bound to the well detected by addition of chromogenic substrate. The intensity of the coloration is proportional to the hormone concentration in the sample.ELISA technique for the quantitative determination of Tri-iodothyroxine (T_3), Tetra-iodothyroxine (T_4), thyroid Stimulating Hormone (TSH), prolactin (Prol.), Luteinizing hormone (LH), Follicle Stimulating Hormone (FSH) and (Testo.) concentration was used. In present work the minimum detectable concentrations (MDC) of hormones by this technique were estimated to be:

Hormone	MDC
T_3	0.275 ng/ml
T_4	0.347 µg/dl
TSH	0.250 µlu/ml
Prol.	2.20 ng/ml
LH	1.90 mlu/ml
FSH	2.90 mlu/ml
Testo.	0.08ng/ml

The study was conducted in Al-Khadmia Teaching Hospital from November 2006 to June 2007 and aimed recovery, precision, accuracy and minimum detection limit of analytical technique ELISA for the determination of hormones in (35) samples of human serum.

تقنية المناعة الأنزيمية لتقدير الهرمونات في المصل البشري

حسين حسن خرنوب أيوب علوان جاسم

المستخلص

أساس عمل تقنية المناعة الأنزيمية هو تفاعل مناعي بين الهرمونات والاجسام المضادة ويكون عبر مرحلتين, المرحلة الاولى هو احاطة الهرمونات بالاجسام المضادة الموجودة المثبتة على خلية التحليل, يعقب ذلك تكوين معقد ملون بين الهرمون والكاشف في المرحلة الثانية ومن خلال شدة اللون يمكن تقدير الهرمون في النموذج. استخدمت تقنية المناعة الانزيمية في هذا البحث للقياس الكمي لهرمونات الغدة الدرقية (T_3, T_4, TSH) وهرمونات الخصوبة الانثوية (LH, FSH, Prol.) والهرمون الذكري (Testo.) وكانت اقل كميات يمكن تقديرها لهذه الهرمونات بهذه التقنية هي ما يلي:-

الهرمون	اقل كميات مقدرة
T_3	0.275 نانو غرام/ مل
T_4	0.347 مايكرو غرام/ مليد
TSH	0.250 مايكرو وحدة عالمية/ مل
Prol.	2.20 نانو غرام / مل
LH	1.90 ملي وحدة عالمية/ مل
FSH	2.90 ملي وحدة عالمية/ مل
Testo.	0.08 نانو غرام/ مل

اجريت هذه الدراسة في مستشفى الكاظمية التعليمي للفترة من تشرين الثاني 2006 الى حزيران 2007 وكان الغرض منها التعرف على دقة, حساسية, مضبوطية تقنية المناعة الانزيمية لقياس تراكيز الهورمونات في (35) نموذج في المصل البشري.

Introduction

Thyroid hormones (T_3, T_4, and TSH) perform many important functions such as growth, cellular metabolism and general hormone balance of the body as well as on the maintenance of metabolic activity and development of the skeletal and organ system [1-3]. The hormones T_3 and T_4 circulate in the blood stream mostly bound to the plasma protein and thyroxine Binding Globulin (TBG). The concentration of T_3 is much less than that of T_4, but its metabolic potency is much greater [4]. T_3 determination is an important factor in the diagnosis of thyroid disease. Its measurement has uncovered of a variant of hyper-thyroidism in thyrotoxic patients with elevated T_3 value and normal T_4 values [5]. In addition to hyper-thyrodism T_3 levels are elevated in women who pregnant and women receiving oral contraceptives or estrogen treatment [6, 7].Increased level T_4 has been found in hyper-thyroidism due to Grave's disease [8], while low level of T_4 has been associated with congenital hypo-thyroidism [9].Thyroid stimulating hormones (TSH) is a glycoprotein with molecular weight of approximately 28,000 daltons consisting of two chemically different sub-units, alpha and beta. Although the concentration of TSH in the blood is extremely low, it is essential for the maintenance of normal thyroid function [10].Human prolactin is secreted from anterior pituitary gland in both men and women. Human prolactin is a single chain polypeptide hormone with molecular weight of approximately 23,000 daltons [11]. Women normally have slightly higher basal prolactin levels than men, the primary functions of prolactin are to initiate breast development and maintain lactation [12]. During pregnancy, prolactin levels increase progressively to between 10 to 20 times normal value [13].Breast-feeding mothers maintain high levels of prolactin and it may take several months for serum concentration to return to non-pregnant levels. The determination of prolactin concentration is helpful in diagnosing hypothalamic pituitary disorder [14]. Prolactin levels are elevated in renal disease, hypothyroidism and increased by drugs such as chloro-promazine [15]. LH is glycoprotein with molecular weight of approximately 30,000 daltons. A lack of secretion by the anterio pituitary may cause lower LH level [16]. Follicle stimulating hormone (FSH) is intimately involved in the growth and reproductive activities of the gonadal tissues, which synthesized and secreted male and female sex hormones, high level of FSH in men may be found in

primary testicular failure, renal failure and hypothyroidism [17].Testosterone (17β-hydroxyandrost-4-ene-3-one) is a C_{19} steroid with an unsaturated bond between C-4 and C-5, keton group in C-3 and a hydroxyl group in the β-position at C-17. This steroid hormone has a molecular weight of 288.4 daltons [18]. Testosterone is responsible for development of secondary male sex characteristics and its measurement is helpful in evaluating the hypogonal states [19]. In women, high levels of testosterone are generally found in hirsutism and virilization. Polycystic ovaries, ovarian tumors, adrenal tumors and adrenal hyperplasia, in men high level of testosterone are associated to hypothalamic pituitary disease, testicular tumors and prostate cancer, while low level of testosterone can be found in patients with hypopituitarism and some auto-immune disease [20].

Experimental, Reagents and results

In the T_3, T_4 and TSH determination, a certain amount of antibody is coated on micro-titer wells. A measured amount of patient's serum and a constant amount of T_3 conjugated to horseradish peroxidase are added to the micro-titer wells during incubation. T_3, T_4, and TSH compete for the limited binding sites on the anti- T_3, T_4 and TSH antibody. After 60 minutes or higher incubation at room temperature, the wells are washed 5 times by phosphate buffer solution to remove unbound T_3, T_4 and TSH conjugate. A solution of tetramethylbenzidine (TMB) is then add and incubated for 20 minutes, resulting in the development of blue color. The color development is stopped with addition of 1N HCl and the materials were used stop solution (1N HCl), tetramethylbenzidine (TMB), Rabbit Anti-hormon reagent, hormone-HRP conjugated reagent, hormone standard solutions, micrometer well, microtiter plate reader, and micropipettes. The absorbance is measured spectrophotometrically at 450 nm.The following procedures were used for the determination of thyroid gland hormones:-

1.0 *determination of T_3*

1- Pipette 50 µl of standard (0, 0.75, 1.5, 3.0, 6.0 and 10 ng/ml), sample and controls in appropriate wells.

2- Dispense 50 µl of antibody reagent into each well, mix thoroughly for 30 seconds.

3- Add 100 µl of working conjugate reagent (0.1 ml of 50 T_3 –HRPO conjugate dilutent) and mix for thirty seconds.

4- Incubate at room temperature for 60 minutes.

5- Remove the incubation mixture by flicking plate contents into washing container.

6- Rinse and flick microtiter well five times with distilled water.

7- Dispense 100 µl of tetramethylbenzidine (TMB) solution in each well for 10 seconds.

8- Incubate at room temperature in the dark for 20 minutes without shaking.

9- Stop the reaction by adding 100 µl of stop solution (1N HCl) to each well. The yellow colour does not absorbs at 450 nm.

10- Read optical density at 450 nm with microtiter well reader within fifteen minutes.

11- The results obtained are listed in Table -1-

2.0 *determination of T4*

The same procedure was used for determination of T_3 except the following:

2.1 Standard concentration of T_4 (0, 2, 5,10,15,25 µg/dl).

2.2 Sample volume, standard and control 25 µl.

primary testicular failure, renal failure and hypothyroidism [17].Testosterone (17β-hydroxyandrost-4-ene-3-one) is a C_{19} steroid with an unsaturated bond between C-4 and C-5, keton group in C-3 and a hydroxyl group in the β-position at C-17. This steroid hormone has a molecular weight of 288.4 daltons [18]. Testosterone is responsible for development of secondary male sex characteristics and its measurement is helpful in evaluating the hypogonal states [19]. In women, high levels of testosterone are generally found in hirsutism and virilization. Polycystic ovaries, ovarian tumors, adrenal tumors and adrenal hyperplasia, in men high level of testosterone are associated to hypothalamic pituitary disease, testicular tumors and prostate cancer, while low level of testosterone can be found in patients with hypopituitarism and some auto-immune disease [20].

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1.0 *determination of T_3*

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2- Dispense 50 µl of antibody reagent into each well, mix thoroughly for 30 seconds.

3- Add 100 µl of working conjugate reagent (0.1 ml of 50 T_3 –HRPO conjugate dilutent) and mix for thirty seconds.

4- Incubate at room temperature for 60 minutes.

5- Remove the incubation mixture by flicking plate contents into washing container.

6- Rinse and flick microtiter well five times with distilled water.

7- Dispense 100 µl of tetramethylbenzidine (TMB) solution in each well for 10 seconds.

8- Incubate at room temperature in the dark for 20 minutes without shaking.

9- Stop the reaction by adding 100 µl of stop solution (1N HCl) to each well. The yellow colour does not absorbs at 450 nm.

10- Read optical density at 450 nm with microtiter well reader within fifteen minutes.

11- The results obtained are listed in Table -1-

2.0 *determination of T4*

The same procedure was used for determination of T_3 except the following:

2.1 Standard concentration of T_4 (0, 2, 5,10,15,25 µg/dl).

2.2 Sample volume, standard and control 25 µl.

2.3 The reagent in applied kit for T_4 determination were used.

2.4 The results obtained are shown in Table -2-

3.0 *determination of TSH*

3.1 Reconstitute each lyophilized standard with 1 ml distilled water and allow the reconstituted material to stand at least 20 minutes.

3.2 Dispense 100 µl of standard (0, 0.5, 2.0, 5.0, 10.0, 25.0 µlu/ml), sample and controls in appropriate wells.

3.3 Repeat the same procedure for T_3 determination except TSH reagents should be applied.

3.4 The results obtained are listed in Table -3-.

4.0 *determination of prolactin*

4.1 Secure the desired number of coated well in the holder.

4.2 Dispense 50 µl of standard (0, 5, 15, 50, 100, 200 ng/ml), sample and controls in appropriate wells.

4.3 Dispense 100 µl of enzyme conjugate reagent into each well.

4.4 Gently mix for 10 seconds.

4.5 Incubate at room temperature for 45 minutes.

4.6 Remove the incubation mixture by flicking plate contents into sink.

4.7 Rinse and flick the microtiter wells five times with distilled water.

4.8 Strike the wells sharp onto absorbent paper.

4.9 Dispense 100µl tetramethylbenzidine (TMP) reagent into each well. Gently mix for 10 seconds.

4.10 Incubate at room temperature in the dark for 20 minutes.

4.11 Stop the reaction by adding 100 µl of stop solution to each well.

4.12 Gently mix for 30 seconds, the color changes from blue to yellow color.

4.13 Read the optical density at 450 nm with microtiter plate reader within 15 minutes.

4.14 The results obtained are listed in Table -4-.

5.0 *Determination of LH*

5.1 Dispense 50 µl of standard (0, 5, 15, 50, 100, 200 mlu/ml), sample and controls into appropriate wells.

5.2 Use the same steps for determination of prolactin except the reagent for LH should be used.

5.3 The results obtained are recorded in Table -5-.

6.0 *Determination of FSH*

6.1 Dispense 50 µl of standard (0, 5, 15, 50, 100, 200 mlu/ml), sample and controls in appropriate well.

6.2 Apply the same steps for determination of prolactin except the reagent in applied kit for FSH should be used.

6.3 The results obtained are listed in Table -6-.

7.0 *Determination of testosterone*

7.1 Secure the desired number of coated well in the holder.

7.2 Dispense 10 µl of standard (0, 0.1, 0.5, 2.0, 6.0, 18.0 ng/ml).

7.3 Dispense 50 µl of rabbit anti-testosterone reagent into each well.

7.4 Mix for 30 seconds.

7.5 Incubate at 37 C^o for 90 minutes.

7.6 Rinse and flick the micro-wells five times with phosphate buffer solution.

7.7 Dispense 100µl tetramethylbenzidine (TMP) reagent into each well.

7.8 Incubate at room temperature (18-25 C^o) for 20 minutes.

7.9 Stop the reaction by adding 100 µl of stop solution into each well.

7.10 Read the absorbance at 450 nm.

7.11 The results obtained are shown in Table -7-.

8.0 ***Determination of Hormones in Human Serum***

8.1 The blood sample (5 cc) was drawn from a healthy person (Age 45 years) after taking his agreement.

8.2 Blood sample was collected in a test tube and centrifuge to obtain serum.

8.3 The determination of hormones was carried out on fresh serum in the same day using the procedure for calibration standard curve of each hormone.

8.4 100 µl of the highest standard concentration of each hormone (100 µl) was added to the serum and the experiment repeated to estimate the recovery of each hormone in serum.

8.5 The results obtained are described in Table -8-.

9.0 ***Statistical Determination of Minimum Detectable Concentration (MDC) of hormones***

9.1 The minimum detectable concentration of hormones by ELISA assay were measured multiple by 2 SD from the mean of zero standard [21].

9.2 Standard Deviation (SD) for each hormone was calculated using the below equation

$$s = \sqrt{\frac{\sum(x - \bar{x})^2}{n - 1}}$$

Where

S = Standard Deviation

X = value obtained from each measurement

$\bar{x}$ = mean value of the measurements

n = number of measurements

9.3 The results obtained are listed in Table -9-.

Table (1):- The standard curve of T_3.

Standard T_3 (ng/ml)	Absorbance at 450 nm			mean
0	2.700	2.420	2.541	2.553
0.75	2.370	2.300	2.490	2.386
1.5	2.025	2.000	1.999	2.008
3.0	1.500	1.600	1.542	1.547
6.0	0.900	0.965	0.898	0.921
10.0	0.500	0.499	0.500	0.499

Table (2):- The standard curve of T_4.

Standard T_4 (µg/dl)	Absorbance at 450 nm			mean
0	2.650	2.600	2.613	2.621
2	1.780	1.702	1.768	1.750
5	1.040	1.030	1.290	1.120
10	0.720	0.700	0.722	0.714
15	0.600	0.600	0.585	0.595
25	0.392	0.400	0.391	0.394

Table (3):- The standard curve of TSH.

Standard TSH (µlu/ml)	Absorbance at 450 nm			mean
0	0.060	0.052	0.055	0.055
0.5	0.155	0.156	0.150	0.153
2.0	0.390	0.388	0.376	0.384
5.0	0.800	0.800	0.799	0.803
10.0	1.400	1.400	1.399	1.399
25.0	2.600	2.600	2.564	2.588

Table (4):- The standard curve of prolactin.

Standard Prol. (ng/ml)	Absorbance at 450 nm			mean
0	0.050	0.052	0.055	0.052
5	0.160	0.166	0.160	0.162
15	0.380	0.383	0.382	0.381
50	1.040	1.047	1.043	1.043
100	1.700	1.730	1.730	1.720
200	2.644	2.645	2.600	2.620

Table (5):- The standard curve of LH.

Standard LH (mIu/ml)	Absorbance at 450 nm			mean
0	0.041	0.040	0.043	0.041
5	0.140	0.146	0.148	0.144
15	0.328	0.325	0.328	0.327
50	0.946	0.940	0.945	0.944
100	1.657	1.650	1.656	1.654
200	2.710	2.700	2.720	2.710

Table (6):- The standard curve of FSH.

Standard FSH (mIu/ml)	Absorbance at 450 nm			mean
0	0.050	0.055	0.053	0.052
5	0.134	0.130	0.136	0.133
15	0.265	0.260	0.265	0.263
50	0.788	0.780	0.788	0.782
100	1.482	1.480	1.483	1.481
200	2.883	2.880	2.887	2.883

Table (7):- The standard curve of Testosterone.

Standard Testo. (ng/ml)	Absorbance at 450 nm			mean
0	3.072	3.045	3.025	3.063
0.1	2.750	2.700	2.690	2.713
0.5	2.175	2.180	2.190	2.181
2.0	1.699	1.700	1.740	1.713
6.0	1.100	1.105	1.120	1.108
18.0	0.500	0.510	0.520	0.510

Table (8):- Determination of hormones in human serum.

Hormones	Concentration of Hormone				%Recovery
	A	Normal value	Addition	B	
T_3	1.50	0.6 – 1.85 ng/ml	1.0 ng	2.25	90
T_4	8.0	5.0 – 13 µg/dl	2.5 ng	8.92	85
TSH	2.0	0.4 – 6.0 µu/ml	2.5 µu	3.60	80
Prolactin	5.0	3.0 – 14.7 ng/ml	2.5 ng	7.05	94
LH	6.4	1.24 – 7.8 mlu/ml	0.5 miu	6.07	88
FSH	10	11 mlu/ml	0.5 miu	8.19	78
Testosterone	3.5	3 – 10 ng/ml	0.5 ng	3.8	95

A = Actual concentration of hormone detected in human serum.

B = The concentration of hormone after the addition of known amount of it.

Table (9):- Statistical determination of (MDC) of hormones by ELISA.

Hormones	Measurements					SD	MDC
	X_1	X_2	X_3	$\bar{x}$	$\sum (x - \bar{x})$		
T_3	2.700	2.420	2.541	2.553	0.019	0.137	0.275
T_4	2.650	2.600	2.613	2.621	0.006	0.173	0.347
TSH	0.060	0.052	0.055	0.055	0.0156	0.125	0.250
Prol.	0.050	0.052	0.055	0.052	1.210	1.10	2.200

LH	0.041	0.040	0.043	0.041	0.902	0.95	1.900
FSH	0.050	0.055	0.053	0.052	2.102	1.45	2.900
Testosterone	3.072	3.045	3.025	3.063	0.0016	0.04	0.080

Discussion

The results in Table (1), (2), and (3) indicate the following:

1-The concentration of T_3 and T_4 are inversely proportional to the absorbance figure (1) and (2).

2-The concentration of TSH is directly proportional to the absorbance figure (3).

3-The results are reliable and reproducible but turbidity and hemolysis should give poor precision.

The measurements of prolactin, LH, and FSH are recorded in Tables (4-6), illustrate that concentration of hormones directly proportional to color intensity of the test samples figure (4-6). The determination of the hormone will give falsely elevated absorbance reading if insufficient wash used.The data in Table -7- indicate that the intensity of the color formed is proportional to the amount of enzyme present and is inversely related to the amount of unlabelled Testosterone concentration in the sample Fig. (7).

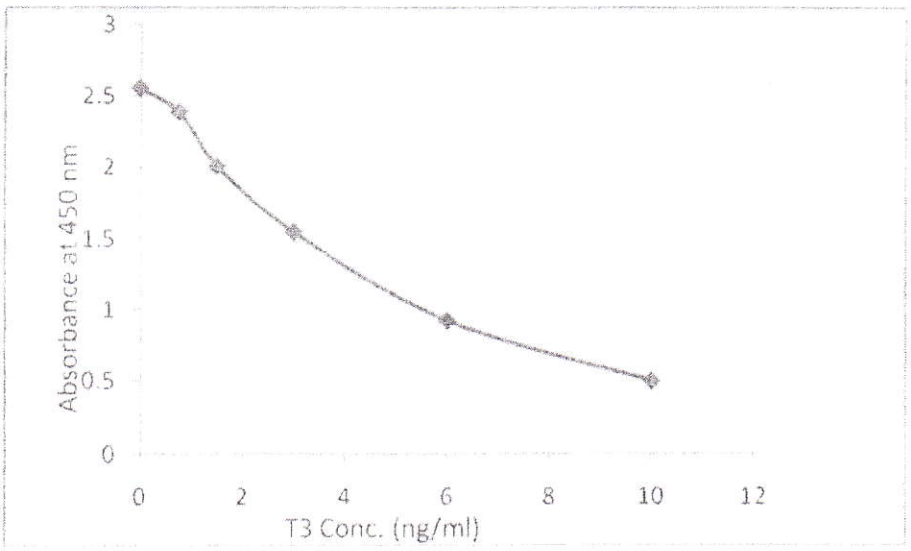
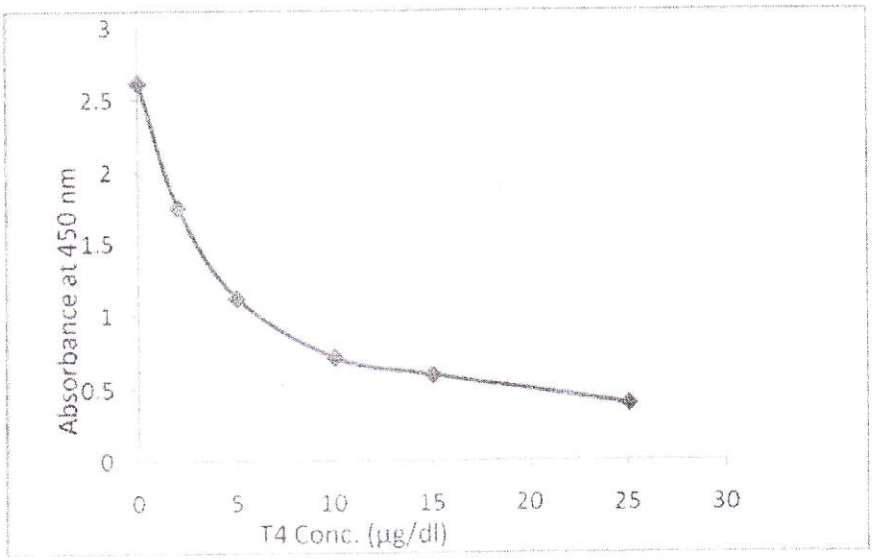


Figure (1):- Calibration curve of T_3.



Figure(2):- Calibration curve of T_4.

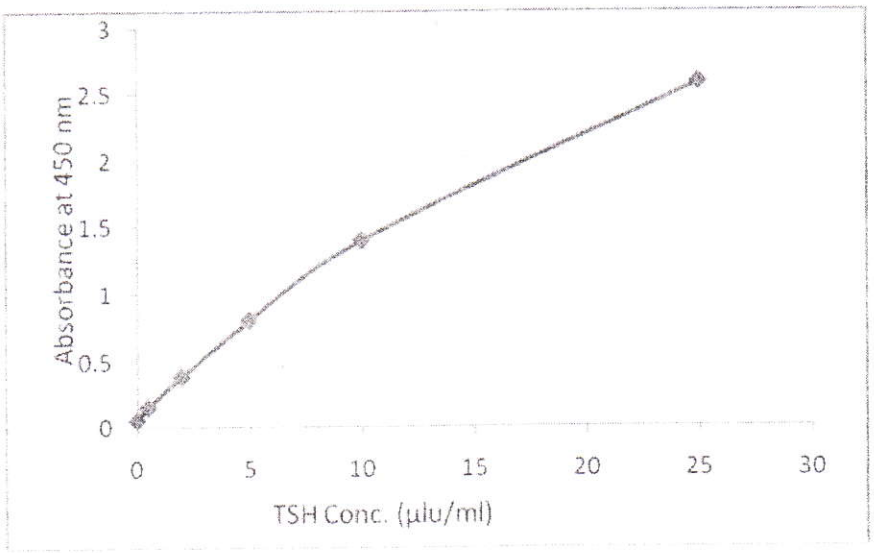


Figure (3):- Calibration curve of TSH.

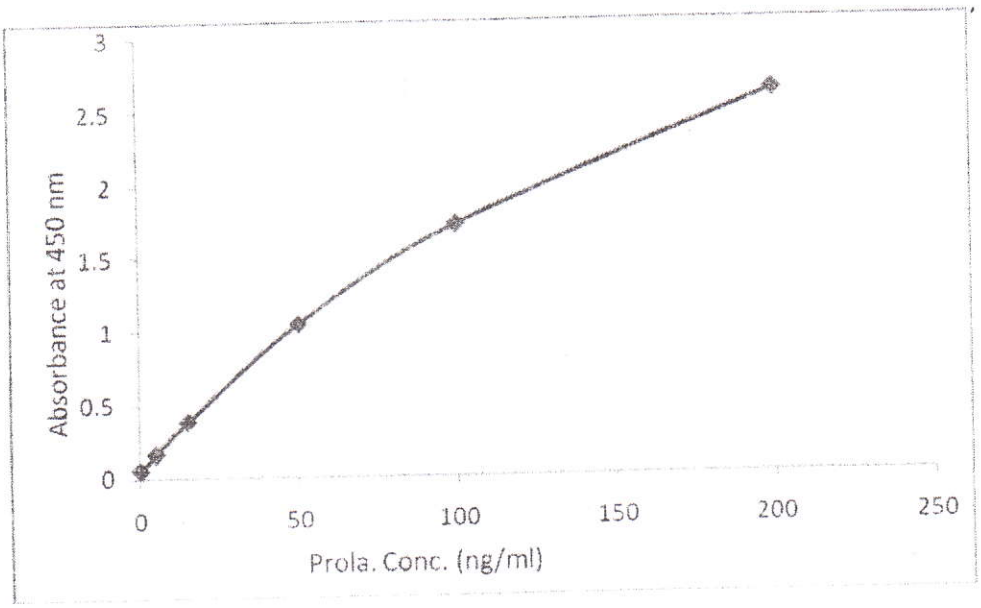


Figure (4):- Calibration curve of Prolactin.

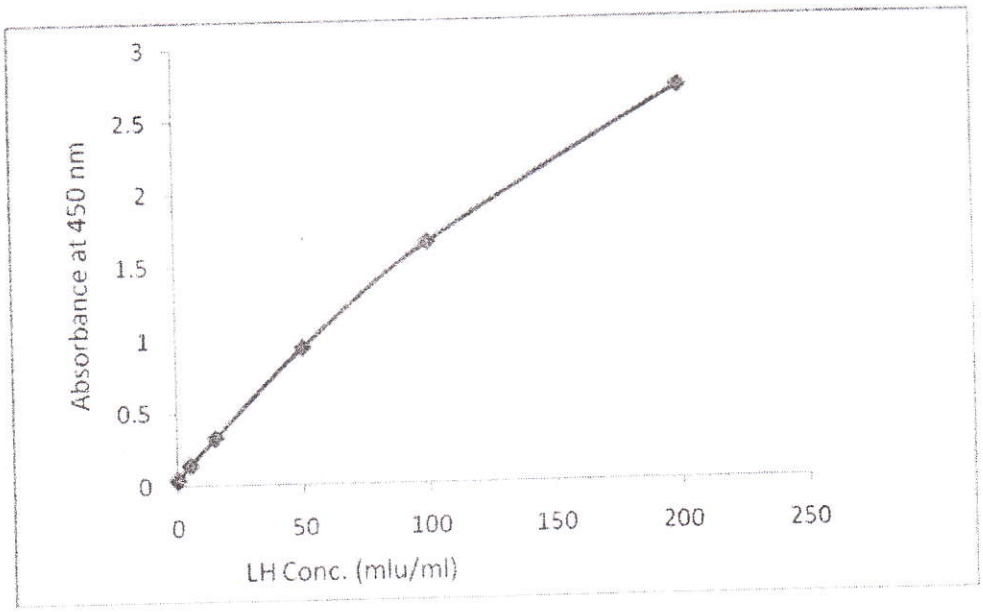


Figure (5):- Calibration curve of LH.

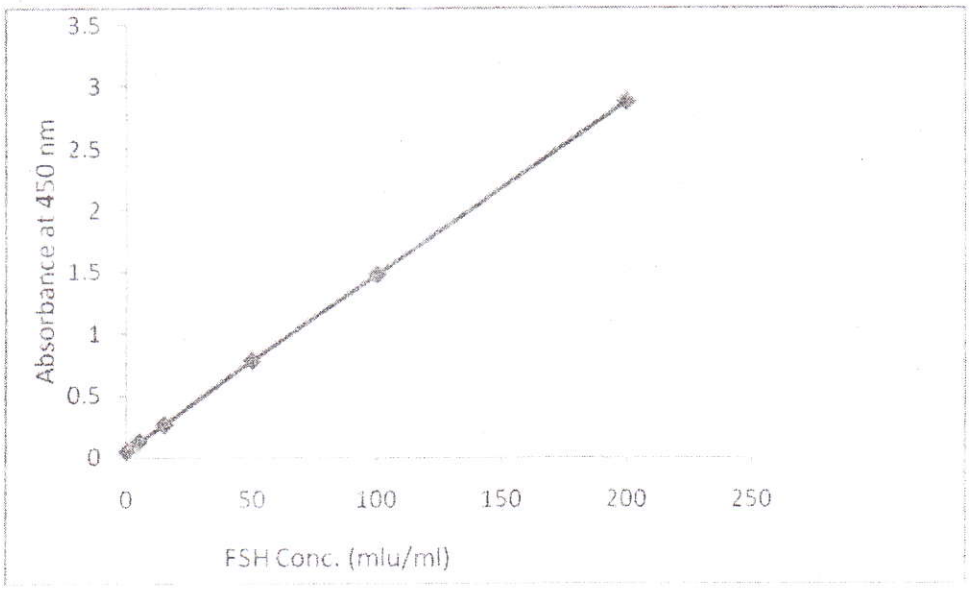


Figure (6):- Calibration curve of FSH.

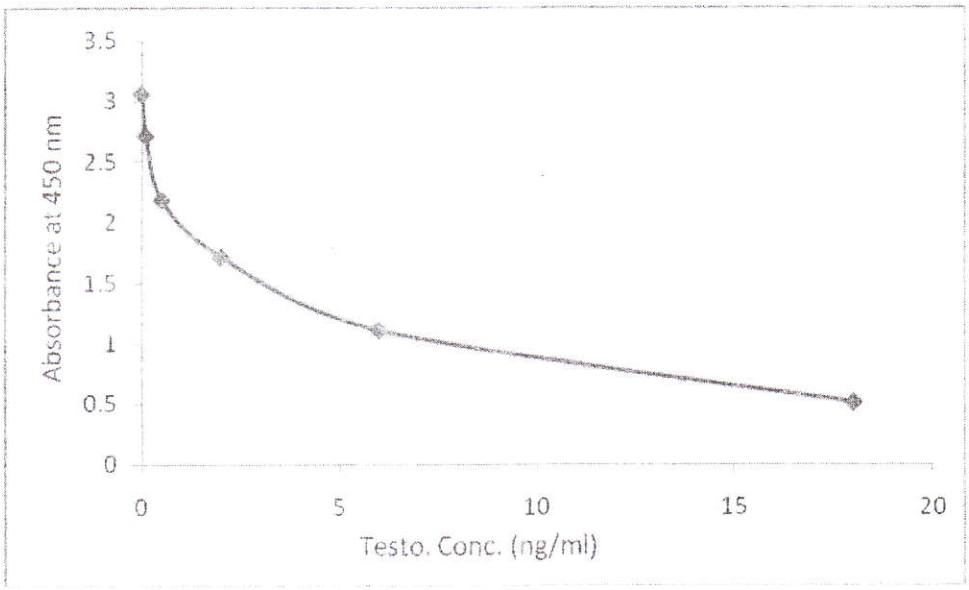


Figure -7- Calibration curve of Testosterone.

Many workers [22-25] estimated the MDC of hormone by ELISA, the results obtained in present work slightly difference from these works as shown in Table -10-.

Table (10):- Comparison between MDC detected by present work and other studies.

Hormones	MDC (present work)	MDC (other studies)
T_3	0.275	0.2 (Ref. 22)
T_4	0.347	0.4 (Ref. 22)
TSH	0.250	0.2 (Ref. 23)
Prolactin	2.20	2.00 (Ref. 24)
LH	1.90	2.00 (Ref. 23)
FSH	2.90	2.5 (Ref. 22)
Testosterone	0.08	0.05 (Ref. 25)

The difference in results is due to random errors, systematic errors and the conditions analysis used in the laboratory.

Conclusion

Reliable and reproducible results obtained from the use of previous procedures in this research. The sensitivity, precision, accuracy, recovery and minimum detection limit of analytical method are good enough for determination of hormones in human serum by enzyme Immunoassay technique. The serum sample demonstrating lipemia, hemolysis or turbidity should not be used in this technique due to elevated absorbance readings will give poor precision and falsely results. We recommended using enzyme immunoassay technique in determination of hormones in human serum because this method of analysis is cheap, available, easy to use and more sensitive than colourimetric method. Radioimmunoassay technique more sensitive than enzyme immunoassay but the first technique is expensive and difficult to use in determination of hormones in human serum.

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