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# Development of a Spectrophotometric Method to Assay Tetracycline Hydrochloride by Coupling with Diazotized p-Aminobenzoic Acid: Application to Pharmaceutical Forms

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# **Article information**

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#### ABSTRACT

This work comprises developing a sensitive and precise spectrophotometric procedure to analvze tetracycline hydrochloride in the bulk and in the drug forms. In this method, p-aminobenzoic acid reagent was diazotized with an equimolar NaNO<sub>2</sub> (sodium nitrite) solution in the existence of HCl (hydrochloric acid) to produce corresponding diazonium salt subsequently coupling with tetracycline hydrochloride in a basic solution of NH<sub>4</sub>OH to produce a yellow azo dye which was water soluble and has a maximum absorption at 422 nm versus a reagent blank. Under optimal conditions, the linearity of Beer's law was estimated to cover the concentration range of  $0.25-17.5 \ \mu g/ml$  with an exceptional determination coefficient ( $R^2 = 0.9993$ ) and molar absorptivity of 1.66×10<sup>4</sup> l/mol.cm. The calculated values of detection limit (LOD) and quantitation limit (LOQ) were equal to 0.012 and 0.040  $\mu$ g/ml, respectively. The values of error percentage and precision (RSD%) ranges were calculated and found in the range of -0.08% to 3.3% and 0.16% to 1.59%, respectively. The stoichiometry of the product was set up to be 1:1 tetracycline hydrochloride: diazotized p-aminobenzoic acid. The mentioned procedure has been utilized successfully to the evaluation of tetracycline hydrochloride in capsules and ointment.

Keywords: Tetracycline, HCl, Diazotization, p-aminobenzoic acid, Spectrophotometric Method.

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#### **INTRODUCTION**

Among broad-spectrum antibiotics, the tetracycline-series antibiotics occupy a leading place. They suppress Rickettsia, acid-resistant bacilli, the reproduction of gram-positive and gram-negative microorganisms and many infections (Mamani *et al.*, 2006). Tetracycline hydrochloride (TCH), is a yellow powder and chemically acknowledged as [(4S, 4aS, 5aS, 6S, 12aS)-4-dim-ethylamino-3, 6, 10, 12, 12a-pentahydroxy-6-methyl-1, 11- dioxo-1, 4-, 4a-, 5, 5a, 6, 11, 12a-octahydro-tetracene-2-carboxamide monohydrochloride] (The Japanese Pharmacopoeia, 2016). Fig. (1).



Fig. 1: The chemical structure of tetracycline hydrochloride  $C_{22}H_{24}N_2O_8.HCl$ M.Wt. = 480.9 g/mol

TCH is a synthetic agent used in human and veterinary therapy for hundreds of years. The study of the literature found that several different methods have been described for the direct and indirect valuation of the TCH, they included: high performance liquid chromatography (HPLC)-diode array detection (Vuran *et al.*, 2021), sulfur quantum dot probes (Lu *et al.*, 2021), HPLC–MS/MS (Pang *et al.*,2021), cyclic voltammetry using the reduced grapheme oxide (rGO) modified electrode associated with the flow-injection analysis (Faria *et al.*, 2019), fluorometric and electrochemical dual-mode nanoprobe for tetracycline (Hu *et al.*, 2020), a potentiometric sensor coupled to HPLC (Gil *et al.*,2022) and kinetic spectrophotometry (Alassaf *et al.*, 2019). However, most of these procedures need a skilled operation and expensive equipment.

For the analytical measurement of TCH in pharmaceutical formulations, UV-Visible spectrophotometry is still regarded as a cost-effective and feasible approach. TCH has also been measured using a variety of spectrophotometric techniques, both in bulk and in pharmacological preparations. Some of these methods included diazotization and coupling reaction which is based on the coupling of TCH with diazotized of p-nitroaniline (Al-Abbasi, 2009), 4-aminoantipyrine in a presence of cetylpyridinium chloride (Othman and Al-Ashow, 2012), anthranilic acid (Abd et al., 2017), sulphanilic acid (Ali et al., 2018). Others based on the oxidative coupling reactions of TCH with p-N, N-dimethylphenylenediamine and sodium meta periodate (Tella et al., 2011), 2,4dinitrophenylhydrazine (2,4-DNPH) with potassium periodate in a basic medium (Hameedi, 2021) and N. N-diethyl-p-phenylenediamine reagent in presence of oxidizing agent of Nbromosuccinimide in the alkaline medium (Mari et al., 2016). Other spectrophotometric methods have also been developed for estimating TCH based on the complex formation with cerium (IV) (Al-Sowdani et al., 2006), zirconium (IV) (Saenjum et al., 2022) and yttrium (III) in the presence of cationic surfactant (Thanasarakhana et al., 2011). As well as, charge transfer complex formation with chloranilic acid (Fahelelbom, 2008) and oxidation reaction with sodium hypochlorite (Ahmed et al., 2018) have been used to determine TCH by spectrophotometric method. However, many of these methods suffer from various limitations, for instance, low sensitivity, low stability of the resulting product and time consuming.

In the recent investigation, a p-aminobenzoic acid (p-ABA) reagent was used to produce an easy, rapid, and inexpensive visible spectrophotometric method such as assaying TCH in both its pure and pharmacological forms. The approach was based on the formation of a yellow dye in a

basic solution by the reaction of TCH with diazotized p-ABA reagent, which has been used to assay TCH in capsules and ointment.

# **EXPERIMENTAL**

# Apparatus

All measurements of absorbance and spectra of absorption were performed using a doublebeam UV-visible spectrophotometer (JASCOV-630) with 1.0-cm glass cells. While a professional Benchtop pH meter (BP3001) was used to record the pH values.

#### **Chemical materials and reagents**

All chemicals used are of the highest purity available. TCH was provided by the state company for drug industries and medical appliances (SDI), Sammara, Iraq.

**TCH working solution, 100 \mug/ml.** A 0.0100 g quantity of TCH was dissolved in 100 ml DW (distilled water) using a standard flask. This solution was stored in a brown container.

**p-ABA solution**, (7.29×10<sup>-4</sup> M). A 0.0100 g p-ABA (Fluka) was weighed and dissolved in a tiny amount of DW in a 100 ml standard flask and with the same solvent the volume was made to the mark. The prepared solution was transferred to a dark container for preservation.

Sodium nitrite (NaNO<sub>2</sub>) stock solution (7.29×10<sup>-3</sup>M). A 0.05 g of NaNO<sub>2</sub> was dissolved in 100 ml of DW.

Sodium nitrite working solution (7.29 $\times$  10<sup>-4</sup> M). A 10.0 ml of the (stock NaNO<sub>2</sub>) solution was diluted with DW to make the final volume 100 ml.

**Hydrochloride acid solution (1M).** This solution was made by taking 8.6 ml of concentrated hydrochloride acid (11.6 M) and the volume was diluted to 100 ml in a standard flask.

**Ammonium hydroxide solution (1M).** A 7.5 ml of the concentrated ammonium hydroxide solution (13.4M) was diluted with 100 ml of DW. The solution was then stored in a plastic container.

#### Essential method and standard curve

Into a sequence of 20 ml standard flasks, 1ml of  $7.29 \times 10^{-4}$ M p-ABA, an equimolar of NaNO<sub>2</sub> and 1.25 ml of 1M hydrochloric acid solutions were added to each flask. The solutions were mixed well and set aside at RT (laboratory temperature) for 20 min. An aliquot 0.05-3.5 ml of TCH working solution (100 µg/ml) was then added and followed by adding 3 ml of 1M ammonium hydroxide solutions. The final solutions were shake well and made to the mark with DW. At 422 nm, the absorbance of the produced azo dye was measured against a blank solution prepared by using the same way but without TCH. A standard calibration curve gives a straight line in the concentration range of 0.25-17.5 µg/ml TCH with R<sup>2</sup>= 0.9993 as shown in Fig. (2). The molar absorptivity and Sandell's sensitivity were calculated and found to be  $1.66 \times 10^4$  l/mol.cm and 0.0289 µg/cm<sup>2</sup>, respectively. LOD and LOQ have been also calculated and found to 0.0120 and 0.040 µg/ml respectively.



Fig. 2: The calibration curve and the absorption spectra for estimating TCH according to the suggested procedure

# Procedure for assaying TCH in its pharmaceutical formulations

## For TCH capsule

The contents of five capsules (each capsule contains 250 mg TCH) were weighed and mixed well. An accurate quantity of powder equivalent to 0.0100g TCH was taken and dissolved in 100 ml DW using a standard flask to obtain a solution of TCH its concentration of 100  $\mu$ g/ml and an aliquot of this drug solution was analyzed by following the suggested method.

# For TCH ointment

The content of three containers of TCH ointment is mixed very well and an accurate quantity equivalent to 0.0100g TCH was weighed and dissolved in a mixture of (3 ml ethanol and 50 ml DW). The mixture was warmed and filtered into 100 ml standard flask, the volume diluted up to the mark with DW (Othman and Al-Ashow, 2012), each ml of the diluted solution contains 100  $\mu$ g of TCH and a portion of it was analyzed using the described method.

#### **Preliminary conditions**

#### **RESULTS AND DISCUSSION**

In this investigation, TCH was coupled with diazotized p-ABA in an alkaline solution to produce a yellow azo dye which shows highest absorption at 422 nm vs. blank solution. The resulting colour intensity was found to be proportional to the amount of TCH that originally exist in the solution.

#### Optimal reaction conditions Influence of the type and amount of acid

The equation below describes how the diazotization process happens:

 $ArNH_2 + NaNO_2 + 2 HX \rightarrow ArN_2X + NaX + 2 H_2O$ 

An acid solution is necessary for the diazotization process. Therefore, the effect of several acid solutions in varying volumes (1M), including HCl, HNO<sub>3</sub>, H<sub>2</sub>SO<sub>4</sub>, CH<sub>3</sub>COOH, and HCOOH, on the dye's absorbance has been studied. The volume of 1.25 ml of 1M HCl is the best, according to the experimental results in Fig. (3), the volume 1.25 ml of HCl was selected for the next experiments.

Development of a Spectrophotometric.....



Fig. 3: Influence of various acids and their quantities on absorbance

#### Influence of p-ABA reagent amount

The influence of varying amounts (0.5 to 1.5ml) of p-ABA reagent (0.01%) on the absorption of the resulting azo dye has been tested. According to (Table 1), the result of 1.0 ml of p-ABA (0.01%) was found to be the optimum and provide highest absorbance with coefficient value ( $R^2$ =0.9995).

ml of (p-ABA)	Absorbance / μg of TCH present in 20 ml							
solution $(0.01\%)$	20	50	100	120	150	200	300	<b>R</b> <sup>2</sup>
0.5	0.0433	0.0742	0.1241	0.1447	0.1816	0.2415	0.3367	0.9984
0.7	0.0512	0.0789	0.1387	0.1552	0.1943	0.2411	0.3314	0.9966
1.0	0.0766	0.1089	0.1619	0.1802	0.2057	0.2571	0.3563	0.9995
1.2	0.0637	0.0913	0.1521	0.1605	0.1917	0.2435	0.3367	0.9980
1.5	0.0441	0.0718	0.1192	0.1438	0.1743	0.2379	0.3398	0.9981

Table 1: Effect of reagent amount on the absorbance

### Influence of diazotization time

The influence of diazotized reaction time on the azo dye's absorption intensity was carried out by measuring the absorption at different periods of time at laboratory temperature  $(25\pm 2C^{\circ})$  and the results are illustrated in Fig. (4).



Fig. 4: Time effect of the diazotization reaction on absorbance

The results in Fig. (4) confirmed that the diazotization process needs 20 minutes to be completed and to give the highest value of absorbance.

# Influence of base type and its amount

The influence of varying quantities (0.5- 4.0 ml) of various base solutions (sodium hydroxide, potassium hydroxide, sodium carbonate, sodium bicarbonate and ammonium hydroxide) on the intensity of the yellow dye have been studied. The data are listed in (Table 2) and reveal that the volume of 3 ml of ammonium hydroxide solution (1M) is the best because it provides the highest sensitivity.

Type of base solution		pН					
( <b>1M</b> )	0.5	1.0	1.5	2.0	3.0	4.0	
NaOH	0.0381	0.0630	0.0931	0.0760	0.0489	0.0355	12.9
КОН	0.0342	0.0404	0.0538	0.0330	0.0233	0.0214	12.9
Na <sub>2</sub> CO <sub>3</sub>	0.1105	0.1680	0.1335	0.1236	0.0943	0.0781	9.44
NaHCO <sub>3</sub>	0.0245	0.0340	0.0861	0.1472	0.1489	0.1453	8.20
NH4OH	0.0324	0.0783	0.1160	0.1479	0.2242	0.2008	9.90

 Table 2: Influence of various bases and their quantities on absorbance

# Time effect on the colour development

Two different amounts 100 and 200  $\mu$ g of TCH were used to investigate the effect of time on the azo dye's color stability at 422 nm. The absorbance was determined at varying times up to 60 minutes. The data in Fig. (5) show that the yellow azo dye that resulted from adding the alkaline solution of ammonium hydroxide appears immediately and is stable for at least 60 minutes at laboratory temperature (RT).



Fig. 5: Influence of colour development time on absorbance.

# Absorption spectrum

By applying the ideal reaction conditions,  $5\mu$ g/ml of TCH was reacted with the diazotized p-ABA reagent solution in an alkaline solution of NH<sub>4</sub>OH to produce a yellow water-soluble azo dye which exhibits a high peak intensity with an absorption maximum at 422 nm versus the reagent blank solution Fig. (6).



Fig. 6: Absorption spectra of 5µg/ml TCH measured against blank solution (A), DW (B) and blank solution against DW(C)

### 115

#### Nature of the resulting azo dye

Under the optimized conditions of the essential procedure, continuous variation and mole ratio methods (Delevic, 1997) were used to clarify the correlation ratio of TCH with the diazotized p-ABA. Both methods were carried out using the same concentrations of TCH and p-ABA solutions  $(2.079 \times 10^{-4} \text{M})$ . The results of both methods are explained in Fig. (7) revealing that the dye was created by the reaction of TCH and diazotized p-ABA compound in a ratio of 1:1.



Fig. 7: Plots of (a) the continuous variation and (b) mole-ratio methods for TCH-diazotized p-ABA azo dye.

# Mechanism of the reaction

A mechanism of interaction has been suggested which includes that p-ABA react with (NaNO<sub>2</sub>) in presence of the strong acid like (HCl) to produce diazonium salt coupled with TCH in presence of NH<sub>4</sub>OH solution and production of a yellow azo dye. The reaction's mechanism is illustrated in (Scheme 1).



Scheme 1: The reaction mechanism

# Quantification

The concentration range of Beer's law, molar absorptivity ( $\epsilon$ ), recovery percent, RSD, LOD, and LOQ were determined and the data were listed in (Table 3). The Linearity of the method was also described by the regression equation and the corresponding determination coefficient ( $R^2$ ) for TCH was estimated by using the suggested method and referred to a good linearity.

Table 5. Summary of optical characteristics and statistical data for the proposed meth	3: Summary of optical charac	cteristics and statistica	l data for the	proposed metho
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Parameter	Information
Range of Beer 's law, µg/ml	0.25-17.5
$\lambda$ max, nm	422
Molar absorptivity, l/mol.cm	$1.66 \times 10^4$
Recovery range, %	99.96% to 101.47 %
Error percent range, %	-0.08% to 3.3 %
Precision (RSD), %	≤ 1.59
Determination coefficient $(R^2)$	0.9993
LOD, µg/ml	0.0120
LOQ, µg/ml	0.040
Slope (a) <sup>#</sup>	0.0346
Intercept (b) <sup>#</sup>	0.0374

<sup>#</sup>Regression equation: (Y = a X + b), X =TCH concentration in  $\mu$ g per ml.

# Application

The suggested method has been applied for the analysis of TCH in its pharmaceutical forms (capsules and skin ointment) from different origins and at three different quantities (50, 100, 150  $\mu$ g). The results listed in (Table 4) indicated that the developed method is a suitable for assaying TCH with an acceptable result.

To evaluate the results of the developed method a t-test has been investigated and the results in (Table 4) reveal that the values of the t-experiment are less than the t-tabulated value at the (95%) confidence level (Christian, 2004). The experimental results indicated that the difference is statistically not significant, which confirms the success of applying the proposed method to estimate TCH in its pharmaceutical forms.

Pharmaceutical Form	Certified value	μg TCH Found*	RE. (%)*	Mean Reco. (%)	RSD (%)*	Measured value	Experim. t-value <sup>a</sup>
A monolimo		49.96	-0.08		0.16		
Apcycline		100.035	0.035	99.98	0.41	249.95 mg	1.91
(India)		149.97	-0.02		0.24		
Totrograling	250 (	49.96	-0.08		0.80		
(Iran)	250 mg/ Capsule	99.98	-0.02	99.96	0.48	249.90 mg	1.48
		149.96	-0.03		0.55		
Somoovolino		50.02	0.04		0.66		
(SDI-Iraq)		99.99	-0.01	100.014	0.43	250.035 mg	1.05
		149.98	0.013		0.27		
Samaavalina	3% skin	50.04	0.08		1.19		
Samacycline	ointment	101.04	1.04	101.47	1.59	3.044 %	2.19
(SDI-II aq)		154.95	3.30		1.45		

 Table 4: Determination of TCH in capsules and skin ointment

\*Average of five estimations, at-value =  $(\bar{\mathbf{x}} - \mu) \frac{\sqrt{N}}{s}$ , atabulated "t" value at 95% confidence level is equal to 2.776, degree of freedom (N=4)

# Evaluation of the results of the proposed procedure using the standard addition method

To evaluate the selectivity of the suggested procedure and its success in estimating TCH in its pharmaceutical forms, the standard addition method was applied for this purpose. The results

#### 117

exemplified in Fig. (8) and listed in Table 5 indicating that the standard additions method agrees well with the results of the proposed method without interferences and within a satisfactory range of error.



Fig. 8: Standard graphs of standard addition method for the analysis of TCH in pharmaceutical forms (capsules and skin ointment)

 Table 5: Estimation of TCH in its pharmaceutical preparations using the standard addition method

Pharmaceutical	Certified	µg of TCH	µg of TCH	Recovery	Measured value
preparation	value	Present	Found	(%)*	
Apcycline		50	50.90	101.8	254.5 mg
(India)		100	99.65	99.65	249.12 mg
Tetracycline	250 mg /	50	49.05	98.10	245.25 mg
(Iran)	capsule	100	100.65	100.65	251.62 mg
Samacycline		50	49.04	98.08	245.20 mg
(SDI-Iraq)		100	101.5	101.5	253.75 mg
Samacycline	3% Skin	50	51.60	103.2	3.096 %
(SDI-Iraq)	ointment	100	103.7	103.7	3.11 %

# CONCLUSION

In this investigation, TCH was coupled with diazotized p-aminobenzoic acid (p-ABA) in an alkaline solution to produce a yellow azo dye. This dye was stable and soluble in water and showed maximum absorption peak at 422 nm vs. blank solution. Beer's law was linear in the concentration range of 0.25-17.5  $\mu$ g/ml with molar absorptivity of  $1.66 \times 10^4$  l/mol.cm. The values of error percentage and precision (RSD%) were calculated and found in the range of -0.08% to 3.3% and 0.16% to 1.59%, correspondingly. The suggested approach for estimating TCH in pharmaceutical samples has the advantages of being sensitive, low-cost, and straightforward because it does not require a solvent extraction step or temperature control. The technique is also accurate and precise enough to replace the current spectrophotometric method and evaluation of TCH in ointment and capsules.

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تطوير طريقة طيفية لتقدير التتراسايكلين هيدروكلوريد بالاقتران مع بارا – أمينو حامض البنزويك المؤزوت – تطبيق في المستحضرات الصيدلانية

> ر**انيه إبراهيم شيت** قسم الكيمياء/كلية العلوم/ جامعة الموصل

> > الملخص

يتضمن هذا البحث تطوير طريقة طيفية حساسة ودقيقة لتقدير التتراسايكلين هيدروكلوريد في الوسط المائي وفي المستحضرات الصيدلانية. تعتمد هذه الطريقة على ازوتة الكاشف بارا- أمينو حامض البنزويك بتفاعله مع كمية ممائلة من نتريت الصوديوم بوجود محلول حامض الهيدروكلوريك لتكوين مركب الدايازونيوم الذي يقترن مباشرة مع التتراسايكلين هيدروكلوريد في وسط قاعدي من محلول هيدروكسيد الامونيوم لتكوين صبغة ازوية ذات لون اصفر ذائبة ومستقرة في الماء تعطي أعلى شدة امتصاص عند الطول الموجي (422) نانوميتر مقابل المحلول الصوري. و كانت الطريقة خطية تتبع حدود قانون بير في مدى التركيز (5.0–17.5) مايكرو غرام/مللتر بمعامل تقدير (10903 = R) و امتصاصية مولارية مساوية الى(1093 = R) و (10×60.6) لتر/ مول. سم على التوالي. و كانت حساب قيمة حد الكشف (LOD) و قيمة حد التقدير الكمي (1000) مساوية الى (1000) و (0.010) على التوالي. و كانت حساب قيمة حد الكشف (2001) و قيمة حد التقدير الكمي (2003) مساوية الى (3.0%) مايكرو غرام/مللتر على التوالي. بينما كان معدل قيم الخطأ النسبي وقيم التوافقية (2008) يترام. مساوية الى (3.0%) مايكرو غرام/ مللتر على التوالي. بينما كان معدل قيم الخطأ النسبي وقيم التوافقية (2000) مساوية الى (3.0%) و (0.00%) الى (0.00%) ماليز و كانت معدل قيم الخطأ النسبي وقيم التوافقية (2008) مساوية الى (3.0%) مع الى (3.0%) مايكرو غرام/ مللتر على التوالي. بينما كان معدل قيم الخطأ النسبي وقيم التوافقية (2008) ميترام. مساوية الى (1.0%) الى (2008) معارك المولية بين التتراسايكلين هيدروكلوريد والكاشف بارا- أمينو حامض مايكرو غرام/ مللتر على التوالي. بينما كان معدل قيم الخطأ النسبي وقيم التوافقية (2008) معاوريد والكاشف بارا- أمينو حامض البنزويك المؤزوت وكانت مساوية الى1.1. طبقت المريقة المقترحة بنجاح في تقدير التتراسايكلين هيدروكلوريد والكاشف بارا- أمينو حامض البنزويك المؤزوت وكانت مساوية الى1.1. طبقت الطريقة المقترحة بنجاح في تقدير التتراسايكلين هيدروكلوريد في الكبسولات والمرهم.

الكلمات الدالة: تتراسايكلين هيدروكلوريد، الاقتران الازوي، بارا أمينو حامض البنزويك، الضوئية.