PERIÓDICO TCHÊ QUÍMICA
ARTIGO ORIGINAL

SÍNTESE DE NOVOS COMPOSTOS DE HIDROCLORETO DE PROCAÍNA SUBSTITUÍDOS E SEU USO NA DETERMINAÇÃO ESPECTROFOTOMÉTRICA DE HIDROCLORETO DE TETRACICLINA ATRAVÉS DE REAÇÕES DE ACOPLAMENTO DIAZÓICO

SYNTHESIS OF NEW SUBSTITUTED PROCAINE HYDROCHLORIDE COMPOUNDS AND THEIR USE IN SPECTROPHOTOMETRIC DETERMINATION OF TETRACYCLINE HYDROCHLORIDE THROUGH DIAZOTIZATION-COUPLING REACTIONS

تحضير مركبات معوضة جديدة لهيدروكلوريد البروكائين واستخدامها في التقدير الطيفي لهيدروكلوريد التتراسايكلين من خلال تفاعلات الازوتة والازدواج

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RESUMO

Introdução: A tetraciclina é um dos antibióticos mais importantes. É usado para tratar muitas infecções bacterianas diferentes da pele, intestinos, trato respiratório, trato urinário, genitais, gânglios linfáticos e outros sistemas do corpo. É frequentemente usado no tratamento de acne grave ou doenças sexualmente transmissíveis, como sífilis, gonorreia ou clamídia. Em alguns casos, a tetraciclina é usada quando a penicilina ou outro antibiótico não pode ser usado para tratar infecções graves, como as causadas por *Bacillus anthracis*, *Listeria, Clostridium e Actinomyces*. **Objetivos**: sintetizar um novo reagente usado para determinar o TCH espectrofotometricamente usando diazônio e reação de acoplamento. **Métodos:** Quatro novos derivados de procaína substituídos foram preparados por métodos orgânicos simples usando derivados de anilina. Uma abordagem espectrofotométrica foi estabelecida para a microdeterminação de TCH. A estequiometria foi investigada usando métodos de razão molar e variação contínua, e a constante de estabilidade também foi estimada. O ΔG, ΔH e ΔS foram determinados como parâmetros termodinâmicos para avaliar o efeito da temperatura na reação. **Resultados**: Derivados de procaína substituídos foram preparados, e o-hidroxi procaína parece ser o melhor reagente usado para determinar TCH por diazotização e reação de acoplamento. O resultado foi um corante amarelo solúvel em água com uma absorbância máxima de 380 nm. As condições de reação foram estudadas e otimizadas. A lei de Beer foi obedecida em uma faixa de concentração (2,5–50) $\mu g.mL^{-1}$ para TCH. A absortividade molar foi de $14{,}4669 \cdot 10^3$ $L \cdot mol^{-1} \cdot cm^{-1}$, e o limite de detecção foi de 0,5052 $\mu g \cdot mL^{-1}$. A estequiometria do produto formado foi encontrada 1:1 (o-hidroxiprocaína:TCH). A constante de estabilidade indicou que o produto formado era estável, os parâmetros termodinâmicos mostraram que a reação do sal de diazônio era preferível para ocorrer em baixa temperatura. **Conclusões**: um método simples, preciso e rápido foi desenvolvido para a determinação de TCH na forma pura e farmacêutica por acoplamento do TCH com um reagente derivado de procaína recentemente sintetizado (o-hidroxi procaína) em um meio alcalino.

Palavras-chave: *Cloridrato de tetraciclina, cloridrato de procaína, reação de diazônio, síntese, espectrofotometria, aplicações farmacêuticas*.

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ABSTRACT

Background: Tetracycline is one of the most important antibiotics. It is used to treat many different bacterial infections. It is often used in treating severe acne, or sexually transmitted diseases such as syphilis, gonorrhea, or chlamydia. In some cases, tetracycline is used when penicillin or another antibiotic cannot be used to treat serious infections such as the ones caused by *Bacillus anthracis, Listeria, Clostridium, Actinomyces*. **Aim**: synthesized a new novel reagent used to determine TCH spectrophotometrically by using diazonium and coupling reaction. **Methods:** Four new substituted procaine derivatives were prepared by simple organic methods using aniline derivatives. A spectrophotometric approach was established for the micro-determination of TCH. The stoichiometry was investigated using mole ratio and continuous variation methods, and the stability constant was also estimated. The ΔG, ΔH, and ΔS were determined as thermodynamic parameters for evaluating the effect of temperature on the reaction. **Results**: Substituted procaine derivatives were prepared, and o-hydroxy procaine seems to be the best reagent used to determine TCH by diazotization and coupling reaction. The result was a yellow water-soluble dye with a maximum absorbance of 380 nm. The reaction conditions were studied and optimized. Beers law was obeyed over a concentration range (2.5–50) $\mu g.mL^{-1}$ for TCH. The molar absorptivity was ($14.4669.10^3$) $L.mol^{-1}.cm^{-1}$, and the detection limit was (0.5052) $\mu g.mL^{-1}$. The stoichiometry of the formed product was found 1:1 (o-hydroxyprocaine: TCH). The stability constant indicated that the product formed was stable, and the thermodynamic parameters showed that the diazonium salt reaction was preferred to occur at a low temperature. **Conclusions**: a simple, accurate, and fast method was developed to determine TCH in pure form and pharmaceuticals by coupling the TCH with a newly synthesized procaine derivative reagent (o-hydroxy procaine) in a basic medium.

Keywords: *Tetracycline Hydrochloride, Procaine Hydrochloride, diazonium reaction, synthesis, spectrophotometry, pharmaceutical applications*.

الملخص

الخلفية: يعتبر التتراسيكلين أحد أهم المضادات الحيوية. يتم استخدامه لعلاج العديد من الالتهابات البكتيرية المختلفة للجلد والأمعاء والجهاز التنفسي والمسالك البولية والأعضاء التناسلية والعقد الليمفاوية وأنظمة الجسم الأخرى. غالبًا ما يستخدم في علاج حب الشباب الحاد أو الأمراض المنقولة جنسياً مثل الزهري أو السيلان أو الكلاميديا. في بعض الحالات ، يتم استخدام التتراسيكلين عندما لا يمكن استخدام البنسلين أو أي مضاد حيوي آخر لعلاج الالتهابات الخطيرة مثل الجمرة الخبيثة والليستريا والمطثيات والأكتينوميسيس. الهدف: تحضير كاشف جديد يستخدم لتقدير هيدروكلوريد التتراسايكلين طيفيا" باستخدام تفاعل الازوتة والاقتران. الطريقة: تم تحضير أربعة مشتقات معوضة جديدة للبروكائين بطرائق عضوية بسيطة باستخدام مشتقات الأنيلين. تم تطوير طريقة طيفية من أجل التحديد الدقيق لـ TCH. وتم فحص قياس مقدار ارتباط الكاشف مع الدواء باستخدام طريقة النسبة المولية والتغيرات المستمرة ، كما تم دراسة ثابت الاستقرار. تم تحديد طاقة كبس الحرة و الانثالبي و الانتروبي كمعلمات ديناميكية حرارية لتقييم تأثير درجة الحرارة على التفاعل. النتائج: تم تحضير مشتقات البروكايين المستبدلة ، ووجد أن o-hydroxy procaine هو أفضل كاشف يستخدم لتحديد TCH عن طريق الازوتة والازدواج. وكانت النتيجة تكون صبغة صفراء قابلة للذوبان في الماء مع أقصى امتصاص عند 380 نانومتر. تمت دراسة ظروف التفاعل وتحسينها. تم الامتثال لقانون بيير على مدى تركيز (2.5-50) ميكروغرام مل $^{-1}$ لـ TCH. كانت الامتصاصية المولية ($14.4669.10^3$) لتر.مول$^{-1}$.سم$^{-1}$ ، وكان حد الكشف (0.5052) ميكروجرام لكل لتر. تم قياس نسبة الارتباط المتكافئ للمنتج المتكون 1: 1 (o-hydroxyprocaine: TCH). أشار ثابت الاستقرار إلى أن المنتج المتكون كان مستقرًا ، وأظهرت المعلمات الديناميكية الحرارية أن تفاعل ملح الديازونيوم كان مفضلًا عند درجة حرارة منخفضة. الاستنتاجات: تم تطوير طريقة بسيطة ودقيقة وسريعة لتحديد TCH في شكله النقي والمستحضرات الصيدلانية عن طريق اقتران TCH مع الكاشف المحضر من مشتق البروكائين الجديد (o-hydroxy procaine) في وسط قاعدي.

الكلمات المفتاحية: هيدروكلوريد التتراسايكلين، هيدروكلوريد البروكائين، تفاعل الازوتة، تحضير، المطيافية، المستحضرات الصيدلانية

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1. INTRODUCTION:

Tetracyclines (TCs) are the most common antibiotic drugs in the world. Its broad-spectrum family of antibiotics is known to inhibit protein synthesis in bacteria and combat various bacterial infections. It is well established that tetracyclines inhibit bacterial protein synthesis by preventing the association of aminoacyl tRNA with the bacterial ribosome, it is used to treat infections of the skin, intestines, respiratory tract, urinary tract, genitals, lymph nodes, and other body systems. (Fiaz *et al*., 2021). Tetracycline Hydrochloride (TCH) is defined chemically as: (4S,6S,12aS)4(dimethylamino)-1,4,4a,5,5a,6, 11,12a-octahydro-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxonaphthacene-2-carboxamide hydrochloride as shown in (Figure 1), (drug bank online, 2021).

Several methods have been reported for the determination of TCH in pure form and in pharmaceutical preparations, including spectrophotometry charge transfer complexes (José *et al*., 2010; Khairi, 2008), Oxidative

coupling reactions (Rokayia and Alaa, 2015, Lamia *et al*., 2016, Roaa and Dawooh, 2020), glassy carbon electrodes (Shirley *et al*., 2019), HPLC method (Roy and Gogoi, 2014) and atomic absorption (Fatima and Rana, 2017).

Visible spectrophotometric methods are available for assessing TCH in pharmaceuticals (Chinar, 2013, Swapna and Radha, 2014, Fatima and Rana, 2016); TCH determination has been reported using a variety of diazotization reaction techniques (Michael *et al*., 2016, Maryam *et al*., 2017, Nabeel and Rasha, 2012, Mouayed and Zaid, 2015, Rasul *et al*., 2018). However, many of these procedures have disadvantages, including low sensitivity, the need for heating, extraction steps, or the use of organic solvents, and critical operating conditions that are incompatible with the routine analysis.

Procaine hydrochloride (PCH) is a local anesthetic drug with an amine group (London, Medicines and Healthcare products Regulatory Agency, 2020), a white or almost white crystalline powder or colorless crystals, very soluble in water and soluble in ethanol. Its chemical structure is described in Figure 2.

The methods used to synthesize and identify four new substituted procaine were described in this paper. The prepared reagents were diazotized and used for the spectrophotometric determination of TCH in an alkaline medium to form a yellow soluble dye measured spectrophotometrically at λ_{max} of maximum absorption.

The newly prepared reagents and their use for TCH determination have not been previously reported in the literature.

The effect of the substituted groups (electron-donating or electron-withdrawing) in procaine HCl on the sensitivity of the determination of TCH and on the stability constant of the product for the reaction were studied.

This study describes simple methods for synthesizing organic reagents (using a drug as a raw material, which is procaine hydrochloride) and then studying the optimum conditions for choosing the best reagent that could be used as a coupling agent in diazotization reactions for the determination of tetracycline hydrochloride in pure form and in pharmaceuticals. It also studies the effect of temperature on the diazotization reaction by studying the thermodynamic parameters free energy changes ΔG, enthalpy of formation ΔH, and entropy ΔS.

2. MATERIALS AND METHODS:

2.1. Apparatus:

2.1.1 Fourier Transform Infrared Spectrophotometer (FT-IR)

For FT-IR spectra measurements, the procaine HCl (PCH) and the synthesized reagents (PCH, PCS, NPC, PCA, and HPC) were recrystallized and dried well then take a very small amount which mixed with KBr-powder in order to make KBr disk that was recorded the spectra on the Shimadzu FT-IR 8400.

2.1.2 *UV-Visible spectra*

The absorbance measurements were performed using a Shimadzu UV-Visible 1240 digital single-beam spectrophotometer. The wavelength range used was between 300 to 700 nm for the dye-complex formed.

The spectra measurements were performed by a Shimadzu UV-1800 digital double beam spectrophotometer, a quartz cell (1 cm path length). This was used to determine the maximum wavelength for the complex formed between the TCH and the chosen reagent at an alkaline medium.

2.1.3 Melting point measurements

The melting points were recorded using the Gallenkamp melting point apparatus. After the procaine HCl (PCH) and the synthesized reagents (PCH, PCS, NPC, PCA, and HPC) were recrystallized and dried well, then take a minimal amount was grind well with glass mortar and fill a little amount with closed tail capillary tube, then measured the melting point gradually.

2.2. Chemicals and reagents

All the reagents used in this work were of analytical grade. Tetracycline Hydrochloride and Procaine Hydrochloride were purchased from the state company for the drugs industry (SDI/Samarra-Iraq).

Pharmaceutical formulations containing TCH were obtained from local pharmacies: Samacycline capsules 250 mg of tetracycline hydrochloride by SDI-Iraq, apcycline capsules 250 mg tetracycline hydrochloride by Ajanta pharma limited-India, samacycline skin ointment 3% tetracycline hydrochloride by SDI-Iraq, and tetracycline hydrochloride eye ointment 1% tetracycline hydrochloride by Kontam pharmaceuticals CO. LTD- Hong Kong.

2.2.1 TCH stock standard solution 1000 (µg/mL)

To prepare the TCH stock standard solution, 0.1000 g of pure TCH was dissolved in 100 mL of reagent water type IV. Then, working standard solutions were obtained by simple dilution of the TCH stock solution in reagent water type IV.

2.2.2 Diazotized procaine hydrochloride (DPCH) reagent solution (5 mM)

The Diazotized procaine hydrochloride was daily prepared. An equal molar procedure was used to prepare the diazotized procaine hydrochloride (DPCH) reagent solution (5 mM) by dissolving 0.1346 g of PCH in a minimum volume of reagent water type IV and adding 2 mL of 1M hydrochloric acid to a 100 mL volumetric flask. The mixture was cooled from 0 to 5 °C for 5 min, using an ice bath. After that, 0.0345 g of sodium nitrite was added and stirred for 5 min. The volume of the mixture was made up to the mark with reagent water type IV.

2.2.3 Diazotized synthesized reagents solutions (5mM)

The synthesized reagents: Procaine sulfonic acid (PCS), Nitro procaine (NPC), Procaine acetamide (PCA), and Hydroxy procaine (HPC) solutions (scheme 1) were diazotized by reacting with sodium nitrate solution and hydrochloric acid (using the equil procedure) as follow:

a (5 mM) of these solutions were prepared daily by dissolving 0.1581 g, 0.1406 g, 0.1466 g, and 0.1261 g of prepared reagents respectively in a minimum volume of reagent water type IV, then (1 mL, 1 mL, 2 mL, and 3 mL) of (1 M) hydrochloric acid (37%) was added in a 100 ml volumetric flask for each reagent prepared respectively. By using an ice bath, the mixtures were cooled between 0 to 5 ^{o}C for 5 min, a weight of 0.0345 g of sodium nitrite was added to each mixture and stirred. After 5 min the volume was made up to the mark (100 ml) with reagent water type IV.

2.2.4 The hydrochloric acid solution (1 M)

This solution was prepared by appropriately diluting the standard concentrated volumetric solution (5 M) with reagent water type IV in a volumetric flask of 100 mL. Then, the prepared acid solution (1 M) was used to prepare azo salt (formed from the reaction between the amino group in the reagent and sodium nitrate and hydrochloric acid).

2.2.5 The sodium hydroxide solution (0.1 M)

This solution was prepared by dissolving 1.00 g of sodium hydroxide in distilled water (reagent water type IV). The volume was completed to 250 mL in a volumetric flask with distilled water. The sodium hydroxide solution (0.5M) was used to make the medium alkaline, an essential factor in succeeding in the diazonium reaction. In addition, it was used in the spectrophotometric reaction to determine TCH.

2.3. PHARMACEUTICAL PREPARATIONS SOLUTIONS

2.3.1. Capsule solutions of TCH (250 µg.mL^{-1})

The content of ten capsules of TCH were powdered and mixed. A precisely weighed 0.0250 g of the powder was dissolved and transferred to a 100 ml volumetric flask, where the same solvent (reagent water type IV) was used to finish it to the mark. To prepare 250 µg.mL^{-1} of TCH, the solution was thoroughly shacked and filtered (using simple filtration with filter paper). The concentration of the TCH was determined using different volumes of dilute solutions prepared by simple dilution with reagent water type IV.

2.3.2. Ointment solutions

Five tubes of ointment were thoroughly mixed, and an exact weight amount of ointment equivalent to 0.05 g and 0.025 g of TCH were taken for both samacycline skin ointment and tetracycline hydrochloride eye ointment respectively, this amount was extracted three times with hot and filtered (simple filtration with filter paper) into a 100 mL volumetric flask, the residue was washed with hot. The volume was completed to the mark with the same solvent (reagent water type IV), a sample of 1 mL and 2 mL were used to determine TCH in ointments, respectively.

2.4. Synthesis of procaine HCl derivatives

Scheme (1) shows the path synthesis of the newly substituted procaine (PC), which was prepared according to the synthesis of substituted aniline with some modifications (replace the weights and volumes in the original procedures for preparation of substituted aniline with the weights and volumes of the reagent used procaine HCl).

2.4.1. Synthesis of procaine sulphonic acid (PCS)

The synthesis of procaine sulphonic acid (PCS) was conducted as described by Brain *et al*. (1989). It was separated by 2 g (0.00733 mol) of

procaine HCl, which was dissolved in 1.5 ml of sulphuric acid (98%) with a swirl in a cold water bath (15 °C). In a sand bath, the mixture was refluxed from 180 to 190 °C for about 6 hours. After cooling the mixture to 50 °C, it was poured into crushed ice, and the precipitate formed was filtered with simple filtration. Then, it was washed with reagent water type IV, dried, and finally recrystallized in boiling reagent water type IV.

2.4.2. Synthesis of nitro procaine (NPC)

The synthesis of o-nitro procaine (NPC) was conducted according to Arther (1974). Procaine HCl (2 g, 0.00733 mol) was reacted with acetic anhydride (21.5 ml, 0.2148 mol), (20.5 ml, 0.3518 mol) of glacial acetic acid, and (0.1 g) of zinc dust with continuous stirring. The reactant mixture was refluxed for 1.5 to 2 hours at 80 °C. Then, the mixture was cooled with ice water (5 °C). A precipitate was formed, it was filtered and recrystallized from boiling distilled (reagent water type IV). Finally, 2.5 g (0.00898 mol) of compound C (2-(diethylamino)ethyl 4-acetamidobenzoate) (as shown in Scheme 1) was dissolved in 2.5 mL of glacial acetic acid (99.7%) in a cryogenic bath of ice and salt. Later 3 mL of sulphuric acid (98%) was added under constant stirring. When the temperature of this solution fell between 0 to 5 °C, a cold solution of 1 mL concentrated nitric acid (65%) + 0.5 ml of sulphuric acid (98%) was added dropwise. The temperature was maintained below 10 °C. After cooling with crushed ice, the precipitate was filtered and was recrystallized with hot ethanol.

Furthermore, the nitro compound was prepared by dissolving 1.5 g (0.00463 mol) of compound D (as shown in Scheme 1) in 4 ml of sulphuric acid (70%), and the reactant mixture was refluxed for 3 hours at 80 °C. The hot solution was then poured into 20 mL ice water and precipitated by adding 4 to 5 mL 10% sodium hydroxide. Finally, the precipitate was filtered and recrystallized with hot reagent water type IV.

2.4.3. Synthesis of Procaine acetamide (PCA)

The synthesis of procaine acetamide was conducted as described by Manaf (2012),1 g (0.00309 mol) of compound D (2-(diethylamino)ethyl 4-acetamido-3-nitrobenzoate) (Scheme 1) was slowly dissolved with stirring in 5 mL of concentrated hydrochloric acid (37%). After that, zinc powder (0.1 g) was added. The mixture was poured into an ice-salt bath with stirring after acetylation of procaine HCl and substitution of the nitro group in the ortho position. The reaction mixture was warmed for 30 mins, and 10 mL of sodium hydroxide (6 M) was slowly added, and the product formed was extracted with 10 mL of ether. A small amount of potassium nitrate was added, and the precipitate formed was filtered with filter paper and recrystallized with hot reagent water type IV.

2.4.4. Synthesis of o-hydroxy procaine (HPC)

According to Manaf (2012), the synthesis of o-hydroxy procaine (HPC) was conducted by separating 1.2 g (0.00408 mol) of o-amino procaine acetamide that was added to 1 mL of hydrochloric acid (37%) and 10 mL of reagent water type IV. After cooling the mixture to room temperature (25 °C), a solution of 0.5 g of sodium nitrate in 2 mL water was added to form the azo compound. After that, a cold solution (10 °C) of 5 mL of 10% sodium hydroxide was slowly added to the azo compound formed, and the precipitate formed was filtered. Finally, 1.5 g (0.00509 mol) of compound H (Scheme 1) was hydrolyzed in 4 ml of sulphuric acid (70%), and the reactant mixture was refluxed for 3 hours at 80 °C. The hot solution was then poured into 20 mL ice water, and the product was precipitated by adding 4 to 5 mL of a 10% sodium hydroxide solution. Finally, the precipitate was filtered and recrystallized with hot reagent water type IV.

2.5. GENERAL TECHNIQUE

Five aliquots of TCH standard solution containing 500 $\mu g.mL^{-1}$ of TCH were transferred into a sequence of 20 mL standard flasks. Next, 1 mL of a 5 mM of diazotized PCH, PCS, NPC, PCA, and 2 mL of diazotized HPC 5 mM was added. A sodium hydroxide solution (0.5 M) was added to the aliquots (1, 2, 0.5, 1, 1) mL respectively (Table 4). The contents of the flasks were diluted to a final volume (20 ml) volumetric flask with reagent water type IV, mixed well, and left for a particular time (Table 4). At 25 °C (room temperature), the absorbance of each synthesized reagent (Table 4) was measured against a blank reagent containing all materials except TCH. The regression equation was calculated after a calibration graph was drawn. A 1000 µg solution was used at a final volume of 20 mL to optimize conditions and in all subsequent experiments (50 $\mu g.mL^{-1}$).

2.6. STABILITY CONSTANT

The stability constant (K) was calculated by comparing the absorbance of a solution

containing the stoichiometric amount of TCH ($1.0397.10^{-3}$ M) and diazotized HPR ($1.0397.10^{-3}$ M) in a final volume of 20 mL (As) with that of a solution containing a five-fold excess of diazotized HPC in an alkaline medium and with a final volume of 20 mL (Am).

2.7. THERMODYNAMIC STUDIES

The free energy changes ΔG, enthalpy of formation ΔH, and entropy ΔS have been studied by calculating the stability constant (K) for the dye formed of the stoichiometric amount of drug and diazotized reagent ($1.0397.10^{-3}$ M) in an alkaline medium at different temperatures (5 °C, 25 °C, and 50 °C.

3. RESULTS AND DISCUSSION:

3.1. Results

Four new substituted procaine (PCS, NPC, PCA, and HPC) derivatives were prepared (Scheme 1) to increase the sensitivity of TCH determination and the stability of the compound produced from the reaction of the diazotized substituted procaine with TCH. The four new compounds were identified using their physical properties and FT-IR spectra (Tables 2 and 3).

TCH forms a yellow-colored product (λ_{max} = 380 to 450 nm) with diazotized substituted PC in an alkaline medium. The absorption spectra of the colored product are shown in Figure 3.

The optimum conditions were established by changing one-factor-at-a-time (OFAT) and keeping the others fixed by observing the effect produced on the absorbance of the colored species. Different types of 1M acids (HCl, HNO_3, H_2SO_4, and H_3PO_4) were used for the diazotization of the substituted PCH. It was found that the best experimental conditions for the determination of TCH were established using 1M HCl (from 0.5 to 5 mL, which was used for the diazotization of substituted PCH reagent, 5 mM from 0.3 to 5 mL to a fixed concentration of TCH (50 $\mu g.mL^{-1}$). In contrast, the others were kept constant in a final volume of 20 mL, as described in Table 4.

The yellow-colored product, formed between TCH and the diazotized substituted PCH, was developed only in an alkaline medium. Therefore, the effects of different alkaline solutions like (NH_4OH, NaOH, CH_3COONa, and Na_2CO_3) in the intensity of the color formed in the diazotization reaction were studied. The maximum sensitivity and stability were achieved only after the reaction was carried out in the presence of 0.5M sodium hydroxide (0.3 to 5 mL) by varying OFAT (Table 4).

The colored product was formed immediately and became stable after 10 min and remained stable up to 200 min depending on the type of diazotized substituted PCH used (Table 4). The addition order of the reagent was mentioned under the general technique, which gave a maximum color intensity and a minimum absorbance of the blank and was used in all subsequent experiments. In addition, the effect of the temperature on the color intensity of the dye was examined; a high absorbance was found when the color was developing at room temperature (25 °C) rather than if the flasks that were placed in an ice bath (5 °C) or placed in a water bath at (45 °C).

Under proper conditions, the absorption spectrum of the colored dye versus the corresponding reagent blank and the reagent blank were measured in the wavelength region of 300-700 nm (Figure 3). The yellow-orange azo dyes show maximum absorbance at 424, 390, 450, 382, and 380 nm for TCH with PCH, PCS, NPC, PCA, and HPC reagents. On the other hand, the reagent blank has practically minimum absorption at these wavelengths.

After the optimum conditions were fixed (Table 4), calibration graphs of TCH were constructed using the new diazotized substituted PCH, and their analytical, statistical data was incorporated into Table 5.

The precision of the method was evaluated by analyzing a pure sample of TCH with diazotized HPC, and good recovery was obtained (Table 6); the limit of detection (LOD) and the limit of quantitation (LOQ) (Hadjiionannou *et al*., 1993) were calculated using the relationships 3SDB/b (0.5049) and 10SDB/b (1.6831), respectively, where SDB ($5.0662.10^{-3}$), equal to the standard Table 6, the accuracy and precision of the suggested method.

The reaction stoichiometry between TCH and diazotized o-hydroxy procaine reagent was investigated using molar ratio and continuous variation methods (Oxtoby *et al*., 1999). The results were shown in Figures 4 and 5, illustrating that a (1:1) azo dye was formed between the drug and the reagent (HPC). The proposed product of the reaction between TCH and diazotized HPC is given in Scheme 2.

The dye formed was soluble in water. Therefore, the seeming stability constant (K) was

calculated according to (Equations 1 and 2) (De Levie, 1997), and it was equal to $19.0205.10^6$ $L.mol^{-1}$.

$$\alpha=(Am-As)/Am \quad \text{(Eq.1)}$$

and,

$$K=(1-\alpha)/\alpha^2C \quad \text{(Eq.2)}$$

Thermodynamic studies, according to Van't Hoff equation (Atkins and De Paula, 2006) (Equation 3), is a useful expression that shows the relationship between the temperature and the stability constant (K). For example, a plot between lnK versus 1/T gave a straight line with a slope equal to $-\Delta H/R$ (where ΔH = enthalpy changes and R is the gas constant of 8.314 $J.mol^{-1}.k^{-1}$) as shown in Figure 6, the intercept was equal to the standard entropy changes divided by the gas constant $\Delta S/R$:

$$\ln K=(-\Delta H)/RT+\Delta S/R \quad \text{(Eq. 3)}$$

Gibbs free energy (ΔG) of the reaction also calculated from Gibbs-Helmholtz (Equation 4) (Mouayed and Hind, 2014):

$$\Delta G=\Delta H-T\Delta S \quad \text{(Eq.4)}$$

All thermodynamic parameters are summarized in Table 7.

The effect of five common excipients (Starch, Lactose, Talc, PVP, and Mg stearate) was studied by analyzing a synthetic sample solution containing 25 $\mu g.ml^{-1}$ of TCH and excess amounts of 10-fold of each excipient.

3.2. Discussion

FT-IR spectral data of PCS (Table 2) shows the formation of a new band in the fingerprint zone at 684, which refers to forming a new bond (C-S) and bands at 1166 and 1035 assigned to SO_2 and SO groups, respectively. In compound NPC, the FT-IR spectral data shows asymmetric and symmetric stretching bands of NO_2 at 1566 and 1348. Also, the FT-IR spectrum showed a stretching band at 3161 for the NH group. For compound PCA, asymmetric and symmetric bands for the NH_2 group are at 3325 and 3296, respectively. The FT-IR spectrum for compound HCP broadband was assigned to 3417 due to the substitution of the OH group for a benzene ring.

The parameters influencing the sensitivity and stability of the colored dye were investigated and optimized, according to Table 4.

The results showed that diazotized HPC had the highest molar absorptivity, the best correlation coefficient, and the lowest Sandell sensitivity and could be used for simple and accurate spectrophotometric determination of TCH drugs in pure form and pharmaceutical formulations.

The proposed method was applied effectively to investigate some drug formulas containing TCH (capsule) and gave decent exactness and accuracy, as demonstrated in Table 7. And after studying the stability constant for the complex formed, the results showed a high value of the stability constant, indicating that the reaction product is stable.

The effects of some substances revealed that none of these substances were adversely affected by the proposed method for determining TCH (Table 8).

The results obtained by the proposed and standard method (Medicines and Healthcare products Regulatory Agency, 2020) for dosage forms were compared statistically employing the F-test and t-test (Peter and Richard, 2000). They were found to differ fundamentally in accuracy and precision between the proposed and official methods (Table 9).

The negative values of ΔG indicate that the diazonium reaction is spontaneous (Nicholas *et al*., 2018), while the positive value of ΔS means increasing the disorder of the reaction because of the thermal decomposition of the complex as the temperature rises (Dilek, 2020). The negative value of ΔH, on the other hand, indicates that the release of heat accompanies the reaction and that the process is exothermic, proving that the diazonium salt reaction is preferred to occur at a low temperature (Alexander *et al*., 2019).

4. CONCLUSIONS:

The present work was directed towards synthesizing new coupling reagents (as shown in Scheme 1) substitution with different groups using procaine hydrochloride as the starting material. Four new substituted procaine HCl were prepared with simple organic methods. After optimizing them, it was observed that o-hydroxy

procaine was the most sensitive reagent for determining TCH (Molar absorptivity $14.4750 \cdot 10^3$ $L \cdot mol^{-1}.cm^{-1}$). Moreover, it gives a stable soluble dye product ($K_{stability} = 19.0205 \cdot 10^6\ L \cdot mol^{-1}$). This is probably due to the effect of the donating electron of the hydroxyl group substituted on the benzene ring in the structure of the coupling reagent. A simple and accurate method for determining TCH using o-hydroxy procaine as a chromogenic reagent via diazotization and coupling reaction in the alkaline medium was proposed and applied successfully for the analysis of pharmaceutical formulations containing TCH. Also, studying the thermodynamic parameters showed that the diazonium reaction was spontaneous, and the process was exothermic. A review of some analytical characteristics of the suggested and selected reported methods was illustrated in Table 10.

Finally, the selected reagent for the proposed method offered a sensitive, simple, fast, and economical method that matched the other analytical methods. The literature involved many spectrophotometric methods to determine TCH, but some of these methods were either not sensitive or needed expensive reagents. The suggested method has proved to be rapid and sensitive for TCH determination, uses a drug compound as a nontoxic reagent, and uses safe materials to support green chemistry.

5. DECLARATIONS

5.1. Study Limitations

No limitations were known at the time of the study.

5.2. Acknowledgements

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5.3. Funding source

This research was funded by the authors.

5.4. Competing Interests

There is no potential conflict of interest in this publication.

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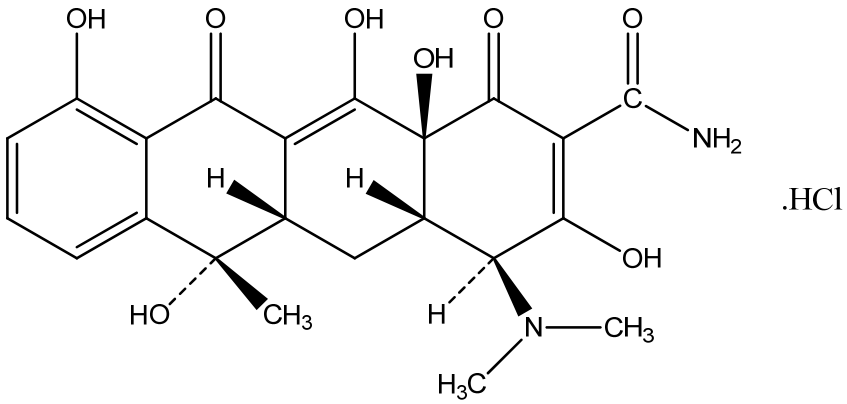


Figure 1. *Chemical structure of Tetracycline Hydrochloride*

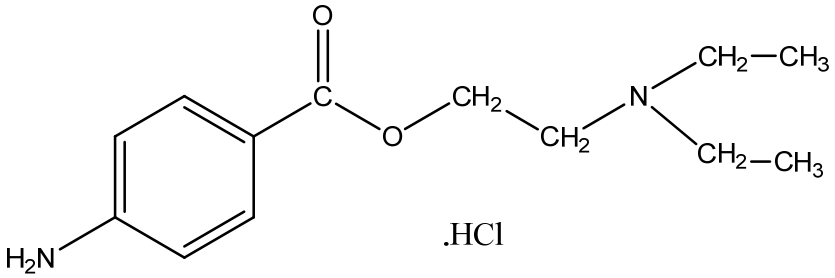
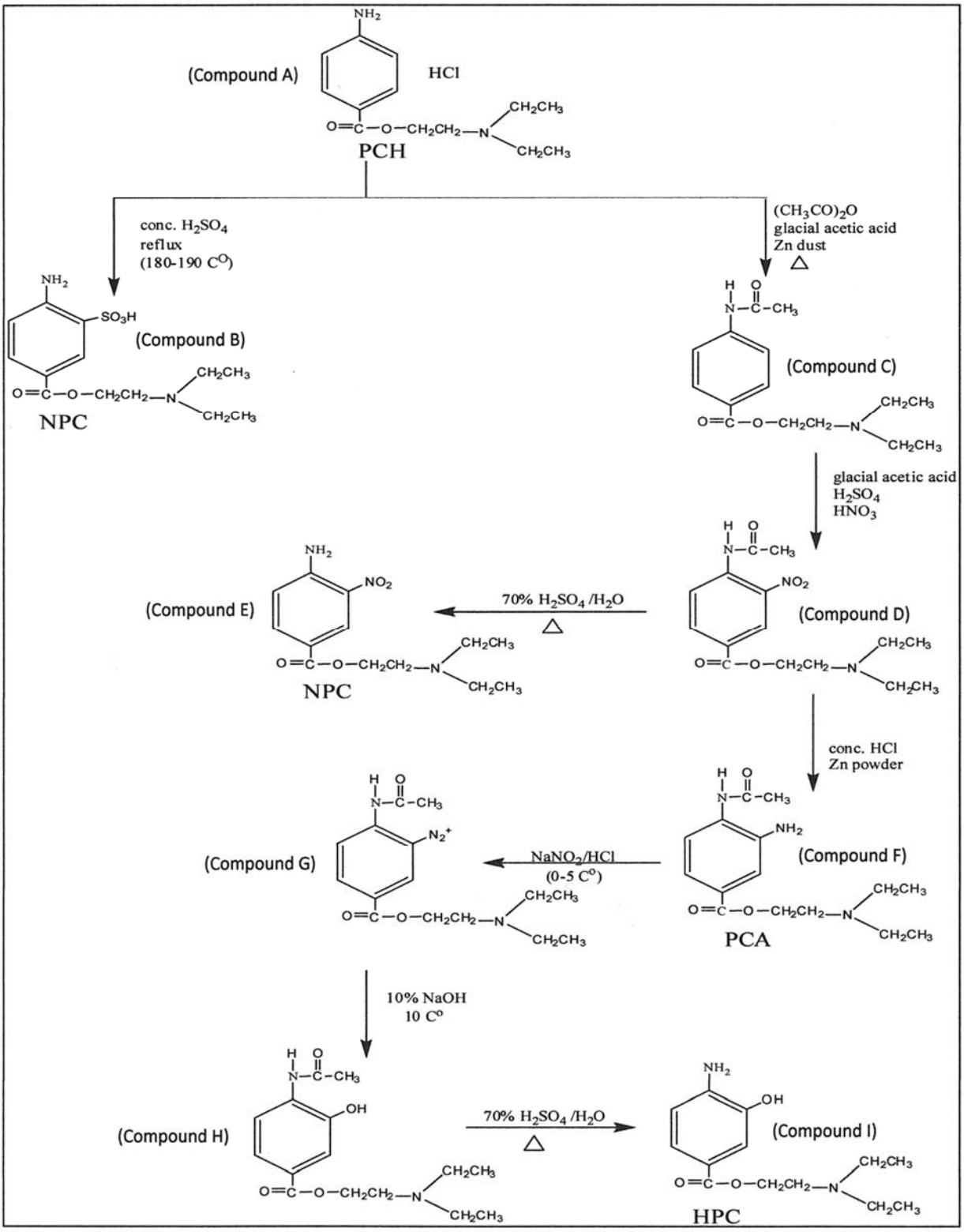
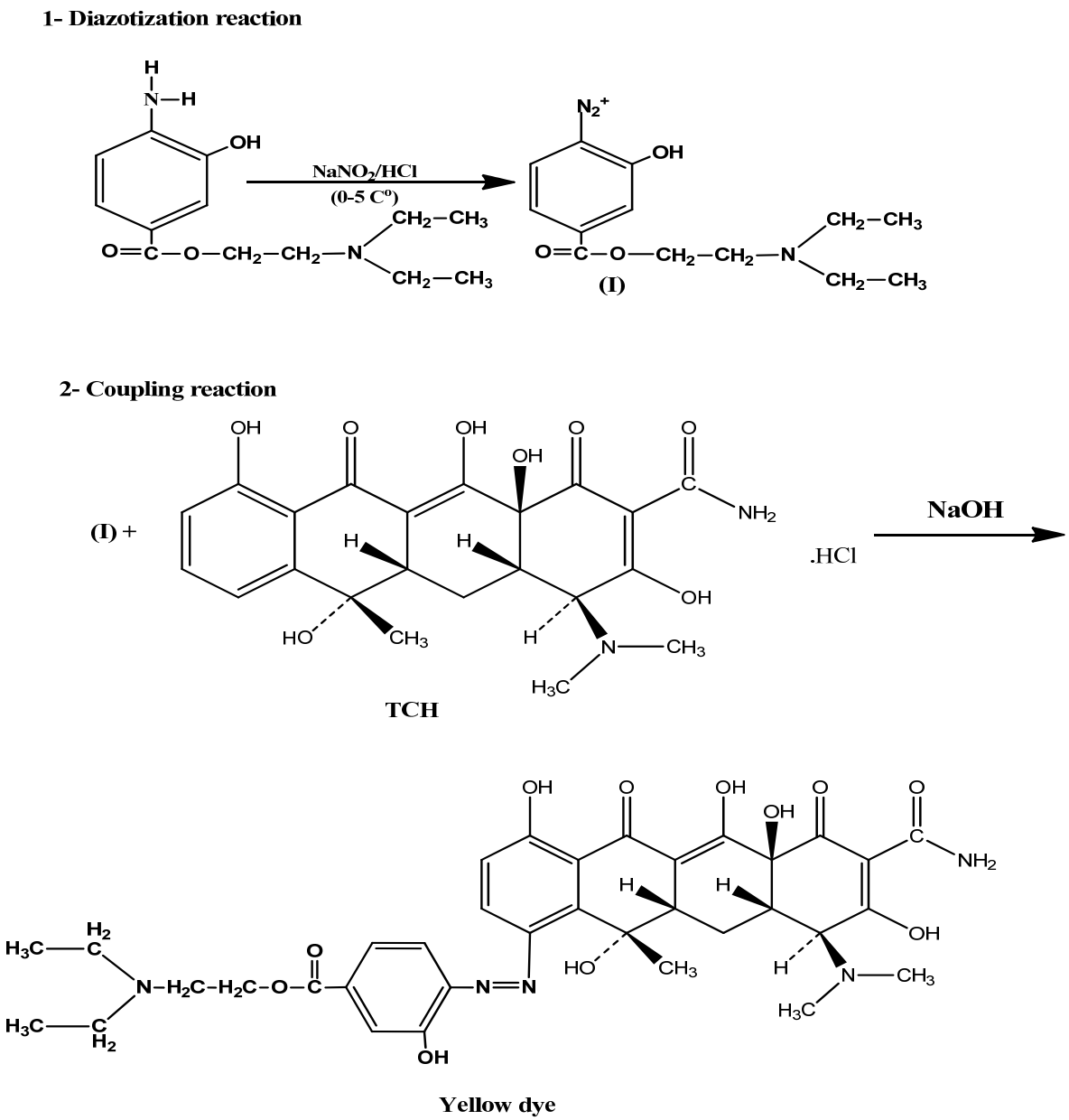


Figure 2. *Chemical structure of Procaine Hydrochloride*



Scheme 1. *Path synthesis of the derivative procaine reagents.*



Scheme 2. *The proposed reaction between TCH and diazotized o-hydroxy procaine.*

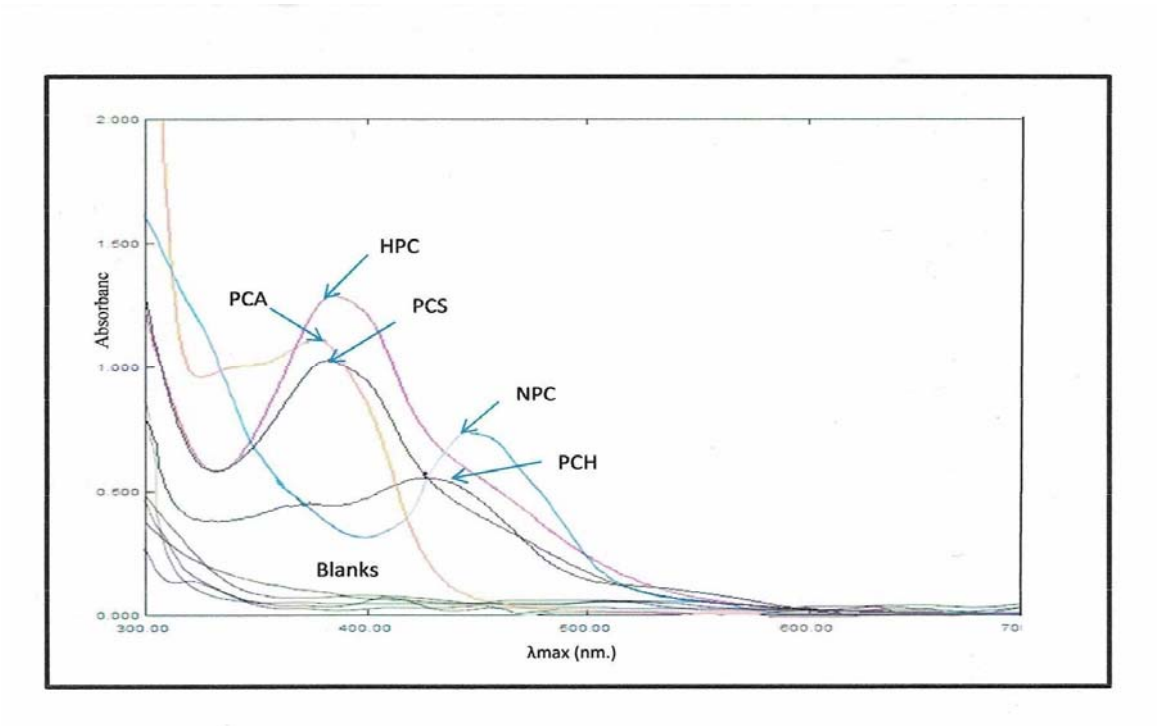


Figure 3. *The absorption spectrum of the colored products was obtained when TCH drug and diazotized PCH, PCS, NPC, PCA, and HPC reagents were reacted in the presence of sodium hydroxide and measured against reagent blank, and other spectrums were blank against reagent water type IV.*

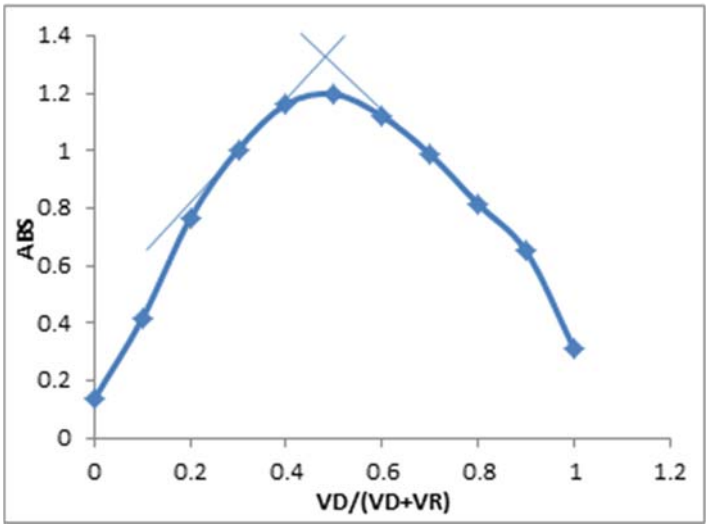


Figure 4. *Continuous variation plot of the reaction between TCH drug (1.0397.10^{-3} M) and diazotized HPC prepared reagent.*

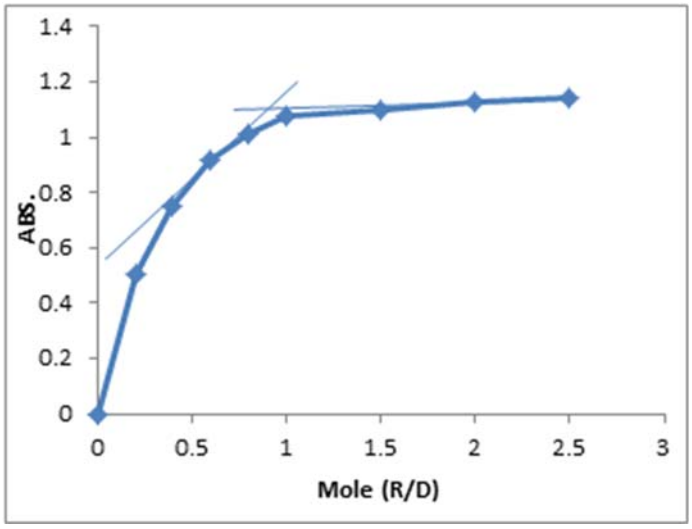


Figure 5. *Mole ratio plot of the reaction between TCH drug ($1.0397.10^{-3}$ M) and diazotized HPC prepared reagent.*

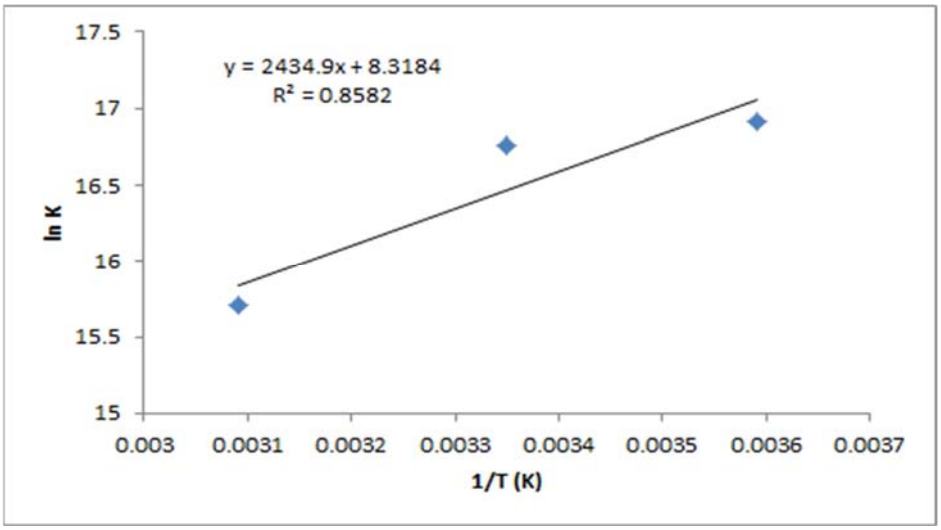


Figure 6. *Van't Hoff plot (the relationship between the temperature and the stability constant (K)).*

Table 1*: preparation of 100 mL (5 mM) of diazotized substituted procaine reagents using 0.0345 g of sodium nitrate.*

Name of compound	M.wt	Weight (g)	Volume (mL) of (1M) HCl added
Procaine sulfonic acid (PCS)	316.37	0.1581	1
Nitro procaine (NPC)	281.31	0.1406	1
Procaine acetamide (PCA)	293.36	0.1466	2
Hydroxy procaine (HPC)	252.31	0.1261	3

Table 2: *FT-IR spectral data (cm^{-1}) of compound (1-5).*

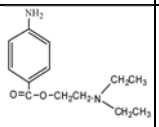
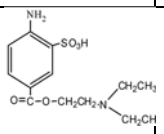
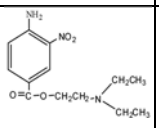
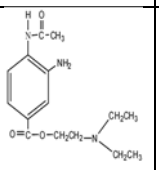
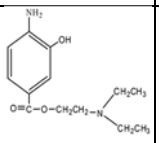
NO.	Compound structure	υ (C=O) ester	υ (C=O) amide	υ (C=C)	υ (C-N)	υ (NH_2)	Others
1	 PCH	1741	-	1604	1400	Asym-3352 Sym-3315	-
2	 PCS	1749	-	1600	1419	Asym-3467 Sym-3446	υ (C-S) 684 υ (SO_2) 1166 υ (S=O) 1035
3	 NPC	1776	-	1602	1402	Asym-3313 Sym-3282	υ (NO_2) Asym 1566 Sym 1348
4	 PCA	1735	1654	1610	1396	Asym-3325 Sym-3296	υ (N=H) 3161
5	 HPC	1743	-	1612	1391	Asym-3325 Sym-3257	υ (O-H) 3417

***Table 3**: physical properties of procaine HCl and its derivative.*

Reagent	Formula	M.wt g/mol	M.P °C	Color	Yield %
PCH	$C_{13}H_{20}N_2O_2$.HCl	272.8	154-158	White	-
PCS	$C_{13}H_{20}N_2O_5S$	316.3	>330 decomposed	Silver shiny crystals	73%
NPC	$C_{13}H_{19}N_3O_4$	281.3	196-198	Yellow-orange	81.4%
PCA	$C_{15}H_{23}N_3O_3$	293.3	211-*213*	gray	80%
HPC	$C_{13}H_{20}N_2O_3$	252.3	179-181	Brown	73.1%

***Table 4**. The optimum conditions for the determination of TCH.*

Parameters	Range selected	Optimum conditions
λmax (nm.) PCH, PCS, NPC, PCA and HPC reagents	300-700 nm	424, 390, 450, 382, 380 nm respectively
Effect of the type of acids	HNO_3, H_2SO_4, H_3PO_4, HCl	HCl
Effect of the type of base	NH_4OH, NaOH, CH_3COONa, Na_2CO3	NaOH
Effect of the volume of HCl (1M) solution is required with PCH, PCS, NPC, PCA, and HPC reagents	0.5-5.0 mL	(2 , 1, 2, 3 ,1) mL respectively
Effect of volume of sodium hydroxide (0.5M) solution required with PCH, PCS, NPC, PCA, and HPC reagents	0.3-5.0 mL	(1,2,0.5, 1, 1) mL respectively
Effect of volume of (5mM) diazotized PCH, PCS, NPC, PCA and HPC reagents in basic medium	0.3-5.0 mL	1 mL for PCH, PCS, NPC, PCA reagent and 2 mL for HPC reagent
Effect of addition order for TCH with diazotized PCH, PCS, NPC, PCA, and HPC reagents in basic medium	1-TCH+Diazotized reagent+NaOH 2-TCH+ NaOH +Diazotized reagent	No.1 order
Effect of temperature	5°C, 25°C and 50°C	25 °C
Stability period after final dilution for TCH with diazotized PCH, PCS, NPC, PCA and HPC reagents in basic medium	5-240 min	PCH= 15-60 min PCS= 20-55 min NPC= 30-45 min PCA=15-90 min HPC= 10-200 min

Table 5. *Analytical values and statistical analysis for calibration graphs of the drug with diazotized reagents in the presence of a basic medium.*

Parameters	**Value PCH**	**Value PCS**	**Value NPC**	**Value PCA**	**Value HPC**
Regression equation y = b x + a ; y= absorbance , x= concentration (μg.mL^{-1})	y=0.0287x+0.0863	y=0.0217x+0.0892	y=0.0177x+0.0455	y=0.0291x+0.0436	y= 0.0301x+0.0191
Correlation coefficient, r	0.9968	0.9963	0.9967	0.9967	0.9991
Linearity percentage, r^2% (r^2% = r^2 × 100)	0. 9938	0.9927	0.9935	0.9936	0.9983
Dynamic range (μg mL^{-1})	5-60	5-60	5-50	2.5-50	2.5-50
Molar absorptivity, ε (L. mol^{-1}.cm^{-1}) ε = b×M×1000 ; M = Molecular weight (g.mol^{-1})	13.7846.10^3	11.2049.10^3	8.5119.10^3	13.9941.10^3	14.4750.10^3
Slope, b (mL μg^{-1}) $b = \Sigma_i [(x_i - \bar{x})(y_i - \bar{y})] / \Sigma_i (x_i - \bar{x})^2$	0.0287	0.0217	0.0177	0.0291	0.0301
Intercept, a (a = y– b x)	-0.0863	-0.0892	+0.0455	+0.0353	-0.0191
Sandell's sensitivity, S = M / ε S (μg cm^{-2})	0.0197	0.0282	0.0330	0.0209	0.0174

Table 6. *Accuracy and precision of the suggested method.*

Concentration (μg.mL^{-1})				
Present	**Found***	**E.%**	**Rec.%**	**R.S.D.%**
5	5.1196	+2.3920	102.3920	0.3605
20	19.7818	-1.0908	98.9091	0.3535
40	40.000	0.0000	100.0000	0.4942

*Average of five determinations

Table 7. *Thermodynamic parameters for the determination of TCH at different temperature*

Temperature, K	**K,L.mol^{-1}**	**ΔH, KJ.mol^{-1}**	**ΔS, J.mol^{-1}.K^{-1}**	**ΔG, KJ.mol^{-1}**
278	22.1101.10^6	-20.2437	+69.1591	-39.4699
298	19.0205.10^6			-40.8531
323	6.6162.10^6			-42.5820

Table 8. *Effect of excipients (250 µg.mL^{-1}) on the recovery of TCH (25 µg.mL^{-1}).*

Excipient	Conc. of TCH (µg.mL^{-1})*	E. %	Rec. %
Starch	24.3670	-2.5316	97.4683
Talc	24.6592.	-1.3631	98.6368
Lactose	25.0486	+0.1947	100.1947
PVP	24.7802	-0.8763	99.1208
Mg stearate	25.2677	+1.0710	101.0710

* Average of five determination.

Table 7. *Application of the proposed method for the determination of TCH in capsule samples.*

Pharmaceutical preparations	Conc. of TCH (µg.mL^{-1})*		E%	Rec%	RSD%
	Present	Found			
Samacycline capsules - Iraq	5	5.0889	+1.7699	101.7699	0.4000
	20	20.1292	+0.6463	100.6460	0.7211
	40	39.6235	-0.9411	99.0588	2.1400
Apcycline capsules - India	5	4.9225	-1.5486	98.4513	0.5916
	20	19.8265	-0.8670	99.1329	0.7449
	40	39.6235	-0.9411	99.0588	1.6822

* for five determinations.

Table 8. *Application of the proposed method to determine TCH (25 µg.mL^{-1}) in ointment sample by calibration method and standard addition method.*

Ointment samples	Calibration method		Standard addition method	
	%E	%Rec.	%E	%Rec
samacycline skin ointment-Iraq	-9.5375	90.4624	+1.9607	101
tetracycline HCl eye ointment- Hong Kong	+9.1412	109.1412	-0.4022	99.5977

Table 9. *The comparison of the proposed method with standard methods using t-and F-statistical tests.*

Drug forms	Proposed method		Standard method		Statistical values
	Rec.% $(xi)_1$	$(xi-x^-)_1^2$	Rec.% $(xi)_2$	$(xi-x^-)_2^2$	
TCH pure	100.4915	0.1835	100.2100	0.51222	S_1^2=1.0745 S_2^2=2.9681 S=1.4217 F*=19.000 t*=2.776
Samacycline capsules – Iraq	98.8810	1.3973	100.7440	1.5617	
Apcycline capsules – India	100.8170	0.5683	97.5290	3.8624	
	$(x^-)_1$= 100.0631	$\sum (xi-x^-)_1^2$= 2.1491	$(x^-)_2$ = 99.4943	$\sum (xi-x^-)_2^2$= 5.9363	

* Theoretical value at 95% confidence limit, $n_1=n_2=3$.
t=0.4900, where t has degree of freedom= $n_1+n_2-2=4$
F= 2.762, where F has degrees of freedom= $n_1-1= n_2-1 =2$.

Table (10). *Comparison of the proposed method with some spectrophotometric methods for the determination of TCH.*

Methods	Reagent used	λmax (nm)	Linear range $\mu g.mL^{-1}$	LOD $\mu g.mL^{-1}$	Remarks	Ref.
Oxidative coupling	2,4-dinitro phenylhydrazine	360	0.1-9	0.0123	Narrow linear range	(Roaa and Dawooh, 2020)
Formation of chelate complex with Rh(II)	Rhodium(II)	430	2-50	0.140	Uses of 2-heptanon a solvent for extraction of the complex	(Fatima and Rana 2016)
Oxidation with NBS	NBS and Celestine blue dye	540	0.2-5	0.37	Narrow linear range	(Chilukuri *et al*., 1996)
Charge transfer complex	Chloranilic acid	540	2.5-30	0.4	Use of acetonitrile for dilution	(Khairi, 2008)
Diazonium reaction	o-hydroxy procaine	380	2.5-50	0.525	Good linearity, sensitivity, stable	**Present work**