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Spectroscopic Studies in UV region of Different Purified Isoforms of Testosterone Receptors in Human Ovarian Tumor Homogenates.

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Abstract :The aim of this study is to characterize the testosterone receptors spectrophotometrically in ovarian tissue homogenates of patients with benign and malignant ovarian tumors. Two groups of ovarian tumor patients were included in this study .Group I contained 31 patients with benign ovarian tumor .Group II consisted of 18 patients with ovarian cancer.Spectroscopic characterization in the UV region was carried out on purified testosterone receptors in human ovarian tumors and the effects of pH and polarity on the spectra were also studied.Spectrophotometric pH titration and several spectral changes were obtained in the presence of different polar and non-polar solvents, like the alteration of λmax position and intensities of protein spectrum, and the appearance of new chromophores on the surface of protein molecule .These chromophores where embedded in an interior region of the protein in the absence of the solvent. The difference in pH and polarity of the solvents is very important thing to characterize the protein molecules spectrophotometrially because they change the positions and values of molecules λmax in the UV region.

Key words : UV region , Isoforms of Testosterone Receptors ,Human Ovarian Tumor

Introduction

The receptors of testosterone are nuclear receptors .The nuclear receptors are ligand-controlled transcriptional regulators that function as activators or repressors of transcription (1) . The nuclear receptors are involved in the regulation of numerous physiological processes and are therefore important medical targets (2).
The ultraviolet absorption maxima of proteins undergo shifts to shorter wavelengths and slight decreases obtained in intensity when the secondary and tertiary structures of the molecules are disrupted (3, 4) .In the interpretation of this effect attention has been directed particularly to the tyrosyl side chains, because of the possible contribution of their phenolic hydroxyl groups to the stability of the tertiary structure of proteins via hydrogen bonding (5).
The absorption spectrum of a chromophore is primarily determined by the chemical structure of the molecule .However, a large number of environmental factors produce detectable changes in λmax and Ɛ. Environmental factors consist of pH, the polarity of the solvent or neighboring molecules, and the relative orientation of neighboring chromophores .It is precisely these environmental effects that provide the basis for the use of absorption spectroscopy in characterizing macromolecules (6, 7). The solvent influences the conformation by acting as a polarizable macroscopic dielectric medium, by copeting with intramolecular interaction, and by contributing a "solvent force" (8-10).
One of the main assumptions of the solvent perturbation technique is that solvents alter the peak positions and intensities by altering the energy and probability of electronic transitions . Other considerations include the following (11-14):
polarization effect,(b) Changes in permanent dipole moment during excitation i.e., the dipole hydrogen banding, which will tend to produce either a short wave or long wave shift depending on the nature of the electronic transition and weather the solute is the hydrogen donor or hydrogen acceptor.
Accordingly, the aim of the work in this paper includes the spectroscopic studies on testosterone receptors in ovarian tissue homogenates of patients with benign and malignant ovarian tumors.

Experimental

Chemicals

All laboratory chemicals and reagents were of analar grade and were used without further purification .Tris (hydroxy methyl) aminomethan, dimethyl sulfoxide, were obtained from **Fluka Company, Switzerland .**NaCl, PEG-10000, glycerol, hydrochloric acid, NaOH, ethanol, were obtained from **BDH limited pool, U.K .** Sepharose CL 6B, dextran T-70, blue dextran 2000 were obtained from Pharmacia Fine Chemicals, **Switzerland .**Kit of radioactive testosterone)^{125}I-testosterone (was purchased from CIS Bio International (**France)** .The activity of the labeled testosterone was approximately 5µci.

Instruments

The instruments used in this work were, LKB gamma counter type 1270-rack gamma II, LKB spectrophotometer ultraspec type 4050, Pye-unicom pH meter, Varian DMS 100 UV -visible spectrophotometer, LKB ultracentrifuge type 2332, Memmert water bath, Memmert incubator.

Patients

Two groups of ovarian tumor patients were included in this study .Group I contained 31 patients with benign ovarian tumor .Group II consisted of 18 patients with ovarian cancer .All patients were admitted for treatment to the Medical City and Al-Arabe Hospital under the supervision of specialists .The patients were newly diagnosed and not underwent any type of therapy .Patients did not suffer from any disease that may interfere with our study were excluded.

Collection of Ovarian Tissue Specimens

The tumor tissues were surgically removed from ovarian tumor patients by oophorectomy . The specimens were cut off and immediately rinsed with ice-cold isotonic saline solution .They were collected individually in plastic receptacles and stored at -20°C until homogenization.

Preparation of Ovarian Tumor Tissue Homogenate

The frozen tissues were weighed, pulverized finely with a scalpel in petri dish standing on ice bath, and then homogenized at 4°C in buffer solution with a ratio of 1:5 (weight :volume), using a manual homogenizer .The buffer used was Tris-EDTA (Tris-HCl 0.01M, pH 7.4, containing 0.15 mM EDTA, 2-mM mercaptoethanol and 10 %glycerol) .

The homogenate was filtered through several layers of nylon gauze to eliminate fibers of connective tissue, and then centrifuged at 2000 xg for 30 minutes at 4°C .The sediment was suspended in 10 volumes of TEMG buffer for 15 minutes at 4° C and then suspension was used to obtain the crude nuclear fraction.

Solutions

All buffer solution was prepared [15] by dissolving the appropriate amount of salts in distilled water and the required pH was adjusted . The stock solution of 0.2 M Tris (hydroxymethyl aminomethane) was prepared; other reagents were prepared as following [16] :

1. TEMG buffer (pH 7.4) :Tris (0.01 M, pH 7.4) buffer containing 0.15 mM Na_2EDTA, 2 mM mercaptoethanol and 10 %glycerol.
2. Dixtran-coated charcoal (DCC)solution :Tris (0.01M) buffer, pH 7.4 containing 1.25 % -charcoal, 0.6 %dextran-70, and 0.2 %gelatin .

Methods

Purification of nuclear testosterone receptors using gel filtration technique

Preparation of sepharose CL-6B Gel

The swollen gel was suspended and carefully poured into the glass column) with diameter of 1.5 cm) down the wall .After the gel has set, the column outlet was opened; continuing packing till the gel reached a stable bed height of (29 cm) and equilibrated with Tris-buffer pH 8.0.

Void volume (Vo) determination

Void volume of the gel column was determined by blue-dextran 2000 with concentration of 1mg per ml of Tris-buffer, pH 8.0. One milliliter of blue dextran solution was applied to the column surface carefully, then elution was carried out with the same buffer using a flow rate of 24 ml/hr., fractions of 2 ml were collected, and their absorbances were measured at 600 nm to estimate V_o.

The preparation of nuclear salt extracts

The frozen tissues weighed, pulverized finely with a scalpel in Petri dish standing on ice bath and then homogenized at 4°C in TEMG buffer solution with a ratio of 1: 5 (weight: volume) using a manual homogenizer. The homogenate was filtered through four layers of nylon gauze to remove tissue clumps and fibers of connective tissues. The filtrate fluid was transferred by Pasteur pipette to low-speed centrifuge tubes and prepares a crude nuclear pellet by centrifugation at 2000xg for 15 min. The supernatant was decanted and the pellet was resuspended in 10 volumes of TEMG buffer pH 8.0 for 15 min. Nuclei then allowed to swell at 4°C for 30 min in the same buffer. The nuclei then ruptured by the exposing them to sonic waves for forty, 30 seconds intervals. The tubes were kept immersed in ice during the entire procedure. Sonically ruptured nuclei solution was then sedimented in a refrigerated centrifuge at 2000xg for 30 min. The supernatant was then used as a source of nuclear testosterone receptors [17, 18].

Purification procedure

One milliliter of the nuclear salt extract (5 mg protein) was applied to the surface of Sepharose CL 6B column (1.5 × 29 cm) equilibrated with TEMG buffer pH 8.0. The sample was eluted using the same buffer. Fractions of 2 ml were collected at a flow rate of 24 ml/hr. The absorbance of the fractions collected was measured at 280 nm and the protein contents were determined by the method of Lowry et al [(19)].

The preliminary test of the binding of ^{125}I-testosterone to the purified fractions separated by gel filtration

Two-hundred microliters of purified fractions were added to 100 µl (31.2 PM) of ^{125}I-testosterone with and without the addition of 250 fold excess of unlabeled testosterone in a final volume of 1 ml completed with TEMG buffer. The tubes were incubated for 8 hrs at 4°C for malignant tumor homogenate and 25°C for benign one, the bound testosterone was measured as follows [(20)]:

1. The counted radioactivity in each tube (expressed in CPM) represents the total binding (TB).
2. The counted radioactivity (expressed in CPM) in the tubes contained labeled hormone and excess of unlabeled hormone represents the non-specific binding (NSB).
3. The specific binding (SB) was calculated by subtracting the radioactivity (CPM) obtained in the presence of unlabeled hormone from that produced in the absence of unlabeled hormone.
   SB (CPM) =TB (CPM)-NSB (CPM)
4. The percent of specific binding (SB%) can be calculated from the following formula:

Where:
SB%= [SB/TC]× 100.
SB: Specific binding (CPM), and
TC: Total count of ^{125} I-testosterone (CPM) used, in each tube.

Dialysis for concentration

The fractions that contained high level of testosterone receptors were pooled and concentrated by dialyzing against sucrose at 4°C for 30 min to get the needed concentration.

Calculations

1. The values of SB% for the eluted fractions were calculated in the same method as that of the previous experiments.
2. The values of SB% and absorbance at 280 nm were plotted against the fraction number.

Spectroscopic studies of different purified forms of testosterone receptors

The UV spectra of purified nuclear testosterone receptor in human benign and malignant ovarian tumors

One hundred microliters (350 µg protein) of each purified receptor was completed to 1 ml with TEM buffer pH 7.4, then placed in a 1 cm cuvette in sample beam and the absorption spectrum was immediately measured against TEM buffer as a reference.

Factors affecting the absorption properties of purified nuclear testosterone receptors in human benign and malignant ovarian tumors

pH effect

One hundred microliters (350 µg protein) of each purified receptor was completed to 1 ml with TEM, glycine-HCl buffer, and glycine-NaOH buffer at pH 7.4, 2.7, and 10.7 respectively. Then each of which was placed in test tube cell and the buffer in each case was placed in reference cell and the absorption spectra of different purified receptors were measured immediately.

Polarity effect

A) The effects of 20% ethanol on the testosterone receptor spectra

One hundred microliters (350 µg protein) of each purified receptor were completed to 1 ml with TEM buffer contains 20% ethanol at pH 7.4, then each of which was placed in the test cell and the buffer containing 20% ethanol was placed in the reference cell using 1 cm cuvette. The absorption spectrum of each sample was immediately measured.

B) The effect of 20% ethylene glycol on the testosterone receptor spectra

One hundred microliters (350 µg protein) of each purified receptor were completed to 1 ml with TEM buffer contains 20% ethylene glycol at pH 7.4, then each of which was placed in the test cell and the buffer containing 20% ethylene glycol was placed in the reference cell using 1 cm cuvette. The absorption spectrum of each sample was immediately measured.

C) The effect of 20% polyethylene glycol on the testosterone receptor spectra

One hundred microliters (350 µg protein) of each purified receptor were completed to 1 ml with TEM buffer contains 20% polyethylene glycol at pH 7.4, then each of which was placed in the test cell and the buffer containing 20% polyethylene glycol was placed in the reference cell using 1 cm cuvette. The absorption spectrum of each sample was immediately measured.

D) The effect of 20% DMSO on the testosterone receptor spectra

One hundred microliters (350 µg protein) of each purified receptor were completed to 1 ml with TEM buffer contains 20% DMSO at pH 7.4, then each of which was placed in the test

cell and the buffer containing 20% DMSO was placed in the reference cell using 1 cm cuvette. The absorption spectrum of each sample was immediately measured.

E) The effect of 20% urea on the testosterone receptor spectra

One hundred microliters (350 μg protein) of each purified receptor were completed to 1 ml with TEM buffer contains 20% urea at pH 7.4, then each of which was placed in the test cell and the buffer containing 20% urea was placed in the reference cell using 1 cm cuvette. The absorption spectrum of each sample was immediately measured.

Spectrophotometric pH titration of purified nuclear testosterone receptor in human benign and malignant ovarian tumors

A series of purified receptor (350 μg protein in 100 μl) were completed to 1 ml with distilled water at pH range from 6.0 to 12.0. The maximum absorbance of each sample was measured at a wavelength of 295 nm; the absorbance of λ_{max} at each pH value was plotted versus the corresponding pH.

Another series of purified receptors were completed to 1 ml with distilled water at pH range from 1.0 to 8.0. The maximum absorbance of each sample was measured at a wavelength of 211 nm. The absorbance of λ_{max} at each pH value was plotted against the corresponding pH.

Results and discussion

Purification and isolation of nuclear testosterone receptor using gel filtration technique

Purification and isolation of nuclear testosterone receptors were performed by gel exclusion chromatography technique. Benign and malignant homogenates were applied to Sepharose CL 6B (1.5 × 29 cm) column. The void volume of this column was (24 ml), as predicted from the elution profile of the blue dextran as shown in Fig. 1 A. The resultant fractions of each homogenate type were collected, detected for the binding with ^{125}I-testosterone pooled, concentrated and then subjected to protein determination.

This experiment revealed as shown in Fig. (1B &C) (Appendix II) the presence of two different eluted components. These two components eluted with different elution volume corresponding to their different molecular weights. From benign tumors homogenate, the first one eluted with about (22 ml) while the second one eluted with about (fraction 27), i.e. about (54 ml).

From malignant tumor homogenate, the first one eluted (22-ml) and the second one eluted with (56 ml).

These are two-androgen receptor protein isoforms (androgen receptor-B, apparent molecular weight approximately 110 KDa and androgen receptor-A, apparent molecular weight approximately 87 KDa) [21]. The fact is similar to the results obtained. The second components in benign and malignant tumor homogenate represent the purified testosterone receptors with a lower molecular weight than the first one.

The two types of androgen receptor protein isoforms are present in adult reproductive tissues (prostate, endometrium, ovary, uterus, fallopian tube, testis, seminal vesicls, myometrium, and ejaculatory duct) [21].

Spectroscopic studies on human ovarian testosterone receptors

The UV spectra of purified testosterone receptors in human benign and malignant ovarian tumor

Fig. 2&3 (Appendix II) illustrate the UV spectra of purified testosterone receptors at pH 7.4. The UV spectra show that the λ_{max} for purified receptor in benign tumor homogenate consists of two peaks; at 205.8 nm and 195.8 nm, in malignant tumor homogenate gives two peaks at 193.6 nm and 264 nm. As a result each human testosterone receptor has a characteristic spectrum and can be identified by their peaks. 195.8 And 193.6 are assigned to tyrosine residues, while the vibrational structure as a small "wiggles" at 205.8 and 264 nm is assigned to phynlalanine [22, 23].

Also it was found from the fig. 2&3 that tryptophan residues do not occur on the surface of benign and malignant receptors. It seems that each of tyrosine and phenylalanine residues in the testosterone receptor in the two cases of benign and malignant is located in a way that part of it is on the surface of the protein molecule while the other part is buried. The condition used for this experiment made the receptors intact and in the three dimension molecules to do their action, and that can be supported by binding reaction, therefore the absorbance peaks indicate that the chromophores are faced the radiation beam [24-26]. The folded case here means that the tyrosine and tryptophan residues are on the surface [27].

Factors affecting the absorption properties of testosterone receptors in human benign and malignant ovarian tumors

The absorption spectrum of a chromophore is primarily determined by the chemical structure of the molecule. However, a large number of environmental factors produce detectable changes in λ_{max} and ε. Environmental factors such as pH and polarity of the solvent provide the basis for the use of absorption spectroscopy in characterizing macromolecules [22].

PH effect

The pH of the solvent determines the ionization state of the ionizable chromophore in the protein molecule. Table 1 (Appendix I) shows the λ_{max} values of human testosterone receptors at

different pHs (2.7, 7.4, and 10.7). At pH 7.4, two λ_{max} were obtained for every case (benign and malignant tumor homogenate). At an acidic pH 2.7, benign purified receptor has a λ_{max} at 189.6 nm which were assigned to phenylalanine. In malignant purified receptor, two λ_{max} were obtained. The first one was at 192.5 nm, which assigned to tyrosine. The second was at 258.5 nm that assigned to phenylalanine.

When the pH value was increased from (7.4 to 10.7), an increase in the λ_{max} of tyrosine residue has been shown in all receptor types, this result is due to the dissociation of phenolic OH of tyrosine (pK_a = 10.07) giving an ionized form of this amino acid which absorps at higher wavelength (red shift) [22].

The spectral shifts of protein produced by pH cannot be simply attributed to the inductive effects of vicinal charges, such spectral changes must therefore be attributed mainly to rearrangement of secondary and tertiary structure, although the possibility of field effects due to unusually close conjunction of charges to aromatic groups is not excluded [23, 28].

Polarity effect on UV testosterone receptors spectra

The importance of this study comes from studying of the internal configuration of protein [29].

A) The effect of 20% ethanol

Table 2 (Appendix I) shows the effect of 20% ethanol at pH 7.4 on the testosterone receptors spectra. In benign case, purified testosterone receptor has two λ_{max}; at 278 nm and 291.8 nm. In malignant case, two λ_{max} have been shown in purified testosterone receptor spectrum, that is, at 274.2 nm and 211 nm. The values of λ_{max} 278 nm and 274.2 nm are assigned to tyrosine residues, while 291.8 nm assigned to phenylalanine residues and 211 nm is refered to histidine residues.

The appearance of new λ_{max} values indicates that the protein was folded to change in the secondary and tertiary structure of the protein that bring the histidine to expose to absorbance as well as other aromatic amino acids. It was found that testosterone receptors are highly sensitive to change in the polarity of the solvent [6].

B) The effect of 20% ethylene glycol

Table 3 (Appendix I) shows the λ_{max} values of testosterone receptor at pH 7.4. In benign ovarian homogenate, purified receptor gave additional λ_{max} values, that is, 218.8 nm which are assigned to tyrosine, but in malignant case no significant change in λ_{max} values. According to these results, the λ_{max} is rather shifted toward longer wavelength (red shift) due to hydrogen bonding of OH groups of tyrosine with the solvent or with the π-electron system of the benzene ring where tyrosine was functioned as a hydrogen donor [29]. The appearance of new chromophores indicates that the protein was fplded to the presence of ethylene glycol at this concentration.

C) The effect of 20% polyethylene glycol

Table 3 shows the λ_{max} values of testosterone receptor at pH 7.4. In malignant ovarian tumor homogenate, purified testosterone receptor shows additional λ_{max} at 212.8 nm and increase in the absorbency. In benign tumor, purified testosterone receptor has additional λ_{max} at 215.8 nm and increase in absorbency. The results obtained in the presence of 20% polyethylene glycol indicate that the protein structure has been changed and bring more residues of tyrosine on the surface of protein and expose tryptophan residues to the absorbance. The change in λ_{max} value may indicate that the protein is sensitive to changes in the polarity of the solvent, which indicate that a certain amino acid may be on the surface of the protein [30].

D) The effect of 20% dimethylsulfoxide

The presence of dimethylsulfoxide in the buffer of this concentration doesn't show significant changes on the purified testosterone receptor in two cases (benign and malignant). The difference in polarity of solvents differ the spectrum of receptor from one to another. This is another indicator that is the receptor protein is sensitive to changes in polarity of the solvent.

E) The effect of 20% urea

Table 3 shows the effect of urea on the testosterone receptor UV spectra at pH 7.4. The results obtained indicate that the chromophores (i.e., the tyrosine and phenylalanine residues) were buried inside the receptor molecules and tryptophan residues as well as histidine residues were appeared on the surface. Similar effect has been shown in benign and malignant tumor homogenates.

The results indicate that urea affects the testosterone purified receptors structurally, since many chromophores which were embedded in interior region of the receptor molecule where they were inaccessible to the solvent came into contact with it due to the unfolding the molecule, and hence, different spectra were obtained [28,29].

Spectroscopic pH titration of purified testosterone receptors in human benign and malignant ovarian tumor

Spectrophotometric pH titration is the following of the change in absorbance of the chromophore with increasing pH [22]. Many studies of protein structure require the determination of pK values for proton dissociation from ionizable amino acid side chains, because these values give an indication of the location of the amino acid in the protein. This can often be done spectrophotometrically because

dissociation often changes the spectrum of one of the chromophores, the observation of tyrosine dissociation was performed by measuring the absorption at 295 nm (λ_{max} for the ionized form of tyrosine), and the observation of histidine dissociation was carried out by measuring the absorption at 211 nm [(23)].

Fig. 3A&B (Appendix II) shows the titration curve of purified testosterone receptor in malignant and benign ovarian tumor respectively. Curve (A) shows that the pK_a for tyrosine is (10.3) for benign ovarian tumor and (10.1) for malignant one. While the pK_a of histidine is (5.8) for benign ovarian tumor and (5.7) for malignant one, these results are shown in curve B.

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Conclusion

The use of different solvents with different polarity gives good characterization of the protein molecules because some of buried chromophores come to the surface and would absorbe the light, which give complete picture about the molecule by comparison between each two cases of solvents. The spectroscopic titration is very important to complete the characterization of protein molecule.

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APPENDIX I: THE TABLES

Table (1): The pH effect on the $^{a}_{max}$ of testosterone receptor spectra.-

pH	Benign purified receptors ^{a}max (nm)	Malignant purified receptors ^{a}max (nm)
2.7	189.6	192.5, 258.5
7.4	195.8, 205.8	193.6, 264
10.7	192.1	193

Table (2): The effect of 20% ethanol on the testosterone receptor spectra.-

Sample	^{a}max (nm)
Benign	278, 291.8
Malignant	274.2, 211

Table (3): The effect of solvents on $^{a}_{max}$ of testosterone receptor spectra.-

Sample	-Benign $^{a}_{max}$ nm	Malignant $^{a}_{max}$ nm
20% ethylene glycol	195.8, 205.8, 218.8	193.6, 264
20% polyethylene glycol	215.8, 195.8, 205.8	193.6, 264, 212.8
20% DMSO	195.8, 205.8	193.6, 264
20% urea	195.8, 205.8	193.6, 264

APPENDIX II: THE FIGURES

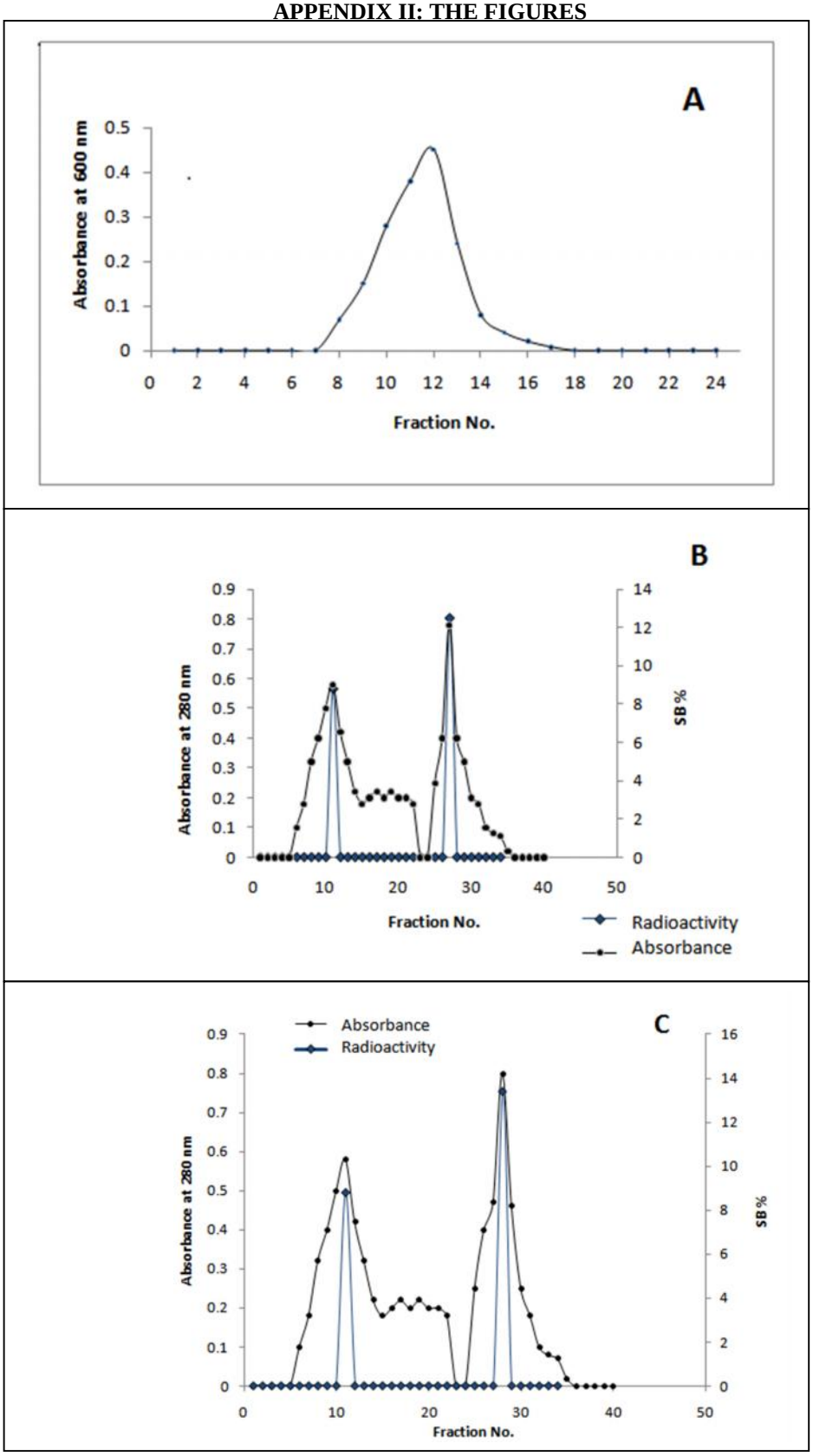

Fig (1) Elution of A) blue dixtran 2000 B) testosterone receptors from ovarian benign tumor homogenate C) testosterone receptors from ovarian malignant tumor homogenate.

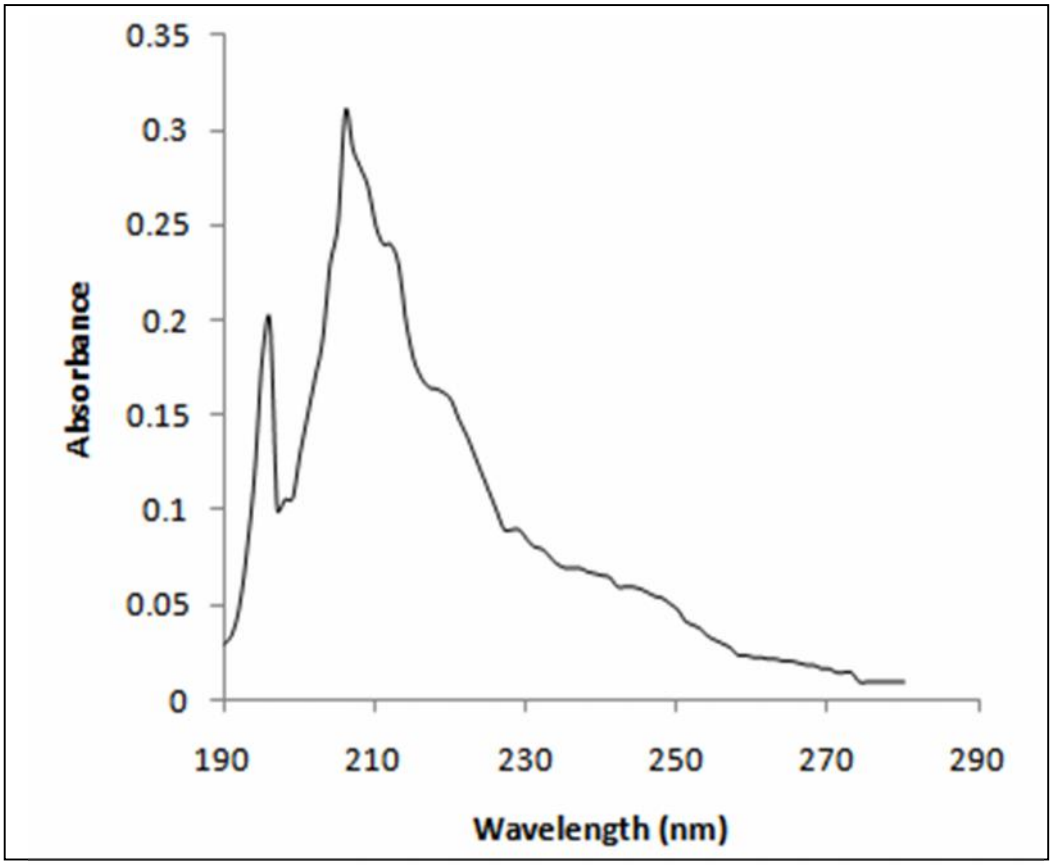

Fig. (2) The UV spectrum of testosterone receptors in benign ovarian tumor

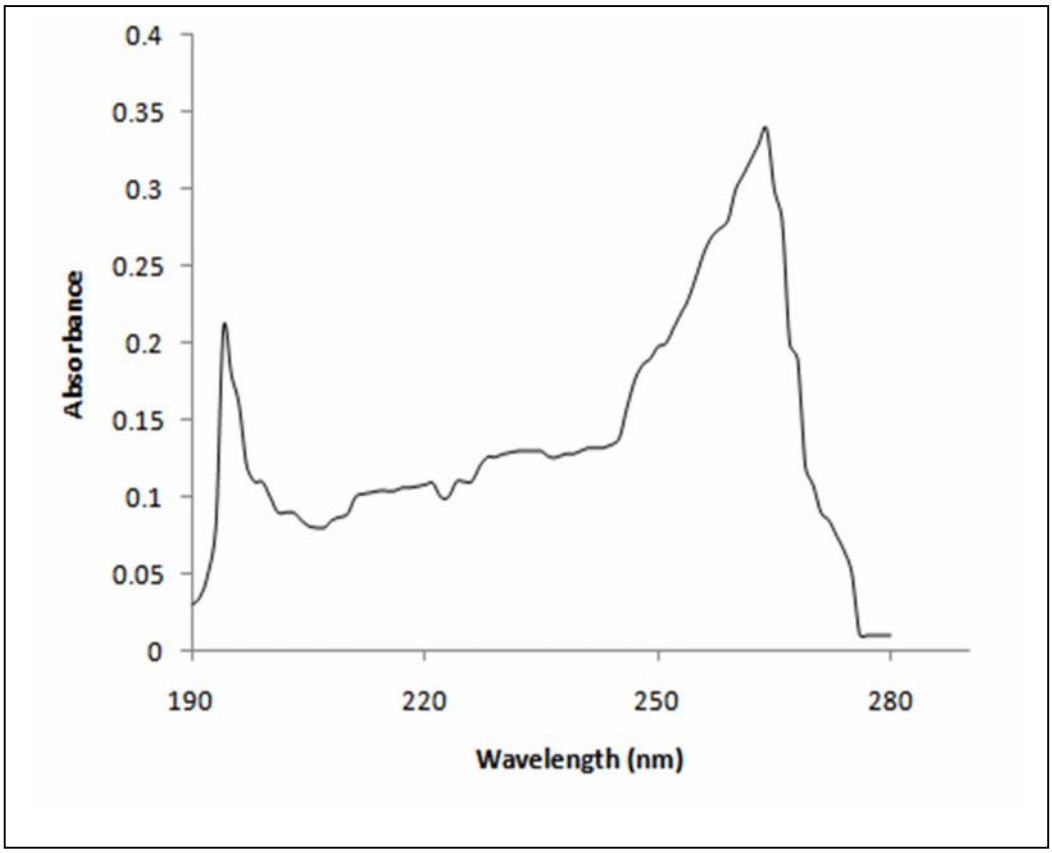

Fig. (3) The UV spectrum of testosterone receptors in malignant ovarian tumor

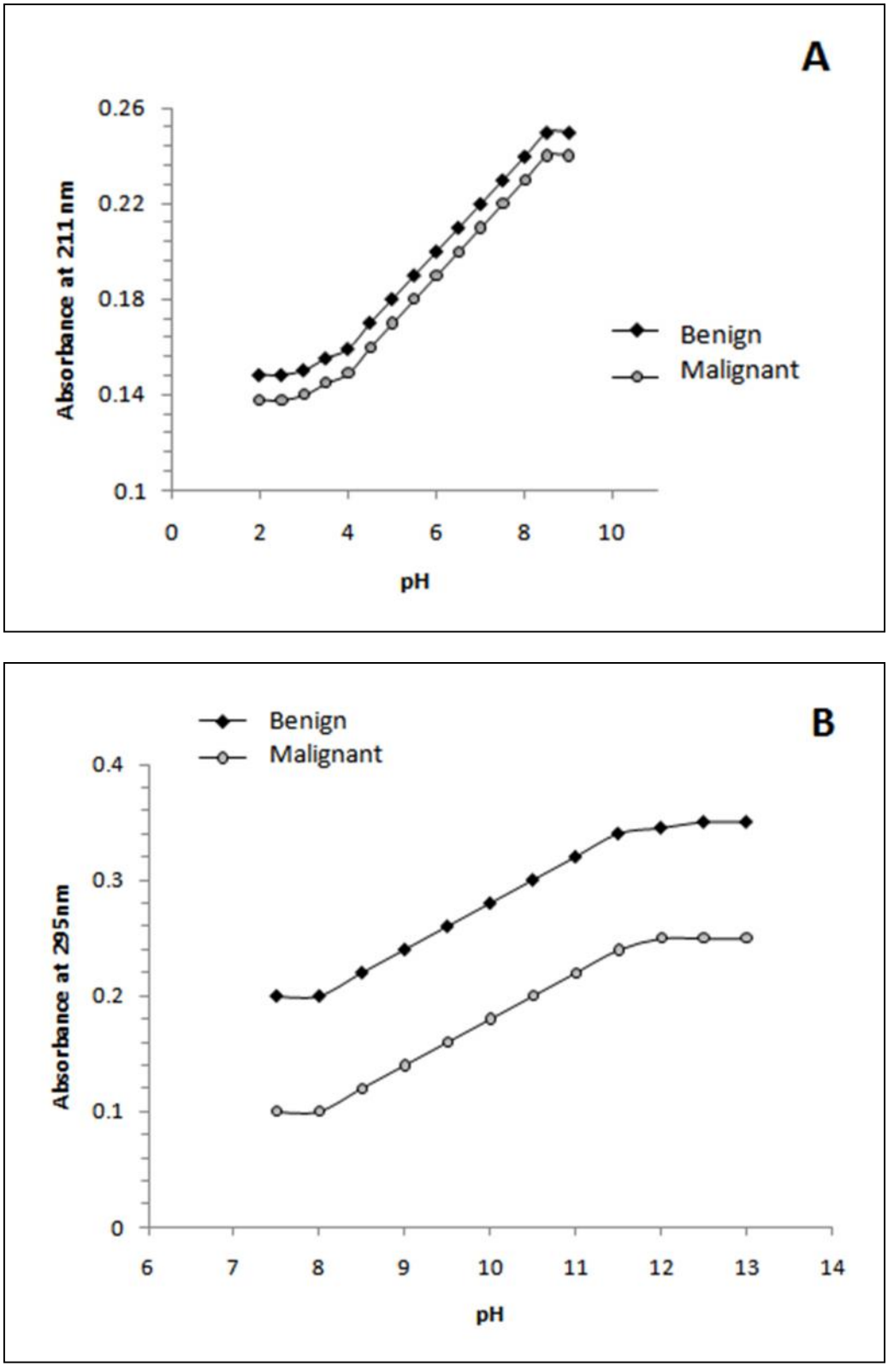

Fig. (4) Spectrophotometric pH titration of purified nuclear testosterone receptors for A) tyrosine, B) histidine in ovarian tumors.

دراسة طيفية للاشكال المنقاة المختلفة من مستلمات هرمون التوستيستيرون في متجانسات اورام المبيض البشرية

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الخلاصة: الهدف من هذه الدراسة هو للتوصيف الطيفي لمستلمات هرمون التوستيستيرون في متجانس اورام المبيض البشرية الحميدة والخبيثة. استخدمت في هذه الدراسة مجموعتين من المريضات. اشتملت المجموعة الاولى على ٣١ مريضة مصابة بورم المبيض الحميد والمجموعة الثانية شملت ١٨ مريضة مصابة بسرطان المبيض. تم اجراء التوصيف الطيفي في منطقة الطيف فوق البنفسجية لمستلمات الهرمون المنقاة كما تم دراسة تاثير الاختلاف في حامضية الوسط وقطبية المذيب على طيف الجزيئات المنقاة من مستلمات الهرمون. تم ملاحظة التغيرات الطيفية الحاصلة على الجزيئات عند استخدام مذيبات مختلفة اذ تغيرت قيم قمم الامتصاص العظمى (λmax) وكذلك مواقع القمم. كما تم ظهور مجموعات قابلة للامتصاص (الكروموفورات) نتيجة للاختلاف في قطبية المذيبات ونتيجة للتسحسح الطيفي دلالة على ان الكروموفورات كانت ضمن المناطق الداخلية للبروتين وبتاثير المذيب وصلت للسطح واصبحت معرضة للشعاع الطيفي. الاختلاف في قيم (pH) والقطبية عوامل مهمة جدا لتوصيف جزيئة البروتين طيفيا لما لها من تاثيرات على قيم حزم الامتصاص ومواقعها ضمن منطقة الاشعة فوق البنفسجية.