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DIRECT COLORIMETRIC ANALYSIS OF STILBESTROL DIPROPIONATE

Nabil M. Omar and Nawal A. EL-Rabbat
Department of Pharmaceutical Chemistry, Faculty of Pharmacy

Abstract- A convenient and specific spectrophotometric determination of stilbestrol and stilbestrol dipropionate is developed by interaction with 2,3,5-triphenyltetrazolium chloride at 50°C for 45 minutes and subsequent measurement of the color formed at 485 nm. Evidence is provided to account for the significance of extended conjugation of 4,4′-stilbenediol derivatives to induce this color reaction. Ideal adherence of color absorption to Beer's law permitted accurate and precise determination of stilbestrol and stilbestrol dipropionate pure forms over a range of 2-22 mcg stilbestrol/ml. Application of the tetrazolium reaction to the analysis of stilbestrol dipropionate oily dosage forms could be satisfactorly effected without prior hydrolytic or extraction processes. This offers a fundamental achievement for utility of the proposed procedure in automated analysis.

I N T R O D U C T I O N

The advocated clinical utility of stilbestrol (α,α′ diethyl-E-4,4′-stilbenediol) for the control of menopausal disorders, prostatic and mammary carcinoma is well documented. Usual formulation of this estrogen in relatively small doses necessitates the adoption of an especially senstive and precise means for the analysis of its

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[x]To whom inquiries should be directed.

dosage forms. Use was made of the reactivity of the two phenolic hydroxyl groups of stilbestrol to develop a diversity of estimation procedures based on acetylation (1,2), nitrosation (3,4), bromination (5) and uv-irradiation (6) processes. Of the several chromogenic reagents reported for phenols, much interest has been focused on the utility of molybdophosphotungstate (7), ferric (8), antimony (9) and vanadium (10) salts to the colorimetric analysis of stilbestrol dosage forms. However, most of these methods lack specificity and are oftenly subject to certain limitations. The compendial uv-irradiation assay procedures (11,12) though apparently specific to stilbestrol, are tedious and time-consuming. Such inconveniences are still more pronounced, when oily formulations of stilbestrol dipropionate are to be analyzed. Essential preliminary hydrolysis to stilbestrol and the further multiple extraction processes oftenly handicap accurate estimation of this ester. Consequently, the need for a rapid, direct and specific method for the determination of stilbestrol and stilbestrol dipropionate can be appreciated.

It has been shown by this laboratory, that 2,3,5-triphenyltetrazolium chloride (I) can be effectively reduced into the correspondingly highly colored formazan derivative, when interacted with o-dihydroxybenzene. This

justified the adoption of (I) for the colorimetric analysis of different pharmaceutical catecholamine congeners (13). The present communication deals with the investigation of possible interaction of (I) with other aromatic diols. As a result of this work, a convenient and accurate method for the determination of 4,4´-stilbenediols is developed. A valuable achievement of this method lies in its utility to the direct analysis of stilbestrol dipropionate oily formulations.

E X P E R I M E N T A L

Instrumentation-A double beam UV-Visible spectrophotometer[1], a pH meter[2], fitted with a sealed calomel and a shielded glass electrodes, and a suitabley thermostated water bath[3]were used.

Materials-Pharmaceutical grade stilbestrol and stilbestrol dipropionate were utilized as the working standards. Ethyl oleate, arachis and sesame oils were chemically pure, other chemicals were analytically pure.

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[1] Spektromom-203 (MOM,Hungary).

[2] Radelkis,OP-401/2 (MOM,Hungary).

[3] T-606,MTA (MOM,Hungary).

As dosage forms, differently markted tablets[4,5] and injections[6-9] of stilbestrol and stilbestrol dipropionate were analyzed.

Reagents-1-Tetrazolium Solution : 0.5 % (w/v) of (I) in aldehyde-free absolute ethyl alcohol[10]. This reagent can be kept stable for at least one week, if stored in a dark cool place

2-Potassium Hydroxide Solution : dissolve 0.1g carbonate-free potassium hydroxide in ca.1-ml of distilled water and complete to 100-ml with absolute ethyl alcohol, filtering if necessary This solution is preferred to be freshly prepared every 48 hours.

Standard Solutions-Dissolve an accurately weighed amount of stilbestrol or stilbestrol dipropionate, previously dried at 80°C in vaccuo for 2 hours, in absolute ethyl alcohol so as to afford a concentration of 200 mcg stilbestrol/ml.

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[4] Stilbestrol, (Misr Co.Pharm., Egypt), contains 5.0 mg of stilbestrol per tablet.

[5] Syntestrin, (G.Richter, Hungary), contains 1.0 mg of stilbestrol dipropionate per tablet.

[6] Stilbestrol, (Misr Co.Pharm., Egypt), contains 1.0 mg of stilbestrol per 1-ml ampul.

[7] Syntestrin, (G.Richter, Hungary), contains 5.0 mg of stilbestrol dipropionate per 1-ml ampul.

[8] Stilbstrol Dipropionate, (Evans, England), contains 10.0 mg of stilbestrol dipropionate per 1-ml solution in ethyl oleate.

[9] Cyren-B, (Bayer, Germany), contains 5.0 mg of stilbestrol dipropionate per 1-ml solution in sesame oil.

[10] A spectro-grade aldehyde-free alcohol (Prolabo-France) was utilized allover the work.

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Assay Samples-Tablets- From a composite of not less than 20 powdered tablets, accurately weigh an amount equivalent to ca. 5 mg of stilbestrol, transfer to a 50-ml volumetric flask, add ca. 25 ml absolute ethyl alcohol and allow to stand for 30 minutes with frequent agitation. Complete to volume with absolute ethyl alcohol, mix well and filter through a dry filter into a dry flask, the first portions of the filterate being rejected. 1-ml of this solution is supposed to contain ca. 100 mcg of the claimed stilbestrol content.

Injections- By means of a 1-ml precision pipet transfer 1.0 ml of the injection solution into a suitable volmetric flask,rinse the pipet with 20 % (v/v) solution of n-heptane in absolute ethyl alcohol,the rinsings being collected in the flask,and complete quantitatively and stepwise with the same n-heptane-alcohol solution, so as to obtain ca. 140 mcg of the claimed stilbestrol per 1-ml of the prepared solution.

Procedure-Pipet 1.0 ml of the standard, or appropriately prepared sample solution into a 10-ml volumetric flask, that contains 3.0 ml of the tetrazolium reagent and 1.0 ml of potassium hydroxide assay-solution,mix well stopper the flask and allow the solution to stand in the dark in a thermostated water bath, maintained at 50 $\pm$0.1^{o}C for 45 minutes.The red colored reaction mixture is cooled well and brought to volume with absolute ethyl alcohol.

Determine the absorbance of this solution as measured in 1-cm glass cell at 485 nm versus a blank prepared from 1.0 ml absolute ethyl alcohol (for tablets) or n-heptane-alcohol mixture (injections).

R E S U L T S A N D D I S C U S S I O N

I-Tetrazolium-Stilbenediol Interaction :

The unique property of tetrazolium salts in providing highly colored formazans upon their reduction advertizes the use of these salts in colorimetric pharmaceutical analysis (13-19). The fact, that (I) could be effectively reduced with o-benzenediol derivatives (13) would presumably ascribe the p-diol isomers with a similar reactivity. The latter was manifested when 0.01 M alcoholic solutions of isomeric benzenediol and triol derivatives were interacted with (I) in presence of potassium hydroxide solution, Table 1.

Selective reduction of (I) by 1,2- 1,4- and 1,2,3- di- and trihydroxybenzenes respectively is mostly influenced by susceptibility of these phenols to oxidation into the corresponding quinones. Such an oxidation of the 1,3- and 1,3,5- di- and trihydroxybenzenes is not known (20). These data would empirically imply, that aromatic

diols can, in principle, reduce tetrazolium salts if the appropriate quinone formation and redox potential are encountered. Since 4,4´-stilbenediol is amenable to oxidation into stilbenequinone (21), it seemed worthwhile to investigate its tetrazolium-reducing effect. When tested as for compounds in Table I, 4,4´-stilbenediol effected the development of a weak red color, that was much augmented by application of heat. Such a feeble reducing tendency is consistent with the high electrode potential(E_o= 0.85 v),reported for the stilbenequinone-stilbenediol system (21).Prominent acceleration of formazan development by heat endowed stilbenediol-tetrazolium interaction with a selective and sensitive feature, that encouraged further investigation of the utility of (I) to the colorimetric analysis of pharmaceutical 4,4´-stilbenediol derivatives.

II-Assay Development :

As expected, interaction of stilbestrol with (I) proceeded in analogy to 4,4´-stilbenediol; warming of the reaction mixture brought about the development of a gradual increase in color intensity. That 2,3,5-triphenylformazan is the chromogen in hand was evidenced by light absorption of the reaction mixture, with minimum and maximum extinction at 405 and 485 nm respectively (15,17,22),Figure 1.The following

reaction variables are shown to affect the rate of formazan development :-

1-Temperature-At fixed experimental conditions, formazan color-absorption was enhanced by rise of the reaction temperature. For the stilbestrol concentration range studied, appropriate absorption readings were attained at 50-60°,Fig.2. However,at higher than 50° temperatures, blank solutions aquired a noticable red tinge, which can adversely lower sensitivity levels of(I)-stilbestrol interaction.Consequently, the latter was carried out at a standard temperature of 50°.

2-Time-Investigation of the color-time curve, Fig.3, reveals maximum formazan development when the reaction mixture was kept in a 50°-thermostated water bath for 45-50 minutes. Whereas heating for longer periods seriously lowered color absorption, interruption of the 45-minutes interaction brought about a marked stability of the formazan formed for not less than three hours, Fig. 3.

3-Reagent Concentration and Mode of Addition-

Under optimal temperature and time effects, maximal formazan development was achieved by addition of 1.0 ml of the working standard solutions to 3-4 ml of (I) reagent in presence of 1.0 ml of potassium hydroxide assay solution, Table II. Other modes of addition lower color intensity.

III-Reaction Interpretation :-

Under the specified reaction conditions, neither of dihydrostilbestrol[11] nor trans-stilbene could induce formazan development. This behaviour would attest the significance of extended n-π conjugation of the trans-stilbenediol structure for the successful reduction of (I). Coplanarity of stilbestrol molecule with subsequent maximum bond delocalization should ultimately motivate electron transfer processes (13). Analogous to hydroquinone, 4,4 -biphenyldiol and 4,4'-stilbenediol, a two-electron transfer can effect the oxidation of stilbestrol into the corresponding quinone (20,21).

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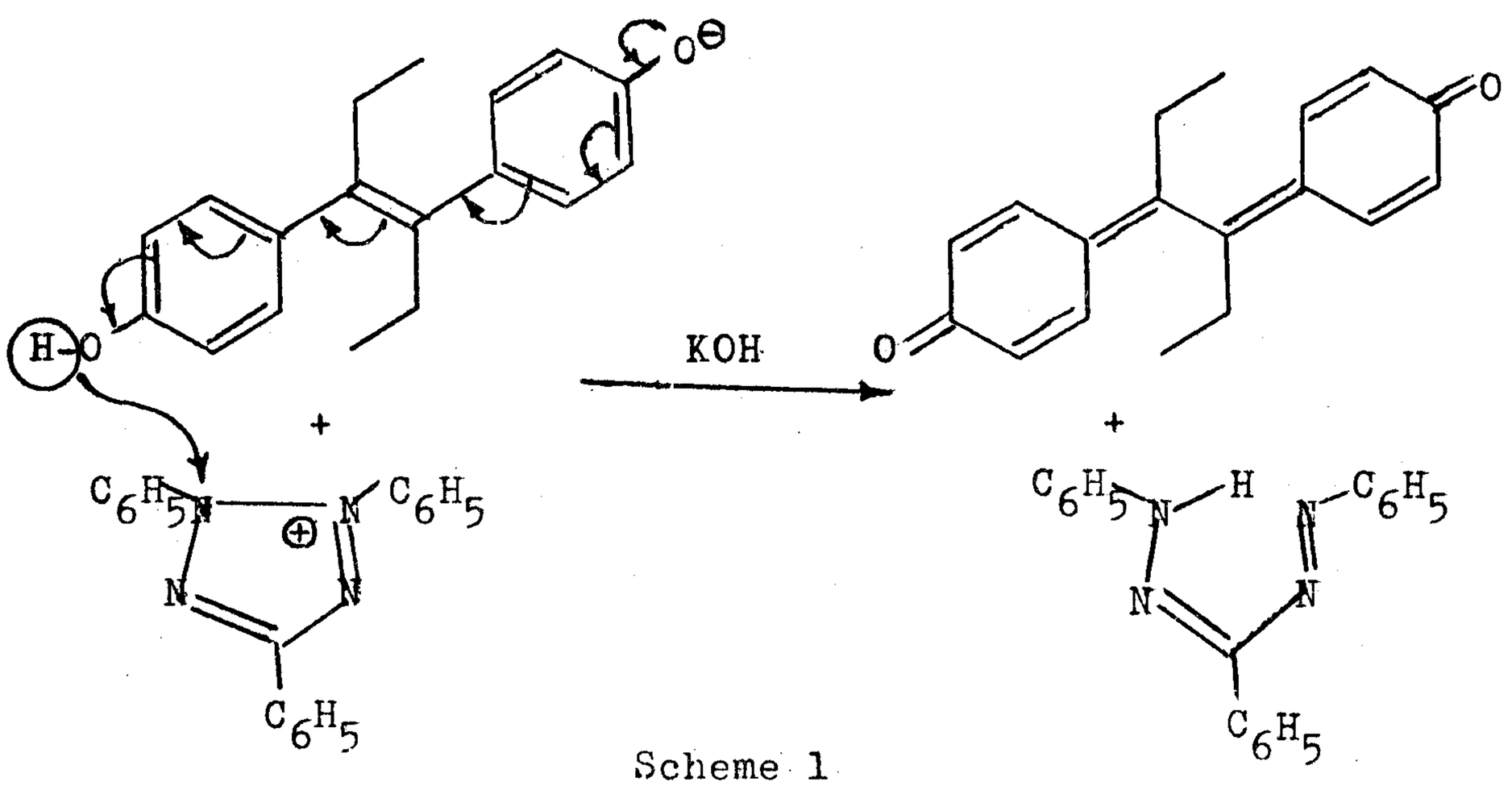
[11] Hexestrol.

As formation of 2,3,5-triphenylformazan involves a two-electron attack of (I), accompanied by a single proton transfer (23), it might be safely suggested, that a stilbestrol-stilbquinone transform can account for the reduction of (I),Scheme 1.



Scheme 1

The fact that light spectrum of the reaction mixture pertained only to formazan absorption, can be easily accepted in terms of the extreme instability of stilbquinone in protic solvents (20).

IV-Quantitative Analysis-Keeping all other experimental conditions constant, the quantity of formazan formed was a function of the concentration of stilbestrol interacted. A linear regression analysis of

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Beer's plot at 485 nm revealed an excellent adherence (r= 0.9996), with a slope value (α) of 0.0465($\pm$1.08 x 10^{-3}SD) and upper limit of sensitivity of 22 mcg (A= 1.02) of stilbestrol/ml of the assayed solution. This permitted the development of the investigated color reaction into a sensitive spectrophotometric analytical procedure on account of the relatively high apparent molar absorptivity, $\mathcal{E}_a$ = 1.277 x 10^4.Replicate analysis of working stilbestrol solutions by the presented method,Table III, proved to be fairly precise, with a relative standard deviation of 2.811 x 10^{-3}.Recovery studies,effected at different concentration levels,Table IV, afforded a mean percent recovery of 99.45($\pm$ 0.93 SD).

Since (I) could be successfully utilized for the colorimetric estimation of Beclomethasone[12] Dipropionate (12, p. 44) without prior hydrolysis, it seemed interesting to develop a possible interaction for stilbestrol dipropionate.Mention should be made,that a direct estimation of this ester is not yet reported.When reacted under the standard stilbestrol assay procedure, stilbestrol dipropionate effected a similar formazan development, with (r) and (α) values of the corresponding Beer's plot of 0.9994 and 0.0328 ($\pm$ 1.05 x 10^{-3}) respectively.Comparison of stilbestrol-stilbestrol dipropionate molecular weights ratio to the slope

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[12] Propaderm

values of the respective. Beer's plot confirmed the quantitative in situ generation of stilbestrol during the base-catalyzed interaction of (I) with stilbestrol dipropionate.

Application of the investigated color reaction to the analysis of stilbestrol and stilbestrol dipropionate tablets was successful, Table V. Potential interference with the assay due to possible existence of reducing sugars in tablet formulations could be eliminated by alcohol extraction prior to analysis. Interference studies of some of the ingredients commonly encountered in stilbestrol dipropionate formualtions revealed their inertness towards reduction of (I). These involved phenobarbitone, amethocaine, benzocaine, sulfathiazole, sulfathiourea and glyceryl trinitrate. The presented tetrazolium interaction can be imparted with specificity.

Noteworthy was the statement, that direct spectrophotometric determination of stilbestrol dipropionate oily dosage forms is unreliable as the oil vehicles contribute a high general absorbance (I, p.475). However, neither of sesame and olive oils nor ethyl oleate 0.8 % (w/v) heptane-alcohol solutions showed a measurable absorbance at 485 nm. In addition, no formazan was produced, when these solutions were interacted with (I) under the standard assay conditions. This motivated direct estimation of stilbestrol dipropionate injections by the proposed tetrazolium interaction, Table V. Concordant results were obtained when these formulations were directly analyzed by (I), or when

preliminarly freed from their oily bases by partition- multiple extraction due to the NF XIV procedure (10).It should be mentioned however, that relatively higher than the claimed stilbestrol dipropionate contents were shown by some intensively yellow-colored batches. Correction for intrinsic light absorption of such samples could be provided by determining initial absorbance at 485 nm.

The presented colorimetric estimation offers several advantages over current methods for the analysis of stilbestrol and stilbestrol dipropionate in terms of convenience and accuarcy.It can be also automated.

Table I-Interaction of (I) with aromatic diols and triols in presence of 0.1 % KOH solution.

Phenol Reacted	Color Intensity[a]
Catechol	++
Recorcinol	--
Hydroquinone	+++
Phloroglucinol	--
Pyrogallol	++

[a]As observed after 30 minutes at 20°C, key; -- = no color , ++ = moderate and +++ = intense red.

TableII-Effect of relative reagent concentration on (I)-stilbestrol rate of interaction.

Mls added/10 mls assay-solution[a]		Absorbance,485 nm
0.5 % (I)	0.1 % KOH	
1.0	0.50	0.210
2.0	0.50	0.315
3.0	0.50	0.385
1.0	1.00	0.125
2.0	1.00	0.375
3.0	1.00	0.475[b]
4.0	1.00	0.473[c]
2.0	1.50	0.280
3.0	1.50	0.330
4.0	1.50	0.425

[a]Containing 10 mcg stilbestrol /ml.
[b,c] pH values of the assay solutions = 10.50 and 9.35 respectively.

TableIII-Replicate analysis of stilbestrol working solutions (10.0 mcg / ml).

Replication	Absorbance,485 nm
1	0.471
2	0.473
3	0.474
4	0.474
5	0.472
6	0.474
7	0.475
8	0.472
Average	0.473
SD	$\pm$ 1.3296 x 10^{-3}
Relative SD	$\pm$ 2.811 x 10^{-3}

Table IV- Recovery-analysis of stilbestrol working solutions.

Sample	Stilbestrol weight,mg	Stilbestrol analyzed[a]	Stilbestrol found[b]	Recovery, %
1	10.0	15.0	9.78	97.80
2	25.0	10.0	24.96	99.84
3	50.0	10.0	50.01	100.02
4	100.0	5.0	99.84	99.84
5	200.0	20.0	199.55	99.78
Mean percent recovery				99.45
SD				$\pm$ 0.93
Relative SD				$\pm$ 9.35 x 10^{-3}

[a]Concentration level in mcg/ml.

[b]Average of five determinations.

Table V-Analysis of stilbestrol and stilbestrol dipropionate commercial formulations.

Preparation[a]	Content, mg/unit		Added	Recovered
	Label claim	Found[b]		
I-Stilbestrol				
1.Tablets	5.0	4.96	10.0	14.94
2.Injection	1.0	0.98	25.0	26.0
II-Stilbestrol Dipropionate				
1.Tablets	1.0	1.10	5.0	6.11
2.Injection	5.0	4.98	5.0	9.96
3.Injection	10.0	10.04	10.0	20.00
4.Injection	5.0	4.85	10.0	14.80

[a]Detailed composition, cf. Experimental.
[b]Average of three determinations.

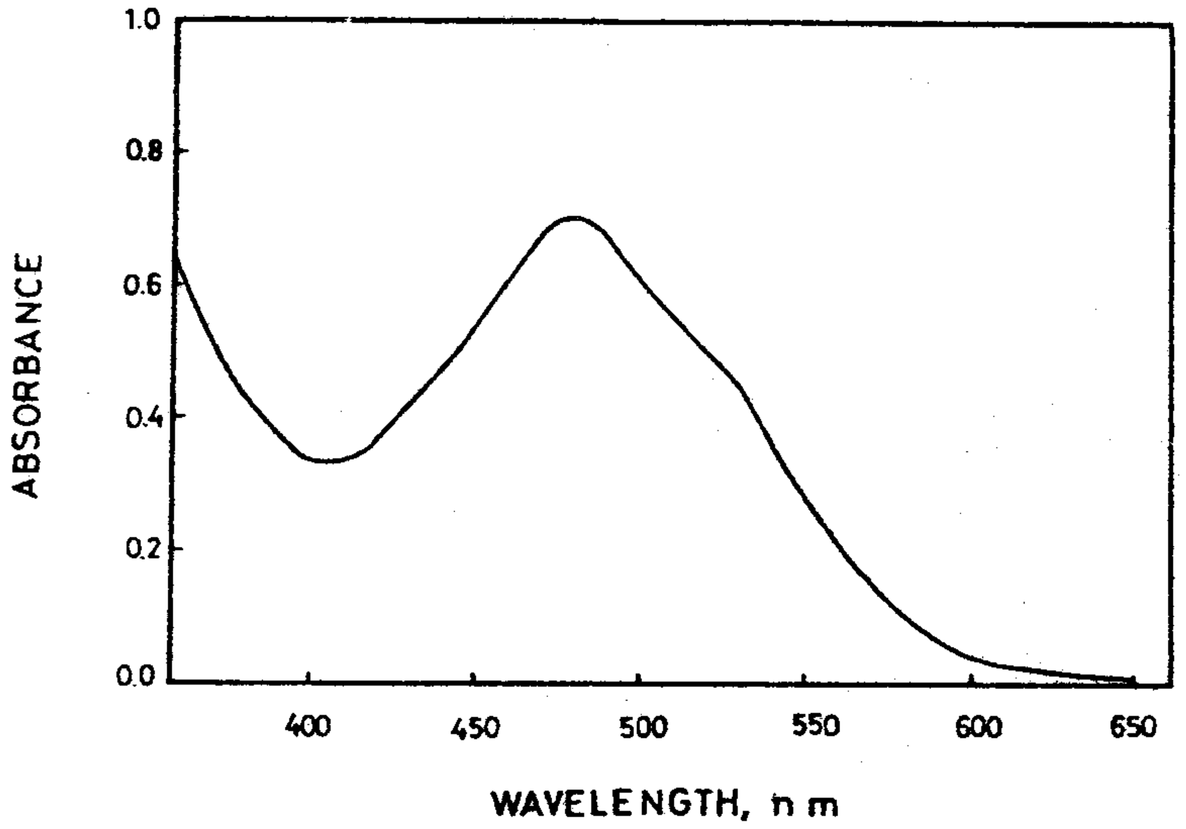

Figure I- Light absorptions spectrum of stilbestrol-(I) interaction mixture, c; I5 mcg/ml.

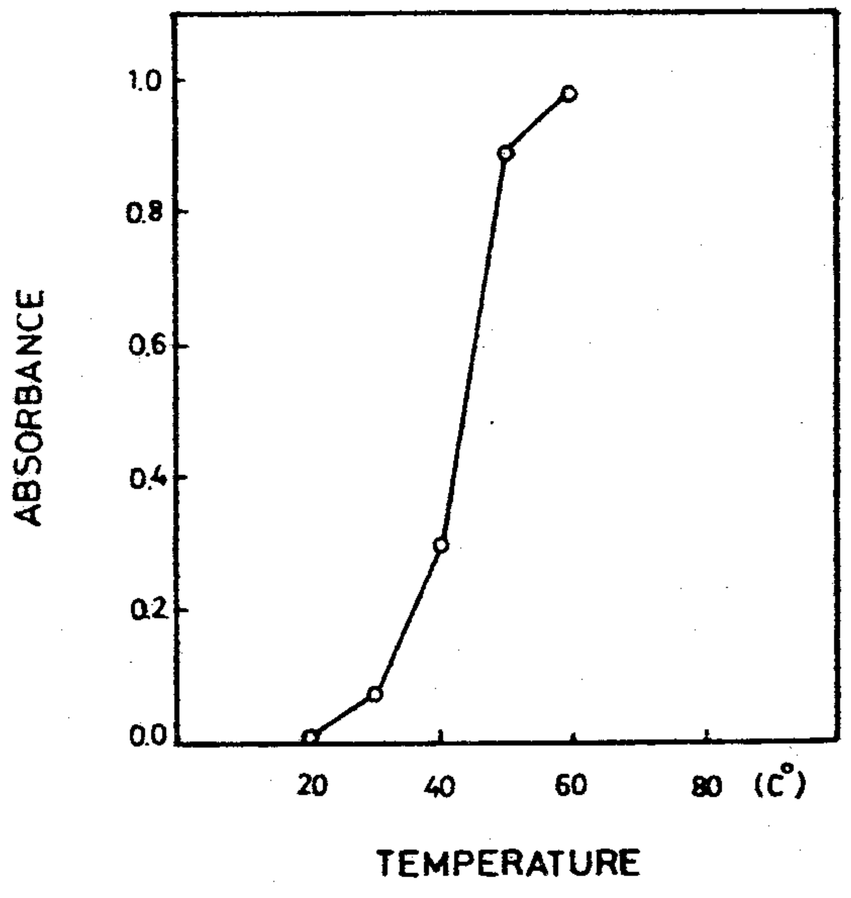

Figure 2- Temperature Effect on color absorption at 485 nm,c; 20mcg stilbestrol/ml.

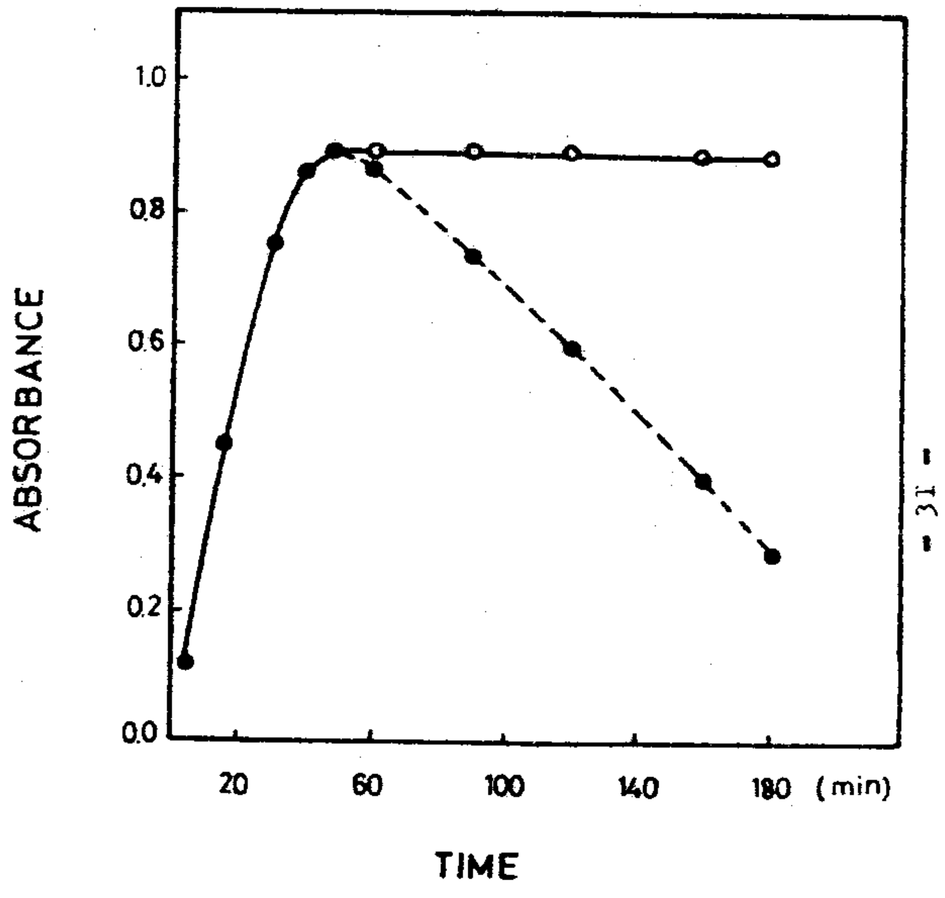

Figure 3- Stilbestrol-(I) interaction color-time curve,c; 20 mcg/ml, Key: ● at 50°C, and o, at room temperature,

R E F E R E N C E S

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تقييم لونى مباشر لثنائى بروبيونات الستلبستيرول
نهيل محمود عمر ونوال على الربـــــاط
قسم الكيمياء الصيدلية كلية الصيدلة ـ جامعة اسيـــــوط

تعكس هذه الدراسة أهمية ماتوصل اليه الباحثان من ضرورة توافر تناظر هيكلى متجاور أو متقابل لمجموعتى الهيدروكسيل العطرية حتى تتاح لمشتقاتها الفينولية فرصة اختزال أملاح التترازوليوم • ولقد أظهر البحث عدم الارتباط المطلق لهذا السلوك بوجود مجموعة " الايندايول ضمن تركيب حلقى واحد ـ فقد تمكن جزئ الستلبين ٤،٤ ـ دايول بفضل التسطح المشترك لحلقتيه وما يتبعه من امتداد للرنين العطرى من إحداث هذا الاختزال •

ونظراً لاحتواء جزئ الستلبستيرول على هذه المقومات البنائية فلقد أمكن استحداث طريقة طيفية لتقييمه وذلك بقياس الفارمازان الناشئ عن اختزاله لكلوريد التترازوليوم • ولقد كان لاحتياج هذا التفاعل الى تنشيط حرارى فعال (٥٠°م) ووجود وسط قاعدى ملائم أثره العميق فى امتداد صلاحية هذه الطريقة الى التعيين المباشر لثنائى بروبيونات الستلبستيرول بفضل حلمأته الموضعية الى الستلبستيرول •

وتحت الظروف القياسية للطريقة المقترحة فانه يمكن معايرة ٢ ميكروجرام من الستلبستيرول الحر أو مايعادله من المشتق البروبيونى بدقة وتكرارية جيدة ـ كما يمكن تطبيق هذه الطريقة بنجاح لتقييم المركبين المعنيين فى تراكيبها الصيدلية المختلفة •

ولعل أبرز ماتميزت به الطريقة المقدمة هو تمكنها من التقيم المباشر لثنائى بروبيونات الستلبستيرول فى تراكيبه الزيتية دون حاجة ما الى أية خطوات استخلاص أو حلمأة تمهيدية •