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Comparative Study between Phenol and Imidazole Derivatives in Radiolabeling of Some Steroid Hormones

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ABSTRACT

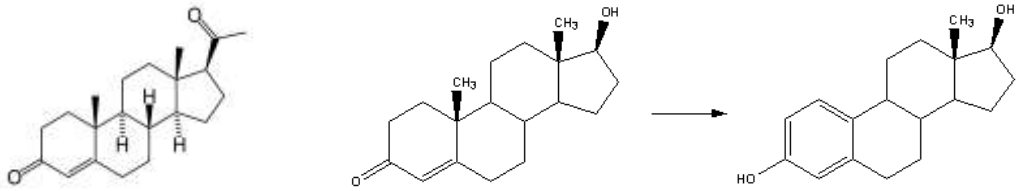
A phenol or imidazole ring is rarely present in steroid hormones, so, the molecule of steroid hormone requires chemical modification by addition of an iodinable residue like phenol or imedazole. So that the comparative study between phenol derivatives, include tyrosine methyl ester (TME) and tyramine, and imidazole derivatives, like histamine and histedine methyl ester (HME), for radiolabeling of some steroid hormones include estradiol, progesterone and testosterone is the aim of the present study. The conjugation step was carried out using mixed anhydride method and followed by radioiodination using iodogen as an oxidizing agent. Purification step was carried out using high performance liquid chromatography (HPLC). Optimization and validation of the tracer were carried out. Immunoreactivity of the all obtained tracers was check by using specific polyclonal antibodies. The results indicated that imidazols derivatives are more suitable from immunoreactivity view and storage period.

Key Words: ^{125}I, tracer, Radioimmunoassay, Steroid hormones, Imidazol, Phenol derivatives.

INTRODUCTION

A radioisotopically labeled steroid is used for the radioimmunoassay purpose and it is not possible to directly radioiodinate the cyclopentanophenanthrene nucleus or its substituents. Furthermore, some authors [1] reported that, although phenolic steroids can be radioiodinated directly at the 2 and 4 positions, this seems to alter the configuration of the molecule and affects the binding of the antibody because the physical dimensions of an iodine atom approximate those of the complete phenolic A ring in the molecule .

In classical iodination of protein it has either a phenol (III) or imidazole (IV) ring which is labeled. These groups are only rarely present in hapten (Scheme1), for example testosterone. Generally, the molecule requires chemical modification by the addition of an iodinable residue [2]. The most commonly used are the tyrosine methyl ester (II), histamine (VI) and Bolton & Hunter reagent. Imidazole (IV) containing molecules: histamine (VI) and histadine (V) is normal to conjugate the residue to hapten before or after iodination. Generally the peptide bond is carried out by the coupling reagent between carboxylic and aminic functions which can be present on either molecule [3, 4].



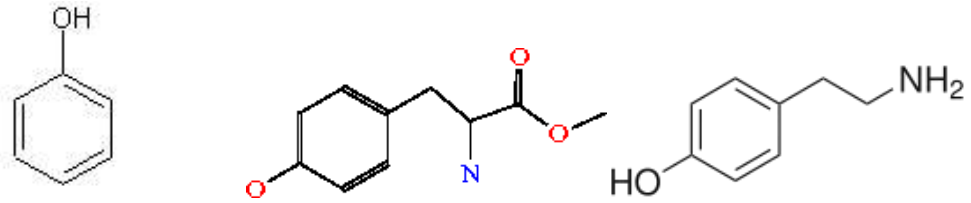
Progesterone **Testosterone** **Estradiol**

I II III

Scheme 1. Progesterone structure (I) and conversion of testosterone to estradiol by aromatase enzyme (II and III).

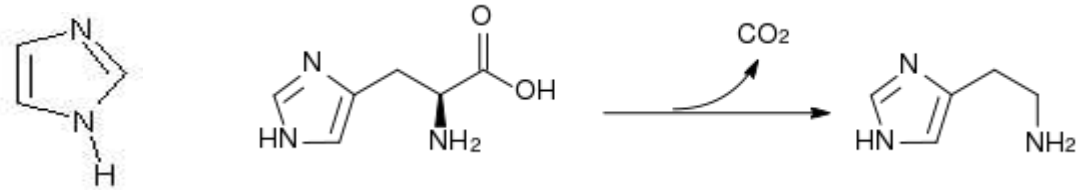
Recently, radioiodination of organic compounds and biomolecules have been the subject of interest of many investigators [2, 4] . This is due to that iodine atom occupies a similar volume to that of methyl or ethyl group and can substitute for an alkyl group in an organic molecule without unduly perturbing the steric or polar configuration. On the other hand, carbon-iodine bond has similar polarities to carbon-carbon bond. In order to quantify the steroid hormone present in assay tube, it is necessary to determine the amount of hormone bound to the antibody [5, 6]. A radioisotopically labeled steroid is used for this purpose and it is not possible to directly radioiodinate the cyclopentanophenanthrene nucleus or its substituents.

To incorporate ^{125}I into a steroid molecule one usually must prepare a conjugate of the steroid and an iodinatable aromatic species (generally histamine, tyramine or tyrosine methyl ester), and this is most conveniently done by using the same bridge structure (eg. hemisuccinate or carboxy methyl oxime) as appears in the steroid-protein conjugate that is used as the immunogen [7]. The greater the number of radioactive atoms incorporated per molecule of labeled hormone, the greater the number of radioactivity decay fragments which could show immunoreactivity. The shelf life can be extended somewhat by reducing the level of incorporation of radioiodine to one atom per molecule [8, 9].



Tyramine **L-tyrosine methyl ester** ***phenol***

I II III



IV **V** **VI**

Scheme 2. Tyramine (I), L-tyrosine methyle ester (II) , Phenol (III), Imidazole (IV) and decarboxylation of histidine (V) by histidine decarboxylase to histamine(VI).

In the present study, two groups one is phenol derivatives and the other is imidazole derivatives were conjugated with each estradiol, progesterone and testosterone followed by radioiodination with ^{125}I using iodogen as oxidizing agent.

MATERIALS AND METHODS

Progesterone 3-(0-carboxymethyle) oxime, progesterone, testosterone 3-(0-carboxy methyl) oxime , 17β-estradiol-17-hemisuccinate, highly purified testosterone, highly purified estradiol were obtained from Steraloids, Inc, UK . $Na^{125}I$ (5 mCi/50 □l) (185 MBq), pH 7-11 (MDS Nordion SA, Belgium), 1,3,4,6-tetrachloro-3 α,6α-diphenylglycouril (Iodogen), 8-Anilino-1-naphthalene sulphonic acid (ANS), histedine methyle ester (HME), histamine, Tyrosine methyl ester (TME) and tyramine hydrochloride, Polyethylene glycol 8000 (PEG), Bovine Serum Albumin were purchased from Sigma Chemical Co., USA. All other chemical reagents were analytical (AR) grade obtained from reputed manufacturers.

A. Radioiodination Methods:

1- Activation and conjugation of testosterone 3-(o-carboxy methyl) oxime with aromatic legands:

Testosterone 3-(0-carboxy methyl) oxime conjugated to HME or histamine was prepared by mixed anhydride coupling [10] .Firstly, 50 µl of testosterone 3-(0-carboxy methyl) oxime (5 mg/ ml in dioxin) was activated by addition 10 µl tri-n-butyl amine (2% dioxan) and 10 µl isobutylchloroformate (1% dioxin) then incubated at 10 ^{o}C for 45 minutes. 200 µl of dioxin were added to the mixture and 50 µl from this mixture were used to conjugate with 50 µl of HME (1.5 mg/ 5 ml of 0.5 M phosphate buffer). 20 µl of 0.2M NaOH were added and the mixture was incubated 1 hour then 1 ml 0.1 M HCL was added and mixed. The product was labeled in microgram quantities by using oxidizing agent and $Na^{125}I$.

Testosterone 3-(0-carboxy methyl) oxime was conjugated to tyrosine methyl ester (TME) or tyramine by mixed anhydride method according to Allen and Redshaw (1978)[10] technique with slight modification. 150 µl of 1:5 diluted tri-n-butyl amine were added to 1.1 ml of anhydrous dimethyl formamide containing 30 mg of progesterone 11α-hemisuccinate and the mixture was stirred at 10^{o}C for a few minutes and then treated with 150 µl of 1:10 isobutyl chloroformate and kept at 10 ^{o}C for 50 minutes. The remaining steps were exactly as aforesaid in conjugation with histamine.

2- Activation and conjugation of progesterone 3-(o-carboxy methyl) oxime with aromatic legands:

Progesterone 3-(0-carboxy methyl) oxime conjugated to HME or histamine was prepared by mixed anhydride coupling [10]. Firstly, 50 µl of testosterone 3-(0-carboxy methyl) oxime (5 mg/ ml in dioxin) was activated by addition 10 µl tri-n-butyl amine (2% dioxan) and 10 µl isobutylchloroformate (1% dioxin) then incubated at 10 ^{o}C for 45 minutes. 200 µl of dioxin were added to the mixture and 50 µl from this mixture were used to conjugate with 50 µl of HME (1.5 mg/ 5 ml of 0.5 M phosphate buffer). 20 µl of 0.2M NaOH were added and the mixture was incubated 1 hour then 1 ml 0.1 M HCL was added and mixed. The product was labeled in microgram quantities by using oxidizing agent and $Na^{125}I$.

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dimethyl formamide containing 30 mg of Progesterone 3-(0-carboxy methyl) oxime and the mixture was stirred at 10°C for a few minutes and then treated with 150 µl of 1:10 isobutyl chloroformate and kept at 10°C for 50 minutes. The remaining steps were exactly as a foresaid in conjugation with tyramine hydrochloride.

3- Iodination of progesterone and testosterone derivatives:

The iodination was carried out and the radiolabeled products were investigated in terms of percent of each of radiochemical yield, radiochemical purity and immunoreactivity [11], [12]. These methods are as follows: One mg of iodogen was dissolved in 2.5 ml of dichloromethane and 50 µl of this solution were dispensed in the bottom of a glass iodination vial and evaporated to dryness under nitrogen gas. The coated film of iodogen was obtained by completely removing dichloromethane and ensures that iodogen doesn't form a suspension which may give variable iodinations. These dried vials may be stored for up to 6 months at -20°C [13].The iodination reactions were directly performed according to Fraker and Speck (1978) [14]. An aliquot (10 µl) of the ethyl acetate containing 2.5 µg of steroid conjugate were evaporated under nitrogen and the residue redissolved in 10 µl of 0.5M phosphate buffer, pH 7.4. Then the mixture was transferred to coated glass tube and treated with 10 µl Na ^{125}I (1.0 mCi, 37 MBq) and 10 µl of 0.5M phosphate buffer, pH 7.4.The reaction mixture were mixed for 10 minutes ensuring that the reactants were in contact with iodogen film. The reaction was terminated by addition of 100µl of 0.05 phosphate buffer and all mixture contents were transferred to another tube. 30 µl of 0.5 mg/ml potassium iodide were added as a carrier.

4- Indirect radioiodination of estradiol:

HME or histamine was iodinated using iodogen as follows: into a iodogen coated glass test tube, 2.4 µg of HME or histamine in ethanol, 20 µl of phosphate buffer (0.05 mol/L, pH 7.4) and 0.5 mCi of $Na^{125}I$ in a volume of 5 µl were added. The mixture was incubated at room temperature for about 10 minutes with occasional stirring, then 20 µl phosphate buffer (0.05 M, pH 7.4) was added. The mixture was transferred into plain glass tube and cooled to 5 – 10 °C on an ice. Estradiol derivatives were prepared by coupling the respective β-estradiol 17-hemisuccinate with ^{125}I-HME, ^{125}I-histamine by a modified method of Stupnicki et al.,(1987) [15] as follows: 10 µg of β-estradiol 17-hemisuccinate and 100 µg of p-(N-dimethyle amino)-pyridine were dissolved in 3 ml of dry dimethyl formamide (DMF). This mixture was added to previous cooled ^{125}I-HME hydrochloride mixture and 20 µl of N-ethyl morpholine was added. The mixture was cooled down to 0 °C, and 0.5 mg of dicyclohexyl carbodiimide in 0.2 ml of DMF was added. The mixture was stirred for 1h at 0 °C, and then 24 h at room temperature. The reaction was monitored by silica gel TLC in choloform: ethanol (9:1). DMF was partially evaporated in a vacuum rotary evaporator. The remaining liquid was transferred to 3.0 ml of water and extracted 3 times with 2.0 ml chloroform. The combined organic phases were extracted with water once, dried with anhydrous $MgSo_4$. Then it was reconstituted in 100 µl of HPLC elution buffer and injected on to HPLC.

Comparative study between different all tracers was carried out in terms of radiochemical purity, radiochemical yield, immunoreactivty, specific activity, storage period and validity of them.

5- Purification of ^{125}I-steroid tracer:

The iodination mixture was purified using HPLC column (ODS-H-OL5-27883, Capital Analytical LTD, England) previously equilibrated with the elution buffer. Thirty-five fractions were

collected on a fraction collector and the radioactivity of each fraction was counted. Then the tracer obtained was diluted with 0.05M phosphate buffer pH 7.4, containing ANS (0.2%) and NaN_3 (0.1%). Elution profile was constructed by drawing activity (μCi) against fraction numbers.

6- Radiochemical purity:

High radiochemical purity was obtained for iodogen methods, (96.7 %), using paper electrophoresis chromatography. The results obtained are in good agreement with Kothari et al., (1995) [16] who reported that radiochemical purity obtained was greater than 95% for different batches of ^{125}I-steroid tracers using iodogen method

7- Molar ratios and specific activity:

Different molar ratios between steroid hormones and aromatic derivatives were investigated followed by determination of specific activity as presented in Table (2).

8- Immunoreactivity check and stability of the tracers:

The immunoreactivity of the tracer prepared was estimated by examining its binding with specific antiserum and the results presented in maximum binding percent (Bo%) , non specific binding percent (NSB %), displacement percent (Bo%-Bs% / Bo% x 100) and sensitivity as indicated in Table (3) . Storage period was studied at storage temperature +4 ^{o}C as shown in Table (4).

RESULTS AND DISCUSSION

A- Characterization of ^{125}I-steroid Tracer:

Extensive studies were undertaken for the preparations of ^{125}I-steroid tracer. The mechanism behind these preparations is the electrophilic substitution reaction to establish an ideal iodination procedure for steroid 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:

Fig. (1), shows three peaks corresponding to free ^{125}I (15.4 %), ^{125}I-tyramine (30.6 %) and ^{125}I-steroid tracer (51.4 %). In the same way, the other results corresponding to another aromatic ligands conjugated with steroids presented in Table (1).

2- Radiochemical purity:

High radiochemical purity was obtained for iodogen methods, (96.7 %), using paper electrophoresis chromatography. The results obtained are in good agreement with Kothari et al., (1995) [16] who reported that radiochemical purity obtained was greater than 95% for different batches of ^{125}I-steroid tracers using iodogen method.

In summary, cumulative data for the preparation of ^{125}I-steroid tracers using different aromatic ligands in terms of percent of each of radiochemical yield and radiochemical purity were presented in Table (1).

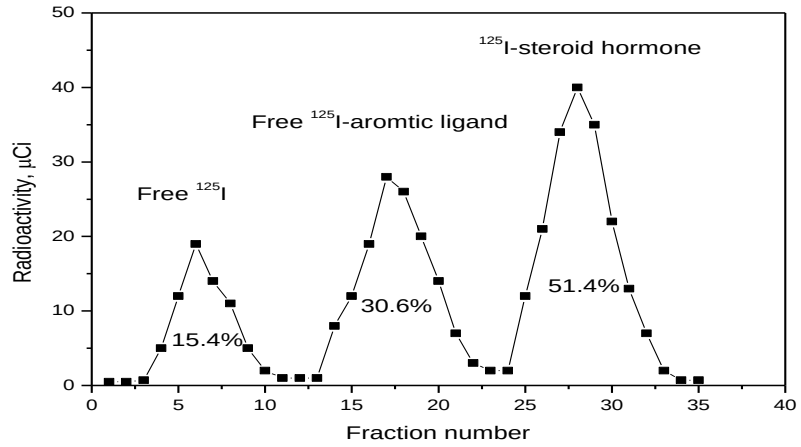

Fig. 1. Purification of ^{125}I-steroid hormones using HPLC technique.

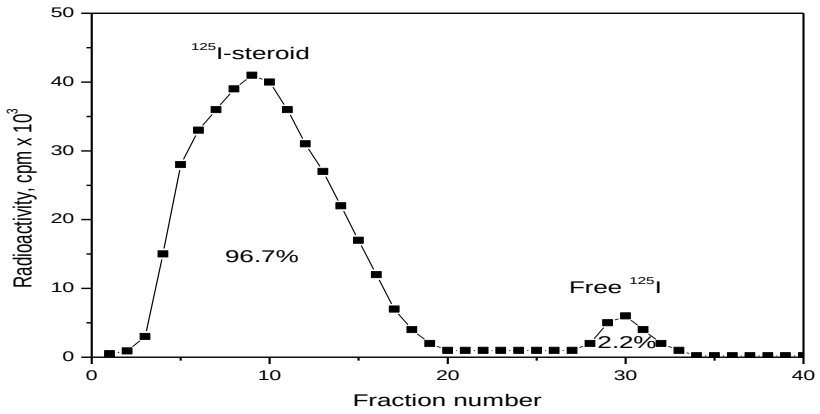

Fig. 2. Electrophortical pattern to determine radiochemical purity of ^{125}I- steroid hormones using paper electrophoresis.

Table 1. Radiochemical yield % and radiochemical purity%

Steroid hormones	Aromatic legends	Free ^{125}I % ±SD	^{125}I-aromtic legends% ±SD	^{125}I-steroid hormones% ±SD	Radiochemical purity % ±SD
Testosterone	**Histamine**	13 ± 1.5	22±3.0	65±4.0	97±1.2
	HME	12 ± 1.6	24±2.5	64±3.0	98±1.3
	TME	14 ±1.2	27±2.5	59±3.5	95.5±1.2
	Tyramine	**15.4±1.8**	**30.6±2.6**	**51.4±2.8**	**94.9±1.1**
Progesterone	**Histamine**	16±2.0	30±3.0	54±3.0	98±1.1
	HME	18±2.1	31±3.0	51±2.7	96±1.4
	TME	18±1.9	29±2.8	53±3.1	97±1.3
	Tyramine	23±2.5	27±2.7	50±2.5	95.5±1.5
Estradiol	**Histamine**	10±1.5	18±2.5	72±3.3	96.4±1.3
	HME	11±1.2	16±2.4	73±3.6	95.2±1.5
	TME	10±2.1	19±2.3	71±2.8	95.3±1.7
	Tyramine	13±2.0	18±2.2	70±2.6	95.8±1.8

3- Immunoreactivity:

The results obtained are presented in Table (2). The results obtained show very interesting and consistent findings. While imidazol derivatives gave a lower binding percents (Bo %) and non specific binding percents (NSB %), a higher sensitivity was obtained.

Table 2. Immunoreactivity check and stability of the tracers

Steroid hormones	Aromatic legends	Bo% ±SD	NSB% ±SD	Displacement% ±SD	Sensitivity ±SD
Testosterone (ng/ml)	Histamine	39±1.2	1.5±0.4	65±2.1	0.22±0.11
	HME	38±1.1	1.4±0.3	66±2.3	0.25±0.12
	TME	42±1.3	1.6±0.5	61±2.4	0.35±0.11
	Tyramine	43±1.2	1.8±0.6	62±2.3	0.42±0.12
Progesterone (ng/ml)	Histamine	38±1.1	1.9±0.5	66±2.5	0.20±0.10
	HME	37±1.2	1.8±0.7	67±2.6	0.21±0.12
	TME	40±1.1	2.1±0.9	60±2.2	0.62±0.12
	Tyramine	41±1.2	2.2±0.8	61±2.4	0.72±0.13
Estradiol (pg/ml)	Histamine	39±1.0	2.1±0.8	68±2.7	1.5±0.14
	HME	41±1.3	2.3±0.7	67±2.5	1.6±0.80
	TME	44±1.5	2.7±0.8	62±2.4	2.0±0.90
	Tyramine	45±2.0	2.5±0.5	63±2.6	2.1±0.90

5- Molar ratios and specific activity.

Using tracers with different specific activities ranged from 100 – 500 □Ci/□g, the maximum binding percent (Bo %) and non-specific binding percent (NSB %) were studied. The results in Table (3) show that with increasing the specific activity □Ci/□g, the maximum binding percent was increased. The results reveal that the tracer obtained with imidazole derivatives have a highest specific activity than phenol derivatives. These results are in agreement with Korde et al., (2001**)** [17]**.**

Table 3. Molar ratios and specific activity

Steroid hormones	Aromatic legends	Expected molar ratios	Optimum experimental molar ratios ±SD	Expected Specific activity (μCi/μg)	Optimum experimental specific activity(μCi/μg) ±SD
Testosterone	Histamine	1 :3.0	1:5± 0.2	250	230 ±10
	HME	1 : 1.5	1:4±0.2	250	220 ± 11
	TME	1: 1.5	1:4±0.3	250	190 ±13
	Tyramine	1 : 2.0	1:3.5±0.4	250	182 ±12
Progesterone	Histamine	1: 3.5	1:6±0.3	300	255 ±12
	HME	1: 1.6	1:4±0.2	300	240 ±14
	TME	1 :1.7	1:4±0.2	300	210 ±11
	Tyramine	1 : 2.0	1:5±0.3	300	200 ±10
Estradiol	Histamine	1: 3.5	1:7±0.3	200	170 ± 8
	HME	1: 1.5	1:4±0.3	200	160 ± 7
	TME	1: 1.5	1.4±0.2	200	150 ± 8
	Tyramine	1 :1.2	1:3.5±0.5	200	145 ± 7

6- Stability of steroid tracers:

The tracer stability was determined by calculating the maximum binding (B_o %) by RIA after 1, 6 and 12 weeks where stored at 4 ^oC. The results of this study, indicated that the binding (B_o %) decreased as the age of the tracer preparations increased. The period which Bo% decrease to 50 % from starting point

was determined and the results presented in Table (4). The results show the stability of tracers whose imidazole derivatives has longer shelf life than phenol derivatives.

Table 4. Stability of the tracers

Steroid hormones	Aromatic legends	Maximum period of Stability (weeks)	Maximum period of Stability (days)	Bo% after this period
Testosterone (ng/ml)	Histamine	14	98	20±1.2
	HME	15	105	19±1.1
	TME	12	84	21±1.3
	Tyramine	12	84	21±1.2
Progesterone (ng/ml)	Histamine	15	105	19±1.1
	HME	15	105	18±1.2
	TME	13	91	20±1.1
	Tyramine	13	91	20±1.2
Estradiol (pg/ml)	Histamine	16	112	20±1.0
	HME	15	105	20±1.3
	TME	13	91	22±1.5
	Tyramine	13	91	23 ±2.0

B- Steroid Standards:

The stock steroid standards were prepared in ethyl alcohol (1 mg/ml) [18, 19]. The dilution was carried out with steroid assay buffer as assay matrixes to get the required assay concentrations.

C- Polyclonal antibody:

The polyclonal antibody which produced in our laboratories before were used.

The assay design can be summarized in the following steps [15]: In 12 mm x 75 mm plain polystyrene tubes, 100 µl of estradiol standards or unknown samples and l00 µl of ^{125}I-estradiol tracer were incubated with 100 µl of estradiol antibody (1: 6000) for 3 hrs at room temperature. At the end of the incubation time, one ml second antibody assisted PEG (8000, 12%) and γ- globulin (1.5%) was added into all assay tubes. After vortex mixing, the tubes were incubated for 30 minutes at room temperature and then centrifuged at 4000 rpm for 20 minutes. The tubes were decanted carefully and the sediment containing the precipitate antibody-antigen complex was counted in a multi-crystal gamma counter and the results were calculated as indicated in Fig.(3).

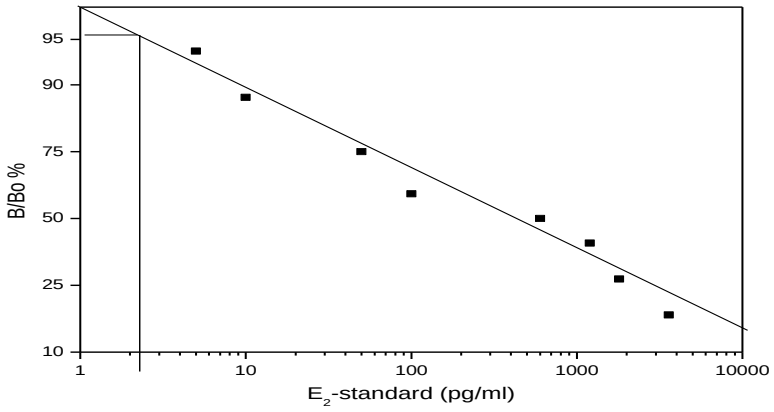

Fig. 3. Typical standard curve of E_2 RIA system.

5- Method comparison: -

The validity of these tracers was tested by comparing the results of steroid hormones levels of 100 serum samples obtained by IZOTOP reagents with those obtained by our technique. Results are comparable with correlation coefficients indicated in Fig. (4). The equation: Y= a + b X which (a) and (b) were calculated from regression line and presented in Table (4). The results show the sample value has a higher value when the imidazole derivatives used as tracer than phenol derivatives.

CONCLUSION

In conclusion the imidazoles derivatives are more suitable to conjugate with steroid hormones for labeling than phenol derivatives. This due to a high sensitivity, a high radiochemical yield %, a high radio chemical purity, more stable, a high specific activity and more accurate method. So these advantages may suggest that imidazoles derivatives are more preferable in the labeling of steroid hormones.

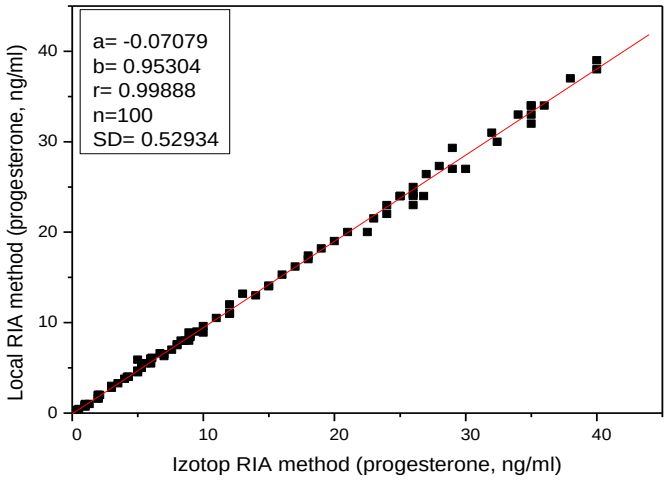

Fig. 4. : Regression line equation and correlation coefficient "r" between testosterone values obtained by IZOTOP method and the local liquid phase.

Table 5. The results of method comparison:

Steroid hormones	Aromatic legends	a	b	r
Testosterone (ng/ml)	Histamine	0.02	0.978	0.9979
	HME	0.015	0.987	0.9977
	TME	-0.070	0.944	0.9987
	Tyramine	-0.066	0.934	0.9976
Progesterone (ng/ml)	Histamine	0.014	0.989	0.9989
	HME	0.013	0.990	0.9987
	TME	-0.069	0.932	0.9987
	Tyramine	**-0.070**	**0.953**	**0.9988**
Estradiol (pg/ml)	Histamine	0.020	0.987	0.9948
	HME	0.025	0.988	0.9986
	TME	-0.045	0.934	0.9987
	Tyramine	-0.055	0.936	0.9988

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