ISSN: 2458-8989

Natural and Engineering Sciences

NESciences, 2025, 10 (2): 169-183 doi: 10.28978/nesciences.1698459

Spectrophotometric Determination of Doxycycline Hydrochloride by Diazotization - Coupling Using Ortho-Anisidine Reagent Application to Pharmaceutical Formulations

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Abstract

The following project aims to produce a sensitive, high-precision and fast spectroscopic method. and cost-effective estimate for the determination of doxycycline hydrochloride in pharmaceuticals preparations), the reaction of Diazotization and coupling , The proposed approach is based on the creation of dyzonium salt by the reaction of In an acidic media, the ortho-anisidine reagent is combined with nitrite before being coupled with doxycycline hydrochloride in an alkaline medium to create a Yellow dye with the highest absorption at 437 nm , a calibration curve within Beer's law and concentration (1-60 µg.ml-1) with a correlation coefficient of 0.9998 of doxycycline hydrochloride, the detection limits were 0.379 (LOD) and 1.26 (LOQ), the Sandell's sensitivity was 0.044 µg.cm-2, and the molar absorptivity was 10.72 x 103 l/mol.cm, relative standard deviation whose value not exceed 0.430 % and the recovery value not less than 99.96%, The method has been applied to pharmaceutical products. in several forms of doxycycline showed that the proposed method has good results within the acceptable error, The validity of the proposed method was proved by a recovery investigation Using the standard addition method. the validated approach is appropriate and extremely helpful for routine quality control of doxycycline hydrochloride since it does not require polluting reagents and is quick and inexpensive.

Keywords:

Doxycycline hydrochloride, ortho-anisidine, determination, spectrophotometric, diazotization and coupling.

Article history:

Received: 12/03/2025, Revised: 27/05/2025, Accepted: 30/06/2025, Available online: 30/08/2025

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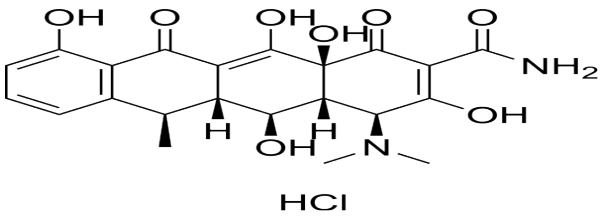
Introduction

Doxycycline (DOX) is a wide- spectrum antibiotic from the group tetracyclines. It is a pharmaceutical compound that was first used in 1967, and is still widely used due to its high activity against all types of bacteria (Korolkovas & Burckhalter, 1981; Ramesh et al., 2010; Al-Abachi & Al-Nedawi, 2015), It is also used in treating chlamydia, infections of the respiratory tract, urinary tract infections, tonsillitis, and nasal sinuses.(Phaechamud & Charoenteeraboon, 2008; Chen et al,2022), For the prevention and treatment of malaria, it is also used as an antioxidant and anti-inflammatory, so it is considered the ideal choice as a treatment against covid-19 virus disease, It is also used as an anti-breast cancer for its strong immune effectiveness (Tan et al., 2011; Chen et al., 2022; Prakash & Meena, 2022; Bhattacharya, 2003; Hashim et al., 2020; Sheeja, 2019),

DOX is advised over other tetracyclines. for specific illnesses due to its rapid absorption and long half-life this reduces dosing frequency and improves therapeutic efficacy at low concentrations (2-4 times MIC) for susceptible bacteria. (Ruiz et al., 2015), Doxycycline Hydrochloride is a yellow-green substance that dissolves in water, there are three forms, namely doxycycline Hydrochloride with molecule weight of 408.90 g. mol-1, doxycycline hyclate with molecule weight of 512.94 g. mol-1 and doxycycline monohydrate with a molecule weight of 462.4 g / mol (Kogawa & Salgado, 2012), its IUPAC name of doxycycline (4S,4aR,5S,5aR,6R, 12aS),-4-(Dimethylamino)-3,5,10,12,12apentahydroxy-6-1 methyl-l,ll-dioxo-l,4,4a,5,5a,6,11,12aoctahydrotetracene-2-carboxamide monohydrate) (Foroutan et al., 2023). Doxycycline has the following structure in figure.1 (Ruiz et al., 2015).



$C_{22}H_{25}ClN_2O_8$

M.Wt = 480.90 g. mol^{-1}

Figure 1. Chemical formula of dox

Several analytical approaches have been used to determine doxycycline in both its pure and prepared forms . Some of these procedures have significant limitations, including low sensitivity, the need for a non-aqueous solution, Heating, solvent extraction, or expensive equipment that requires special training, like liquid chromatography with chromatographic superior performance (NP-HPLC and RP-HPLC). (Ghidini et al., 2018, Verma & Nair, 2025; Singh & Gurudiwan, 2024; Mashru & Koshti, 2021; Dil et al., 2020), HPLC-Block (Permana et al., 2019; Sancho et al., 2022), HPLC (Kumssa., et al 2022), potentiometric sensor (Ali et al., 2018), ratiometric probe (Tian & Fan, 2021), injection flow spectrophotometry (Tawfeeq & Qassim, 2020), fluorometric (Feng et al., 2018), Voltametry (Gürler et al., 2013), Determination and electrochemistry (Zhuang et al., 2021). Flow injection analysis (FIA) (Palamy & Ruengsitagoon, 2017; Rufino et al., 2009), spectrophotometric methods using various reagents (Milan & Žarko, 2018; ALASSAF & Faeza, 2019; Khammas & Rashid, 2016; Ramesh et al., 2011; Amit et al., 2010; Saber & Amin, 2011).

The proposed study aims to create an accurate and exact spectrophotometric method for assaying doxycycline in various dosage forms (Mileva, 2019).

Experimental

Equipment

1. The absorptivity was measured and the absorption spectra were drawn using ultraviolet - visible spectroscopy using a device of the Shimadzu UV-Vis 1900 spectrophotometer Japan type, using glass cells having a 1 cm light path.
2. All weighing operations were carried out using a sensitive electronic scale of the AE ADAM type..... In the measurement of weights of chemicals
3. Use a water bath to conduct the heating process of the type (electro. meg).

Preparation of Reagents

All chemicals compounds uses were of high purity. and obtained from Fluka, BDH, SDI companies.

Solution of Pure Doxycycline Hydrochloride (100 mg.ml-1)

The solution was formed by melting (0.0100) gram of pure doxycycline, which was processed from (S.D.I) in 5 ml of ethanol, stirring for 2 minutes, and then transferred to a volumetric flask of 100 ml and fills with water to the mark. and store the solution in a dark flask.

Ortho-Anisidine Reserve Reagent Solution (100 µg/ml)

Prepare the solution by melting (0.01) g of ethanol by adding 20 ml from ethanol, and then complete the volume to 100 ml of ethanol in a volumetric flask with a capacity of 100 ml and store in a dark flask.

Sodium Nitrite Solution (1%)

Prepared the solution by melting (1.00) g of sodium nitrite with Water that was distilled and then complete the volume with distilled water in a volumetric flask of 100 ml to the mark.

Hydrochloric Acid Solution (1 M)

The chemical compound solution was prepared by diluting (8.57) ml of concentrated hydrochloric acid with distilled water up to the mark in a 100 ml volumetric flask.

Urea Solution (1%)

Prepare the solution by melting (1.00) g of urea with Water that was distilled and then complete the volume in volumetric flask to the mark.

Sodium Hydroxide Solution (1 M)

It was prepared the solution by melting (4.00) g of sodium hydroxide with distilled water and complete the volume in a volumetric flask of 100 ml up to the mark.

Solutions Of Surfactants (0.1%)

The solutions were made by melting. (0.100) g of each (SDS/ CBC/ CTAB / Cetavlone / Triton-X-100) with distilled water and fill the volumetric flask with Water that was distilled up to the mark.

Pharmaceutical Preparation

1 -Doxy-Cyclin Tablets (Doxy-Denk 100)

I took 5 tablets of (Doxy-Denk 100) (processed by the German pharmaceutical company Denk Pharma (100mg Dox/tablet), and the weight of one tablet was approximately equal to 0.2620 and the weight of the combined tablets (1.3155) g (Chinnasamy, 2024). after grinding and mixing well, he took the equivalent of one tablet, which contains 1000 mg of doxycycline and dissolution in 5 mL ethanol and then completes the volume to mark with distilled water, then prepare the sample solution by diluting the required volume using dilution laws and then adding Water that was distilled in a volumetric flask whose volume100 ml.

2- Doxycycline Capsule (Doxycycline Capsules 100mg)

I emptied 8 capsules of (Doxycycline Capsules 100mg) (processed by the English pharmaceutical company accord (100mg Dox/capsules) and the weight of the combined content of the capsules reached (1.602)g. after grinding and mixing well, I took the equivalent of the content of one capsule, which contains 1000 mg of doxycycline and dissolved in 5 ml of ethanol, then complete the volume to the mark with distilled water In a 100ml volumetric flask, then prepare the sample solution by diluting the required volume using dilution laws and then add distilled water in a volumetric flask whose volume100 ml .

The Method's Main Principle

The essential principle of the proposed method involves two steps, where the first step involves the reaction of Ortho-anisidine with an equivalent amount of sodium nitrite in an acidic medium to form a dyzonium salt, second step involves coupling of the ionized ortho- anisidine reagent with the reducing doxycycline hydrochloride in alkaline medium to form a colored AZO dye That provides greatest absorption value at a wavelength of 437 nm.

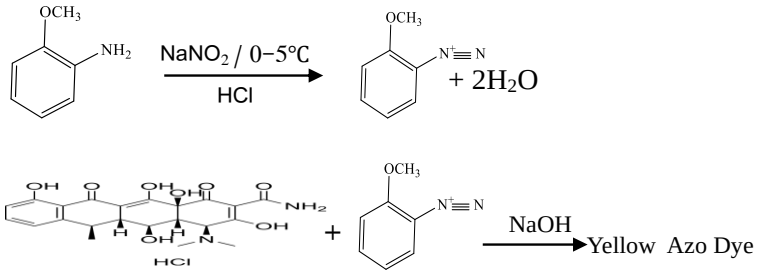


Figure 2. Chemical structure of the colored Azo dye

Results and Discussion

Various experiments have been performed to explore the effect of the reaction components on absorbance, and the ideal conditions that yield maximum absorption have been chosen.

An Initial Study

1.5 ml of the reagent Ortho-anisidine had been added in a 10 ml volumetric flask, then 1 ml of the ozonized reagent sodium nitrite was added to it in an acidic medium by adding 1 ml of acid (HCl)at a concentration of 1M, then 0.5 ml of urea was added , then 2 ml of doxycycline solution (100 μg. ml-1) after making the medium

alkaline by adding 1 ml at a concentration of 1M sodium hydroxide, the yellow color produces the greatest absorption at 437 nm.

Study and Identify of Optimal Conditions

To obtain purpose of obtaining a colored AZO dye with high absorbency and stability, the effects of various variables affecting the intensity of absorption and the color of the resulting dye were studied.2 ml (100 µg. ml-1) of Doxycycline solution was placed in a 10 ml volumetric flask, and the absorbance at 437 nm was measured when compared to the photo solution.

Study the Impact of The Type of Acid Used

The impact of various types of strong and weak acids available at a concentration of 1 M was studied, the acid suitable for the nitrification process, which gives the highest absorption, is hydrochloric acid, so it was adopted in subsequent studies, as the results are shown in the table (1).

Table 1. Impact of the type of acid used on absorbance of azo dye

Acid solution used(1M)	**HCl**	**H2SO4**	**H3PO4**	**HNO3**	**CH3COOH**
Abs	0.410	0.159	0.183	0.221	0.101

Study the Impact of the Amount of Acid used

The impact of adding increasing amounts of acid was studied in the intensity of the resulting absorption, increasing volumes of hydrochloric acid (0.25-3.0) ml were added and the absorption of solutions versus photo Solutions was measured at 437 nm, in Table (2) show that 1 ml of acid used in previous experiments is the best and was installed in subsequent studies.

Table 2. Impact of the amount of acid used on absorbance of azo dye

ml of HCl (1M)		**Abs /ml of Acid used**				
	0.25	0. 5	1.0	1.5	2.0	3.0
Absorbance	0.141	0.345	0.410	0.398	0.391	0.342

Studying the Impact of Nitrite Quantity and Time

The ionizing agent of the hydrolyzed reagent is nitrite, therefore the nitrite impact was investigated adding different amounts of nitrite concentration (1%) and for different periods of time to the hydrolyzed Ortho-anisidine solution and after the nitriding process, The doxycycline solution was added, and the medium was turned alkaline with sodium hydroxide, absorption measurements of colored solutions were taken against their photo solutions at a wavelength of 437 nm.it in Table (3) that adding 1 ml of sodium nitrite and waiting for 5 minutes produced the highest absorption of the generated pigment and was adopted in subsequent studies.

Table 3. Impact of nitrite quantity and time on absorbance of azo dye

V(ml) of $NaNO_2$(1%)	**Abs/min. standing time**			
	0	3	5	7
0.25	0 .060	0 .186	0 .272	0 .203
0.5	0 .197	0 .254	0.289	0 .207
1.0	0 .442	0 .531	0 .604	0 .582
1.5	0 .337	0.362	0.392	0.371
2.0	0 .122	0 .153	0 .185	0 .153

Studying the Impact of Urea and Time

This impact was studied by adding different volumes (0.1-2.0) of urea with a concentration of (1%) and waiting for different periods of time to a constant amount of reagent 1 mL for the purpose of eliminating the residual excess of sodium nitrite, from Table (4) it was shown that the amount of (1.0) ml of urea for 3 minutes gives the highest value of absorbability, so it was adopted in following. studies.

Table 4. Impact of urea and time on absorbance of azo dye

V (ml) of (1%) Urea	**Abs /min. standing time**		
	1	3	5
0.1	0.081	0.101	0.112
0.5	0.210	0.224	0.215
1.0	0.581	**0.625**	0.561
1.5	0.492	0.510	0.487
2.0	0.410	0.420	0.392

Studying the Impact of the Reagent Quantity

This impact was studied by adding different volumes (0.1-3.0 ml) of the reagent Ortho-anisidine at a concentration of 100 micrograms per milliliter in 10 ml volumetric bottles and adding the rest of the additives and dilution to the mark and after the completion of the reaction it turned out that 1 ml of the reagent is the best volume as shown in Table (5), it was adopted and studies.

Table 5. Impact of the reagent quantity on absorbance of azo dye.

Volume of Regent (100 ppm)	0.25	0.5	1.0	1.5	2.0	2.5	3.0
Abs	0.091	0.342	0.654	0.597	0.578	0.533	0.421

Studying the Impact of the Base Type

It turns out through the preliminary study that the colored solution is formed only in the base medium, so the impact of the base type in increasing the absorbency of the colored product was studied, the impact t of different types of bases was studied by adding fixed amounts of 1 ml at a concentration(1 M) to each of them individually and it was found that sodium hydroxide gave the greatest absorbency as shown in table (6) and thus it was used in t studies.

Table 6. Impact of the base type on absorbance of azo dye

Type of base (1M)	**KOH**	**NaOH**	**$NaHCO_3$**	**Na_2CO_3**	**NH_4OH**
Abs	0.245	0.768	0.635	0.336	0.197

Studying the Impact of the Amount of Base

The study was carried out by taked volumes (0.1 -3.0) ml of sodium hydroxide (1M) to the reaction components, then the absorbency of these solutions was taken against the photo solution and the results showed that the volume of 2.0 ml gives the highest absorbency as shown in Table (7), so it was adopted in studies.

Table 7. Impact of the amount of base on absorbance of azo dye

Volume of base (1M)	0.25	0.5	1.0	1.5	2.0	2.5	3.0
Absorbance	0.172	0.390	0.761	0.771	0.785	0.639	0.452

Studying the Impact of Surfactants

Therefore, the impact of these substances on the intensity of absorption of the formed AZO dye was studied, where different volumes (0.5 -2.0) ml of surfactants (neutral, positive and negative) with a concentration of

0.1% each were added to the reaction components. it was found from the results shown in table (8) that surfactants have a negative effect on absorbability and therefore were excluded from the reaction.

Table 8. the impact of surfactants on absorbance of azo dye

Surfactant	Abs /ml of surfactant used			
	0.5	1.0	1.5	2.0
C T A B 0.1%	0 .491	0 .516	0 .562	0 .502
CPC 0.1%	0 .421	0 .459	0 .512	0 .501
S D S 0.1%	0 .659	0 .731	0 .761	0 .710
Triton X- 100 0.1%	0 .710	0 .743	0 .750	0 .721
Cetavlon 0.1%	0 .691	0 .704	0 .733	0 .711
Without Surfactant	**0.785**			

Studying the Impact of Temperature and Time on the Stability of the Formed Pigment

The impact of different temperatures (5-50 M) was investigated based on the optimal settings acquired from earlier research., the intensity of absorption of AZO dye formed at different time intervals was tracked and the results showed that AZO dye gives the highest absorption at Laboratory temperature (20 ± 2) and with the highest sensitivity 10 minutes after dilution and with a stability time of 70 minutes. As shown by the figure (3).

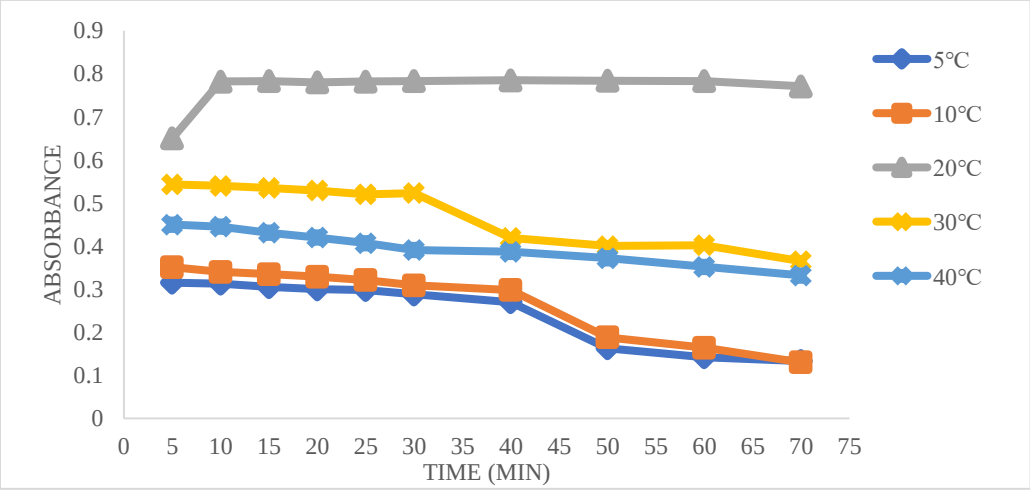


Figure 3. Impact of temperature and time on the stability of the produced pigmen

Table 10. Summery optimal conditions for the proposed way of working.

solution	Concentration	Optimum amount(ml)
Ortho-Anisidine	100 ppm	1
$NaNO_2$	1%	1
Urea	1%	1
NaOH	1 M	2
HCl	1M	1
$\lambda_{max(nm)}$	437	
Color	Yellow	
Temp.℃	Room Temp	
Development Time	10	
Stability period	70 min	

The Final Absorption Spectrum

After experimentally establishing the optimal conditions as in Table (11), the absorption spectrum of the azo dye was taken a model was prepared using 1 ml of doxycycline solution (100 µg/ ml) and adding the rest of the components of the proposed method to form a dye with a yellow color ,the absorption spectrum of the formed product was plotted at wavelengths ranging from 350 to 800 nm and it was found that the formed product provides the greatest absorption. at 437 nm versus the photo solution and Figure (4) shows ultimate absorption spectrum of the formed dye versus its photo solution.

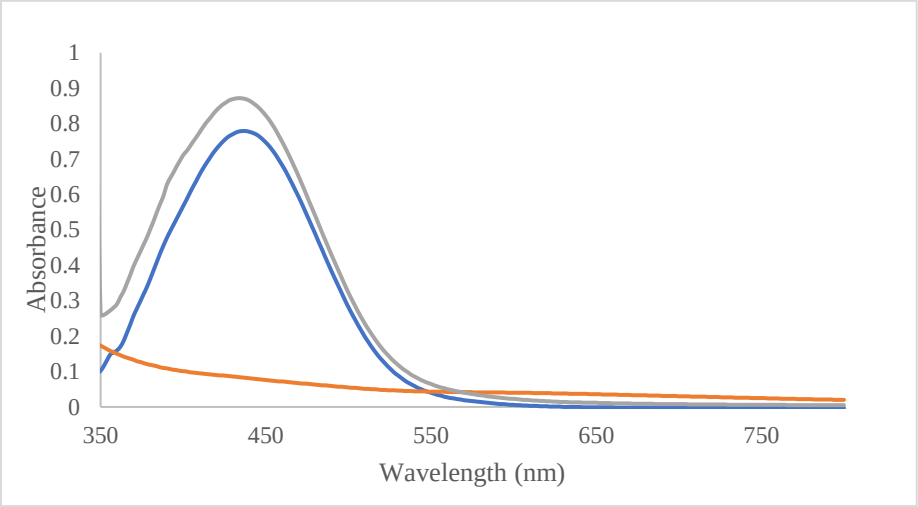


Figure 4. The Absorbtion spectra of (40ppm) DOX solution (A)vs blank, B- vs distilled water, -blank vs distilled water

Calibration Curve

After stabilizing the appropriate conditions, different volumes of the drug are added to the solution containing the reagent, acid, sodium nitrite and urea, which are added in numerous volumetric flasks, followed by the base and water (Phaechamud & Charoenteeraboon, 2008). After 10 minute, the measure is taken against the blank solution at 437nm.

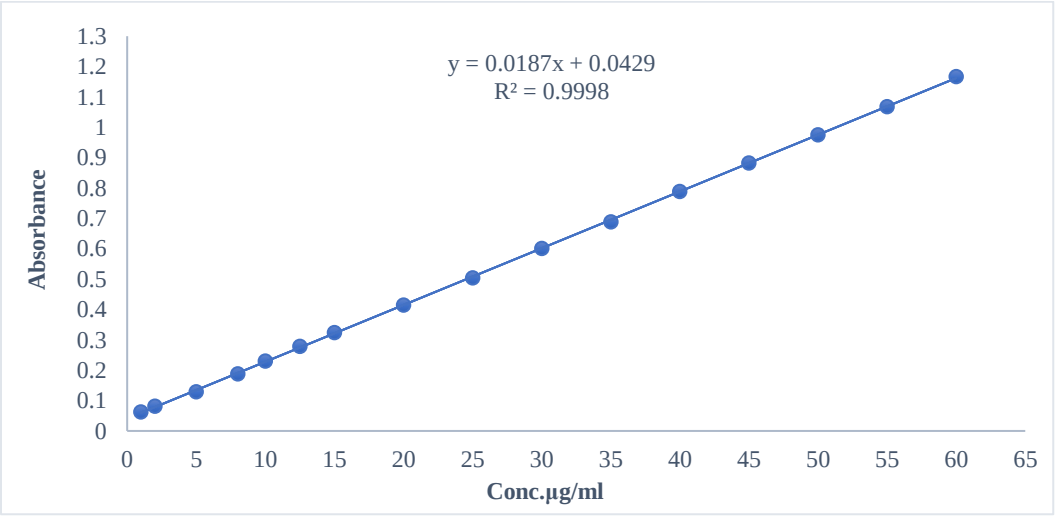


Figure 5. Calibration curve for estimating DOX by the proposed method

Table 11. Linear specification of doxycycline hydrochloride estimation with ortho-anisidine reagent

Beer's law limits (µg/ml)	1-60
(LOD) (µg/ml)	0.379
(LOQ) (µg/ml)	1.26
Molar absorptivity ($I.mol^{-1}.cm^{-1}$)	$10.72x10^3$
Sandell's Sensitivity ($\mu g.\ cm^{-2}$)	0.044
Slope	0.0187
Intercept	0.0429
Correlation Coefficient	0.9998

Method Accuracy and Compatibility

The precision of the technique and its compatibility were studied under optimal conditions, where five repeaters were measured for three different concentrations of doxycycline hydrochloride solution and treated using the approved method. the results listed in Table (13) show that process has high accuracy.

Table 12. Method accuracy and compatibility

Amount of Dox (µg/ml) Present	Amount of Dox (µg/ml) found	Recovery %	Average of recovery	Relative error %	RSD %
10	10.11	101.1	99.96	1.1	±0.430
20	19.87	99.3		-0.050	±0.25
40	39.83	99.5		-0.414	±0.168

The Method of Continuous Changes (job's method)

The nature of the colored azo dye to find out the molar synthetic ratio between the drug compound doxycycline hydrochloride and the reagent Ortho anisidine applied to investigate the nature of the generated the method of continuous changes (job's method) (Skoog et al., 1996), the solutions of the drug compound and the reagent have been generated at same concentrations. (2.07×10-4) M with drug. a number of solutions were prepared by mixing different volumes of doxycycline with the reagent so that the final volume was constant and in the amount of 6 ml and following the optimal conditions shown in table (11) and shown in Figure (6) and dilution to the mark in a 10 ml volumetric flask , the absorption intensity of the solutions was measured against their photo solutions at is 437 nm and it is noted that the coupling ratio of doxycycline with the reagent Ortho-anisidine is 1:1(pharmacological compound: reagent).

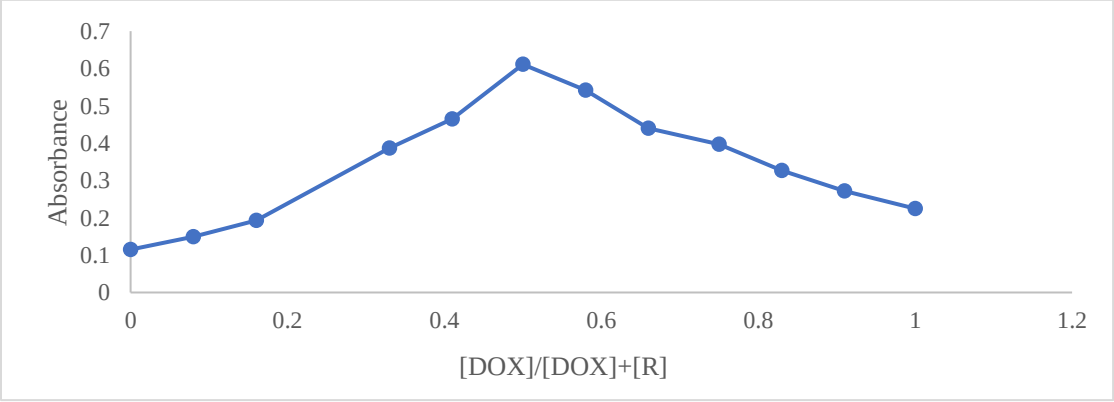


Figure. 6 The curve of continuous changes (Jop's method) of the azo dye resulting from the coupling of (DOX) with the ozonized reagent (Or A)

The Method of Molar Ratios

The molar ratios method (Skoog et al., 1996) was applied to determine nature of the product created. by the reaction of the doxycycline coupling with the reagent Ortho-anisidine ozonized, where a constant volume of doxycycline was taken in the amount of 0.5 ml at a concentration of (2.07×10-4)M and according to the approved method, all the additives installed in the reaction were added according to the optimal conditions shown in Table (11), then gradually adding increasing quantities of the reagent solution. at a concentration of (2.07×10-4) M, then adding the base and dilution to 10 ml volumetric vials, then the absorption intensity of the solutions is measured at a wavelength of 437 nm against the image solutions as shown in the figure (7) the resulting product is formed in a ratio of 1:1(pharmaceutical compound: reagent).

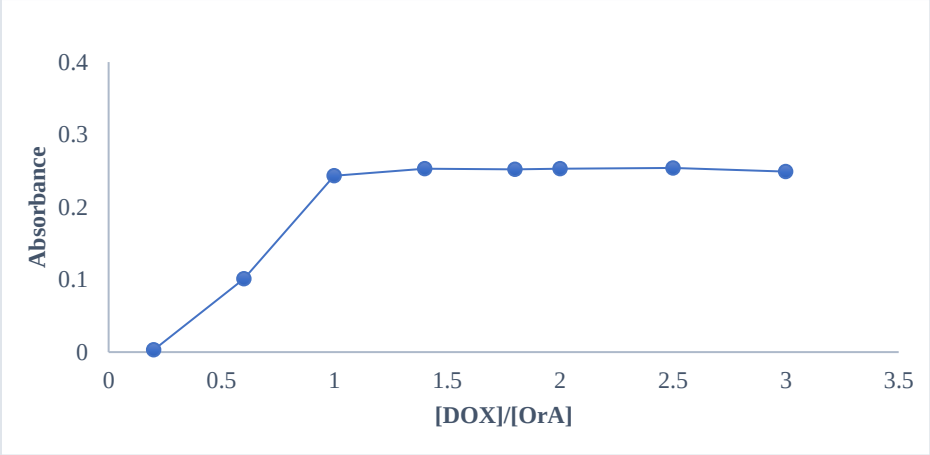


Figure 7. The curve of the molar ratio of the azo dye resulting from the coupling of (DOX) with the ozonized reagent (OrA)

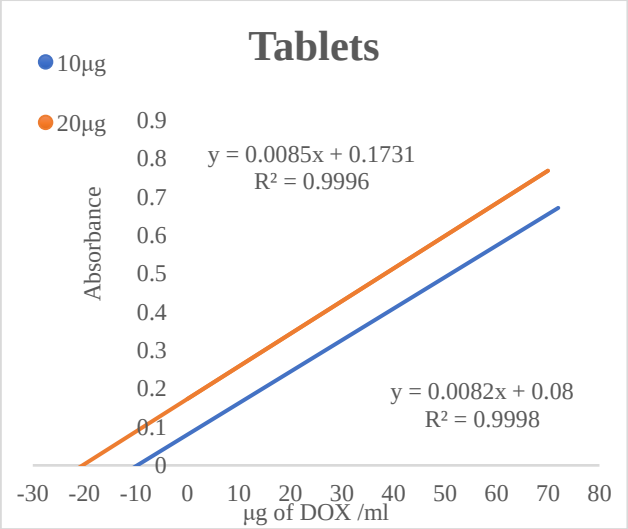
Applying the Method to Medicinal Preparations.

The proposed method for determining the amount of doxycycline present in pharmaceutical products such as pharmaceutical tablets and capsules was applied under optimal conditions, and has given the method of action.

Table 13. Analysis of pharmacological compounds

Pharmaceutical Preparation	Amount taken (µg/ml)	Amount measured (µg/ml)	Recovery* %	-Relative error* %	Relative Standard deviation* %
Doxycycline Capsules 100mg Accord-UK	10	9.59	95.9	-4.04	0.884
	20	19.4	97.3	-2.60	0.390
	40	39.67	99.17	-0.825	0.371
Doxy-Denk Tablets 100mg Denk pharma-Germany	10	9.55	95.5	-4.40	0.679
	20	19.31	96.5	-3.44	0.939
	40	39.23	98.07	-1.92	0.459

Evaluation of the Suggested Method



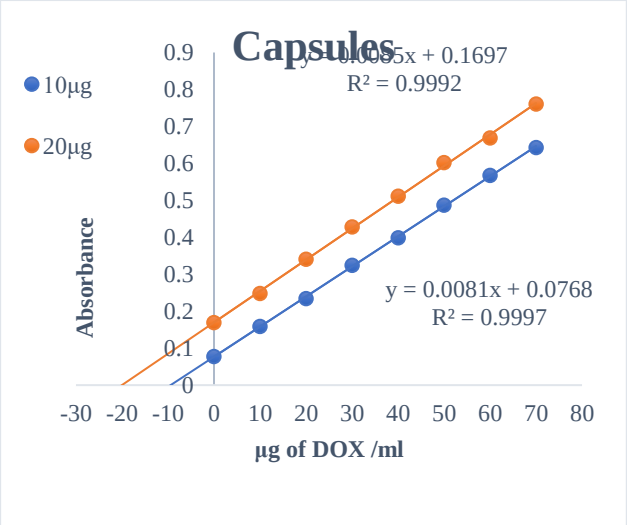


Figure 8. and Figure 9. Standard addition curves for estimation of DOX in pharmaceutical preparations

The standard multiplication method (Delevie, 1997), was applied to the medicines preparation to evaluate the selectivity of the proposed method for estimating doxycycline, the results listed in figure (8), (9), and table (13) indicate that the results of the usual method for adding well agreed. with the results of the proposed method within an acceptable range of error.

Table 14. Evaluation of the suggested method

Drug	DOX Taken (µg/ml)	DOX Measured (µg/ml)	Recovery %
Doxycycline Capsules 100mg Accord-UK	10	9.68	96.8
	20	19.59	97.95
Doxy-Denk Tablets 100mg Denk pharma-Germany	10	9.71	97.1
	20	19.81	99.05

Conclusion

A recently developed spectrophotometric approach has been offered for the measurement of doxycycline hydrochloride in medicines (pharmaceutical tablets-capsule) using Ortho-anisidine by the diazotization and coupling processes.

Author Contributions

All Authors contributed equally.

Conflict of Interest

The authors declared that no conflict of interest.

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