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

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ABSTRACT
This work comprises developing a sensitive and precise spectrophotometric procedure to analyze tetracycline hydrochloride in the bulk and in the drug forms. In this method, p-aminobenzoic acid reagent was diazotized with an equimolar NaNO₂ (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 NH₄OH 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 µg/ml with an exceptional determination coefficient (R² = 0.9993) and molar absorptivity of 1.66×10⁴ l/mol.cm. The calculated values of detection limit (LOD) and quantitation limit (LOQ) were equal to 0.012 and 0.040 µg/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.
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-dimethylamino-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_{2}O_{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 graphene 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), 2,4-dinitrophenylhydrazine (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 N-bromosuccinimide 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 double-beam 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 µg/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^{-4} 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_2) stock solution (7.29×10^{-3} M).** A 0.05 g of NaNO_2 was dissolved in 100 ml of DW.
- **Sodium nitrite working solution (7.29× 10^{-4} M).** A 10.0 ml of the (stock NaNO_2) 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 hydrochloric 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 7.29×10^{-4} M p-ABA, an equimolar of NaNO_2 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^2= 0.9993 as shown in Fig. (2). The molar absorptivity and Sandell's sensitivity were calculated and found to be 1.66×10^4 l/mol.cm and 0.0289 µg/cm², respectively. LOD and LOQ have been also calculated and found to 0.0120 and 0.040 µg/ml respectively.
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 0.0100 g TCH was taken and dissolved in 100 ml DW using a standard flask to obtain a solution of TCH its concentration of 100 µg/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 0.0100 g 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 µg of TCH and a portion of it was analyzed using the described method.

RESULTS AND DISCUSSION

Preliminary conditions
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:

\[
\text{ArNH}_2 + \text{NaNO}_2 + 2 \text{HX} \rightarrow \text{ArN}_2\text{X} + \text{NaX} + 2 \text{H}_2\text{O}
\]

An acid solution is necessary for the diazotization process. Therefore, the effect of several acid solutions in varying volumes (1M), including HCl, HNO₃, H₂SO₄, CH₃COOH, 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.
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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 1.5 ml) 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).

Table 1: Effect of reagent amount on the absorbance

<table>
<thead>
<tr>
<th>ml of (p-ABA) solution (0.01%)</th>
<th>Absorbance / µg of TCH present in 20 ml</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20</td>
<td>50</td>
</tr>
<tr>
<td>0.5</td>
<td>0.0433</td>
<td>0.0742</td>
</tr>
<tr>
<td>0.7</td>
<td>0.0512</td>
<td>0.0789</td>
</tr>
<tr>
<td>1.0</td>
<td>0.0766</td>
<td>0.1089</td>
</tr>
<tr>
<td>1.2</td>
<td>0.0637</td>
<td>0.0913</td>
</tr>
<tr>
<td>1.5</td>
<td>0.0441</td>
<td>0.0718</td>
</tr>
</tbody>
</table>

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 ($25\pm2^\circ C$) 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.

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

<table>
<thead>
<tr>
<th>Type of base solution (1M)</th>
<th>Absorbance / ml of base added</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>NaOH</td>
<td>0.0381</td>
<td>0.0630</td>
</tr>
<tr>
<td>KOH</td>
<td>0.0342</td>
<td>0.0404</td>
</tr>
<tr>
<td>Na₂CO₃</td>
<td>0.1105</td>
<td>0.1680</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>0.0245</td>
<td>0.0340</td>
</tr>
<tr>
<td>NH₄OH</td>
<td>0.0324</td>
<td>0.0783</td>
</tr>
</tbody>
</table>

Time effect on the colour development

Two different amounts 100 and 200 µg 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).

Absorption spectrum

By applying the ideal reaction conditions, 5µg/ml of TCH was reacted with the diazotized p-ABA reagent solution in an alkaline solution of NH₄OH 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. 5: Influence of colour development time on absorbance.

Fig. 6: Absorption spectra of 5µg/ml TCH measured against blank solution (A), DW (B) and blank solution against DW(C)
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 (2.079×10^{-4}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 (NaNO₂) in presence of the strong acid like (HCl) to produce diazonium salt coupled with TCH in presence of NH₄OH 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 (ε), 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 (R²) for TCH was estimated by using the suggested method and referred to a good linearity.

Table 3: Summary of optical characteristics and statistical data for the proposed method

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range of Beer’s law, μg/ml</td>
<td>0.25-17.5</td>
</tr>
<tr>
<td>λ max, nm</td>
<td>422</td>
</tr>
<tr>
<td>Molar absorptivity, l/mol.cm</td>
<td>1.66×10⁴</td>
</tr>
<tr>
<td>Recovery range, %</td>
<td>99.96% to 101.47 %</td>
</tr>
<tr>
<td>Error percent range, %</td>
<td>≤ 1.59</td>
</tr>
<tr>
<td>Precision (RSD), %</td>
<td>0.9993</td>
</tr>
<tr>
<td>Determination coefficient (R²)</td>
<td>0.0120</td>
</tr>
<tr>
<td>LOD, μg/ml</td>
<td>0.040</td>
</tr>
<tr>
<td>LOQ, μg/ml</td>
<td>0.0346</td>
</tr>
<tr>
<td>Slope (a)</td>
<td>0.0374</td>
</tr>
<tr>
<td>Intercept (b)</td>
<td></td>
</tr>
</tbody>
</table>

*Regression equation: (Y = a X + b), X = TCH concentration in μg 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 μg). 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.

Table 4: Determination of TCH in capsules and skin ointment

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

*Average of five estimations, t-value = (X – μ) / s / √N, t-tabulated “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
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.

![Graphs of standard addition method for the analysis of TCH in pharmaceutical forms](image)

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

<table>
<thead>
<tr>
<th>Pharmaceutical preparation</th>
<th>Certified value</th>
<th>µg of TCH Present</th>
<th>µg of TCH Found</th>
<th>Recovery (%)*</th>
<th>Measured value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apcycline (India)</td>
<td>250 mg / capsule</td>
<td>50</td>
<td>50.90</td>
<td>101.8</td>
<td>254.5 mg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>99.65</td>
<td>99.65</td>
<td>249.12 mg</td>
</tr>
<tr>
<td>Tetracycline (Iran)</td>
<td></td>
<td>50</td>
<td>49.05</td>
<td>98.10</td>
<td>245.25 mg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>100.65</td>
<td>100.65</td>
<td>251.62 mg</td>
</tr>
<tr>
<td>Samacycline (SDI-Iraq)</td>
<td></td>
<td>50</td>
<td>49.04</td>
<td>98.08</td>
<td>245.20 mg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>101.5</td>
<td>101.5</td>
<td>253.75 mg</td>
</tr>
<tr>
<td>Samacycline (SDI-Iraq)</td>
<td></td>
<td>3% Skin ointment</td>
<td>50.60</td>
<td>103.2</td>
<td>3.096 %</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>103.7</td>
<td>103.7</td>
<td>3.11 %</td>
</tr>
</tbody>
</table>

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

**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 µg/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.
REFERENCES


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تطوير طريقة طيفية لتقدير التتراسايكلين هيدروكليميد بالاقتراح مع بارا – أمينو حامض البنزويك المؤزوئت

- تطبيق في المستحضرات الصيدلانية

 Eylül İbrahim Şinad
قسم الكيمياء/ كلية العلوم/ جامعة المنصورة

المتخصم

يتضمن هذا البحث تطوير طريقة طيفية حساسة ودقيقة لتقدير التتراسايكلين هيدروكليميد في الوسط المائي وفي المستحضرات الصيدلانية. تم إعداد هذه النظرية على الأوزان الكاففة ببارا- أمينو حامض البنزويك بفاعلية مع كمية مماثلة من نتائج الصوديوم يوجد محلول حامض الهيدروكليميد لتكوين مركب الدايامينيون الذي يقترن مباشرةً إذاً التتراسايكلين هيدروكليميد في وسط قاعدي من محلول الهيدروكسي الأمونيوم ليكون صبغة ازوية ذات لون أصفر ذاتية ومستقرة في الماء تعطي شدة امتصاص عند الطول الموجي (422) نانومتر مقابل محلول الصوديوم. و كانت الطريقة خطية تتبع ححد قانون بير في مدى التركيز (0.25-17.5) مايكروغرام/مللر بالفراغ (R²=0.9999) و انتهاض قياس البالغ (LOQ) و قياس حد التكشف (LOD) و قيم البالغ (0.040) و قيم حد التكشف (0.012) و (0.08%) و (3.3%) و (1.59) و (0.16) على التوالي. و تم حساب نسبة المولية بين التتراسايكلين هيدروكليميد والتفاعل بارا- أمينو حامض البنزويك المؤزوئت وكانت مساوية إلى 1:1. طبقت الطريقة المقتضبة بنجاح في تقدير التتراسايكلين هيدروكليميد في الكبسولات المزروه.

الكلمات الدالة: تتراسايكلين هيدروكليميد، الاقتراح الازوي، بارا- أمينو حامض البنزويك، الصباغة.