ISSN: 0067-2904
GIF: 0.851

Batch and Flow Injection Spectrophotometric Determination of Tetracycline Hydrochloride and Doxycycline Hyclate in Pharmaceutical Preparations

Mouayed Q. Al-Abachi*,Zaid A. Al-Nedawi
Department of Chemistry, College of science, University of Baghdad, Baghdad,Iraq.

Abstract:

New, simple and accurate batch and flow injection spectrophotometric methods have been developed for the determinationsof tetracycline hydrochloride (TCH) and doxycycline hyclate (DCH) in pharmaceutical preparations. The methods are based on diazotization of metchlopramide and coupling reaction with either TCH or DCH in alkaline medium to form yellow–orange water soluble dye with absorption maxima at 414 and 436 nm for TCH and DCH, respectively. A graphs of absorbance versus concentration show that Beer's law was obeyed over the concentration ranges of 1 –52 μgmL^{-1} TCH and DCH for batch method and of 8 – 240 $\mu g\ mL^{-1}$ TCH and 5 – 350 μgmL^{-1} DCH for FIA method. The limits of detection in batchmethods were 0.333 and 0.235$\mu gmL^{-1}$for TCH and DCH respectively, and in FIA methods were 0.895, 0.612 $\mu gmL^{-1}$for TCH and DCH respectively. Samplethroughputs in FIA procedures were 120 and 80samples per hour for TCH and DCH, respectively. Different chemical and physical experimental parameters affecting on the development and stability of the colored product were carefully studied and the proposed methods were successfully applied for determination of TCH and DCH in pharmaceutical preparations.

Keywords: Tetracycline hydrochloride,Doxycyclinehyclate, Metclopramide, Flow injectionanalysis

التقدير الطيفي للتتراسايكلين هيدروكلورايد والدوكسي سايكلين هايكلات في المستحضرات الصيدلانية بطريقة الدفعة والحقن الجرياني

مؤيد قاسم العبايجي، زيد عبد الستار النداوي

قسم الكيمياء، كلية العلوم، جامعة بغداد، بغداد، العراق

*Email:zaid.abdulsatar@yahoo.com

الخلاصة

يتضمنالبحثتطوير طرائق بسيطة وجديدة وذلك باستخدام تقنيةالمطيافالضوئيبطريقةالدفعةوالحقنالجريانيللتقديرالكميلكلمنالتتراسايكلين هيدروكلورايد و الدوكسي سايكلين هايكلاتفي المستحضرات الصيدلانية. اعتمدتالطرائقعلى تفاعل الازودتة والازدواج بين الميتكلوربرمايد المؤزوت والتتراسايكلين والدوكسي سايكلين حيثتتكوننواتج من اصباغ الاوزو الملونةوذائبةبالماءاعطتاعلى قمةامتصاصعندطولموجي414و436نانوميترلكلمنالتتراسايكلين والدوكسي سايكلين علىالتوالي.تشيرمنحنياتالامتصاصمقابلالتركيزبانقانونبيرينطبقضمنمدىالتركيز 1-52و 1-50 مايكروغرام لكل مل لكل من التتراسايكلين هيدروكلورايد والدكوكسي سايكلين على التوالي وبحدودكشف 0.333 و 0.235 مايكروغرام لكل مل للتتراسايكلين هيدروكلورايد والدوكسي سايكلين هيكلات على التوالي لطريقةالدفعة.امابطريقةالحقن –الجريانيفكانمدى التراكيز من 5-280 و5-350 مايكرو غرام لكل مل للتتراسايكلين هيدروكلورايد والدوكسي سايكلين هيكلات على التوالي، وحدود الكشفكانت0.895 و0.612 مايكروغرام لكل مل للتتراسايكلين والدوكسي سايكلين على التوالي، تمت دراسة الظروفالمثلى للمتغيراتالكيميائيةوالفيزيائيةبدقةوطبقتالطريقتينبنجاحعلى المستحضرات الصيدلانية الحاويةعلى التتراسايكلينهيدروكلورايد والدوكسي سايكلين هيكلات،وقورنتالنتائجالتيتمالحصولعليها معنتائجطرائقالتحليل القياسيةللادويةاعلاهواظهرتنتائجالمقارنةعدموجودفرق جوهري بين نتائج الطرائق المقترحة ونتائج الطريقة القياسية .

Introduction:

Tetracyclines (TCs) represent a class of antibacterial compounds which got their name because they share chemical structures that has four rings (Figure-1).Tetracycline hydrochloride (TCH) and doxycycline hyclate (DCH) with all tetracycline drugs have the same broad spectrum antibiotics for their activity against nearly all gram-positive and gram–negative bacteria but differ between them in bioavailability and other pharmacological properties. Due to their broad antibacterial spectrum and economic advantages, TCs have been commonly used in human pathologies as well as in veterinary medicine, animal nutrition and feed additives for cattle growth. TCs are used for many different infections, such as respiratory tract infections and have a role in the treatment of multidrug resistant malaria. [1-3]

Several methods have been reported in the literature for the analysis of TCs including spectrophotometery[4-9];chromatography [10],and highperformanceliquid chromatography [11-13], flourmetry [14-16],chemliumansce[17-19], potentiometry [20-23] and flow injection methods [24-26] have also been reported for the determination of TCs.

In the present paper an automated procedure is proposed for the spectrophotometric determination of TCH and DCH by coupling reaction with diazotized metoclopramide (DMCP) in alkaline medium. The reaction can be carried out in batch and in FIA and in this paper the two approaches are compared. The reaction products have been spectrophotometerically measured at 414, 436 nm for TCH and DCH respectively.

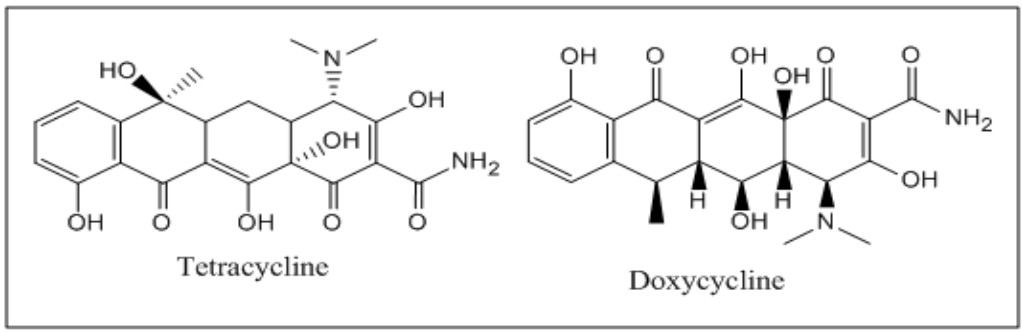


Figure 1-Chemical Structure of Tetracycline and Doxycycline

Experimental part: Materials and methods

Apparatus:

All spectral and absorbance measurements were carried outby using aShimadzu UV – visible – 260 digital double beam recording spectrophotometer (Tokyo – Japan), and using 1 cm quarts cells.A quartz flow cell with 50µL internal volume and 1 cm bath length used for the absorbance measurements. A two channel manifold (Figure-2) was employed for the FIA spectrophotometer determinations of TCH and DCH.A peristaltic pump (IsmatecLobortechnik–Analytic, CH – 8512 , Glatbragg–Zurich, Switzerland, Sixchannels) was used to transport the reagents solutions. Injection valve (Rheodyne, Altex 210, supeko use) was employed to provide appropriate injection volumes of standard solutions and samples, flexible vinyl tubing of 0.5 mm internal diameter was used for the peristalticpump. Reaction coil (RC) was of Teflon with internal diameter of 0.5 mm. The diazotized metchlopramide (DMCP) (A) stream was combined (Figure-2) with injected sample (TCH or DCH) and they merged with sodium hydroxide (B) stream at T – link then mixed in reaction coil (RC) with length (75 cm) for TCH and (100 cm) for DCH, injection loop (200µL), total flow rate 1.5mLmin^{-1}, the absorbance was measured at 414nm for TCH and 436nm for DCH at temperature 25 C^{o}.

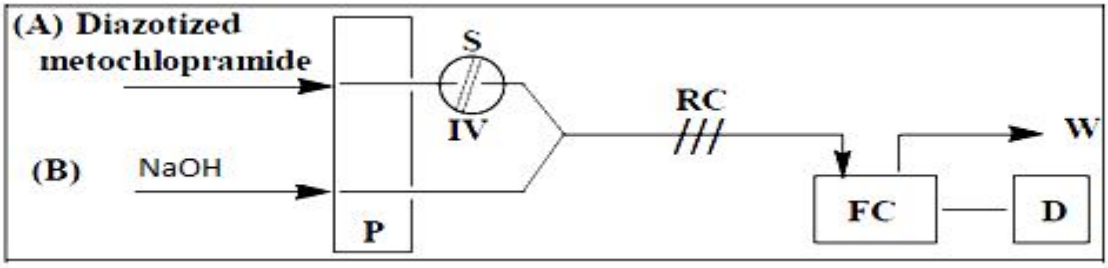


Figure 2 -A schematic diagram of FIA manifold where : (A) & (B) , solutions of diazotized metchlopramide and sodium hydroxide respectively ; P = peristaltic pump ;S= injection sample for TCH or DCH ;IV = injection valve ;RC=reaction coil ;FC = flow cell;D=detector ; W= waste

Reagents and materials

Analyticalreagents grade chemicals and distilled water was used throughout.

A. Tetracycline and Doxycycline stock solutions(500µg.mL^{-1}):

0.05 g amount of pure TCH (SDI-Iraq)orDCH (SDI-Iraq) was dissolved in distilled water then completed to100 mL in a volumetricflask with the same solvent; more dilute solutions were prepared by suitable dilution of the stock standard solutions with distilled water.

B. Hydrochloric acid (BDH-England) (1M):

was prepared by diluting 21.5 mL of concentrated hydrochloric acid (11.64 M) with distilled water in 250 mL volumetric flask.

C. Sodium hydroxide (BDH-England) (0.5M):

A 5.00 g amount of NaOH (BDH) was dissolved in a 250mL volumetric flask with distilled water; 0.1M of sodium hydroxide was prepared by dilution with distilled water.

D. Diazotized metoclopramide(DMCP) (5×10^{-3}M) reagent solution:

0.1772g of pure metoclopramide (SDI-Iraq) was dissolved in distilled water in 100 mL volumetric flask, 3 mL of hydrochloric acid (1M) was added and was placed in an ice bath for 5 min then 0.0345 g of sodium nitrite was added. After 5 min, complete the volume to mark by distilled water and used as stock solution for batch procedure.

For FIA procedure stock solution of DMCP (1×10^{-2}M) wasprepared by dissolving 0.6857 g of metoclopramide (SDI) with amount of distilled water in 250 mL volumetric flask and add 7.5 mL of hydrochloric acid (1M) and put it in ice bath for 5 min then add 0.725 g of sodium nitrite.After 5 min,the volume was completed to the mark with distilled water, more dilute solutions were prepared by suitable dilution of the stock solution with distilled water.

Pharmaceutical preparations:

All pharmaceutical preparations were obtained from commercial sources as follow:

1- Samacycline10-capsules (Samara–Iraq), each capsule contain 250 mg of tetracycline hydrochloric.
2- Apcycline 10-capsules (Ajenta-India),each capsule contain 250 mg of tetracycline hydrochloride.
3- Tetracycline.HCl 10-capsules (MEHECO-China),each capsule contain 250 mg of tetracycline hydrochloride.
4- Doxycycline hyclate8-capsules (Actvis-Barnstaple,UK), each capsulecontain 100 mg of doxycycline.
5- Tabocline10-capsules (Tabuk–K.S.A),each capsule contain 100 mg of doxycycline hyclate.
6- Medomycin10-capsules (MedochemieLtd.-Cyprus), each capsule contain 100 mg of doxycycline.HCl.

General Batch procedure:

Into a series of 25 mL volumetric flasks , an increasing volume of tetracycline and doxycycline working solutions (100 μgmL^{-1}) were transferred to cover the range of the calibration graphs (Table 1), and then add 1mL of DMCP (5mM) and 1mL of NaOH (0.1 M). The solutions were diluted to the mark with distilled water, mixed well and left for 10 min at room temperature (25 C°). The absorbance was measured at 414nm and 436 nm for TCH and DCH respectively versus the reagent blanks prepared in the same waycontaining no tetracyclinedrugs.

A calibration graphs were drawn and regression equations were calculated.For the optimization of conditions and in all subsequent experiments were carried out on 10μgmL^{-1} of TCH and DCH.

General FIA procedure:

Working solution of TCH and DCH in the range (5-240 μgmL^{-1}) for TCH and (5-350 μgmL^{-1})for DCH cited in (Table 1) were prepared from stock solution. A 200µL portion of the drugs solutions were injected into the stream of the 5mM of DMCP and was then combined with a stream of 0.1 M sodium hydroxide with a total flow rate of1.5mL min^{-1}. The resulting absorbance of the colored dye was measured at maximum wave length for each drug. A calibration graphs was prepared over the range cited in (Table 1), optimization of conditions was carried out on 40 μgmL^{-1} for both drugs.

Analysis of pharmaceutical preparations:

An accurate weight (from 10 powdered capsules of 250mg of TCH and 100 mg DCH for each drug), equivalent to 50 mg DCH or TCH of the pure drugwas dissolved in distilled water and was transferred into a 100 mLvolumetric flask (to prepare 500 µgmL^{-1} of drug) and was completed to the mark with distilled water. The flask with its contents was shacked well and filtered.More dilute solutions of pharmaceutical for batch and FIA procedures were made up by simple dilution with distilled water and the measurement was carried out as described earlier under general procedures.

Results and Discussion:

The factors affective on the sensitivity and stability of the colored products resulting from the diazotization-coupling reaction of diazotized metoclopramide with either TCH or DCH in an alkaline medium were carefully studied. A typical spectrum for the 40 µgmL^{-1}of the dye formed was measured versus reagent blank which has negligible absorbance at 414 nm for TCH and 436 nm for DCH (Figure-3).

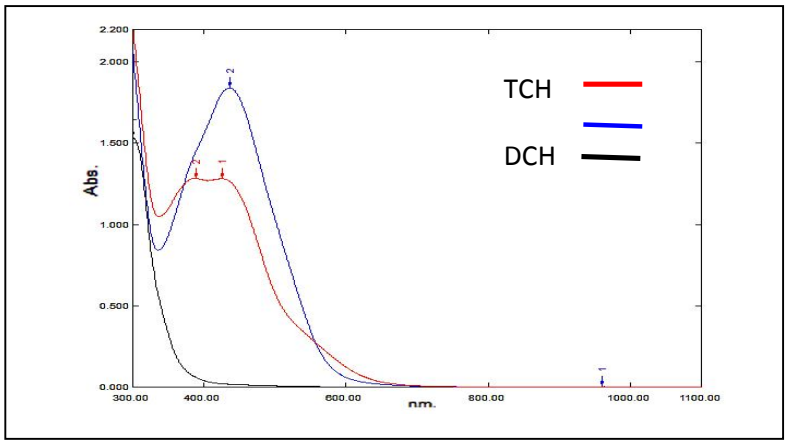


Figure 3- Absorption spectra of the azo dye (40 µg mL^{-1}) of TCH & DCH and blank against distilled water

The colored dye product was only formed in alkaline medium, therefore, the effect of different alkaline solutions were studied such assodium acetate, sodium carbonate, ammonium hydroxide and sodium hydroxide anda maximum sensitivity and stability were obtained only when the reaction was carried out in the presence of sodium hydroxide solutions .

Batch spectrophotometerydeterminations:

The best experimental conditions for the determination of TCH and DCH were establish for DMCP(5×10^{-3})M (from 0.3 to 6 mL), sodium hydroxide 0.1 M (from 0.3 to 5 mL) by adding various volumes of their solutions to affixed concentrations of TCH and DCH (250 µg in a find volume of25 mL) and measuring the absorbance at maximum wave length, also the effect of hydrochloric acid 1M solution was used for preparing DMCP and was studied in the range(from 0.5 to 5 mL).

The obtained results show that 3mL of hydrochloric acid (1 M), 1 mL of DMCP (5×10^{-3}M) and 1 mL of sodium hydroxide are the volumes that can give a higher absorbance intensity and stability of the dye product at 414 nm for 10 µg.mL^{-1} TCH and 436 nm for 10 µg.mL^{-1} DCH. Experimental results recorded that the color intensity reach a maximum after drug solution had been reacted with DMCP in alkaline medium for 5 min therefore a 10 min development time was suggested as the optimum reaction time and remained stable for 60min. The order of addition of the reagents is an essential part of the experiment it was found that the order of addition of the reagent cited under general procedure

gave maximum color intensity and a minimum absorbance of the blank and was used in all subsequentexperiments.

The effect of temperature on the color intensity of the dye was studied, in practice, high absorbance was obtained when the color was developed at room temperature (25C°) then when the calibrated flasks were placed in an ice-bath at (0 C°) or in a water bath at (45 C°) .

The stoichiometry of the reaction was studied using equimolor concentrations of the drugs and DMCP (5.125×10^{-4} M) at constant sodium hydroxide concentrations,adopting a continuous variation (Job's method) and mole ratio methods [27] , a molar ratio of 1:1 drugs to DMCP obtained by applied methods as shown in Figure-4 the proposed reaction mechanism proceed according to (Figure-5). The stability constants of the dye products were calculated [28] by comparing the absorbance of the solution containing stoichiometric amount of TCH or DCH and DMCP with that of solution containing five–fold excess of DMCP reagent. The stability constants of the dye products in water under the described experimental conditions were 3.014×10^{5} and 3.627×10^{4}L.Mol^{-1} for each of TCH and DCH respectively.

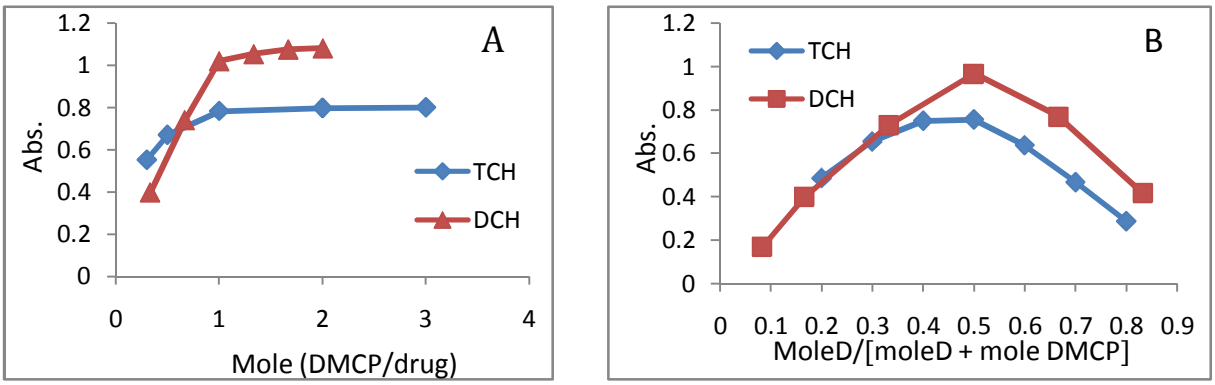


Figure 4- Stoichiometric plots for colored dye products: A: mole ratio plot; B:continues variation plots

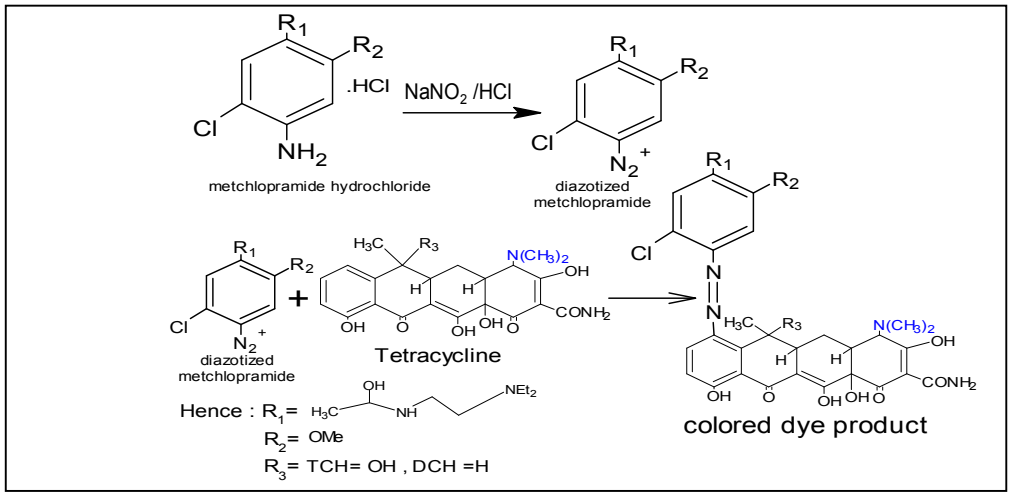


Figure 5-reaction mechanism for producing colored azo-dye

In order to assess the possible analytical applications of the proposed methods the effect ofsome common excipients frequently found with TCH and DCH drugs in pharmaceuticalformulations, such as poly vinyl pyrrolidone(PVP), lactose, talc, starch and magnesium stearate were studied by analyzing synthetic sample solutions containing 10 μgmL^{-1} of either TCH and DCH and excess amounts (10-fold excess of each excipient), none of these substances interfered seriously.

The regression equations obtained from as series of TCH or DCH standards and the analytical features of the procedures are summarized in Table 1. It also summarized the main performance of the flow procedure developed for TCH and DCH determination in order to make an effective compration between thetwoapproaches.

Table 1- Analytical characteristics of the procedures for the determinations of TCH and DCH

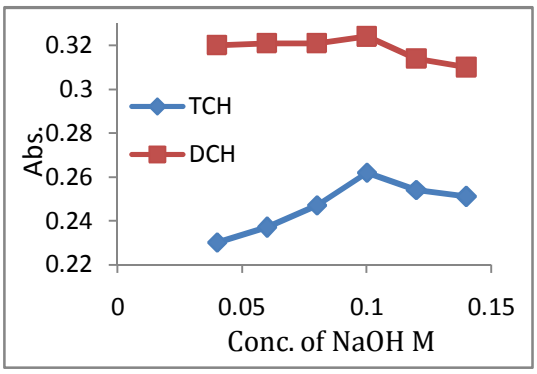
Parameter	Bach method		FIA method	
	TCH	DCH	TCH	DCH
Regression equation	Y=0.03x+0.0211	Y=0.0426x+0.057	Y=0.0067x+0.0135	Y=0.007x+0.0165
Linear range($\mu g\ mL^{-1}$)	1-56	1-52	5 – 240	5 – 350
Correlation coefficient	0.9993	0.9981	0.9993	0.9996
Limit of detection (μgmL^{-1})	0.3339	0.2352	0.6127	0.8955
Average of recovery,%	99.432	102.452	100.414	102.096
Relative standard deviation (RSD), %	1.918	1.876	0.915	0.8015
Sandell's Sensitivity (μgcm^{-2})	0.0333	0.0234	0.1492	0.1428
Through-put (hr^{-1})	6	6	120	80
Molar absorptivity ($L\ mol^{-1}cm^{-1}$)	21.849×10^3	14.427×10^3	3.222×10^3	3.590×10^3

FIA determination:

The batch method for the determination of TCH and DCH were adopted as a basis to develop FIA procedure. The manifolds used for the determination of each of TCH and DCH were so designed to provide different reaction conditions for magnifying the absorbance signal generated by the reaction of TCH and DCH drugs with DMCP in sodium hydroxide medium.Maximum absorbance intensity was obtained when the sample was injected into a stream of DMCP reagent and was combined with the stream of sodium hydroxide.The influence of different chemical and physical FIA parameter on the absorbance intensity of thecolored product was optimized as follows:

Chemical variables

The effect of different concentrations range (1×10^{-3}-1×10^{-2}M) of DMCP was investigated , while keeping other conditions constant , It was found that a 5×10^{-3} M of DMCP was found to be the most suitable concentration for obtaining maximum absorbance (Figure- 6) , and was chose for further use , sodium hydroxide was foundnecessary for developing the colored product and increase its stability the effect of sodium hydroxide was studied in the concentration range (0.04-0.14)M and a greatest absorbance intensity with lower baseline intensity was obtained with 0.1M of sodium hydroxide for determination of TCH and DCH respectively (Figure-7).



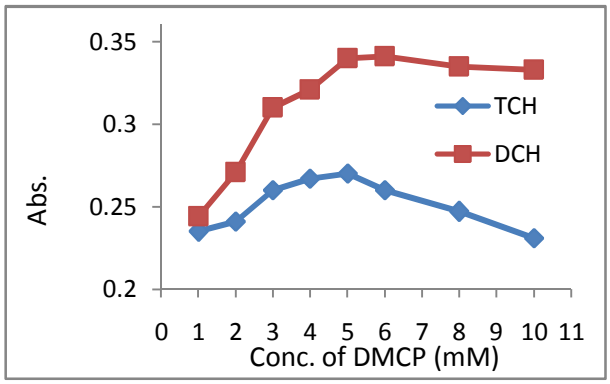
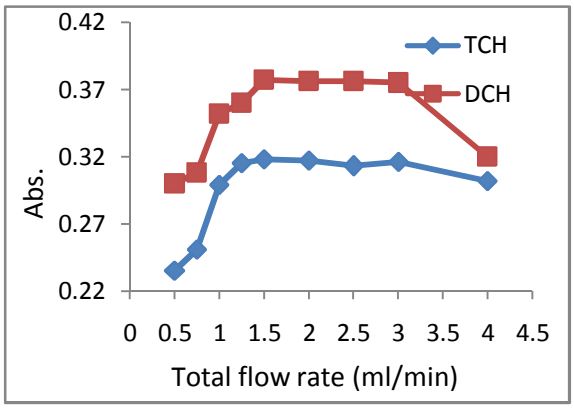


Figure 6-Effect the concentration of NaOH**Figure 7**-Effect the concentration of DMCP

Physical variables

The variables studied under the optimized reagent concentrations were the flow rate, the injected sample volume and the reaction coil length. The effect of total flow rate on the sensitivity of the colored reaction product was investigated in the range (0.5-4)mL.min^{-1} the result obtained showed that a total flow rate of 1.5mL.min^{-1} gave the highest absorbance as shown in (Figure-8) and was used in all subsequent experiments.The volume of the injected sample was varied between 100 – 250 µL using different length of sample loop, the result obtained showed that injected sample of 200µL gave the best absorbance and good reproducibility(Figure-9). Reaction coil is an essential parameter that affected on the sensitivity of the colored reaction product and was investigated in the range of (25 – 250 cm). The result obtained showed that a coil length of 75 cm for TCH and 100 cm for DCH gave the highest absorbance as shown in (Figure-10), and was used in all subsequent experiments. A standard calibration lines, obtained for series of TCH and DCH standards and the main analytical feature of merits of the developed procedures are indicated in Table 1. The accuracy and the precision of the proposed method were studied as shown in Table2.



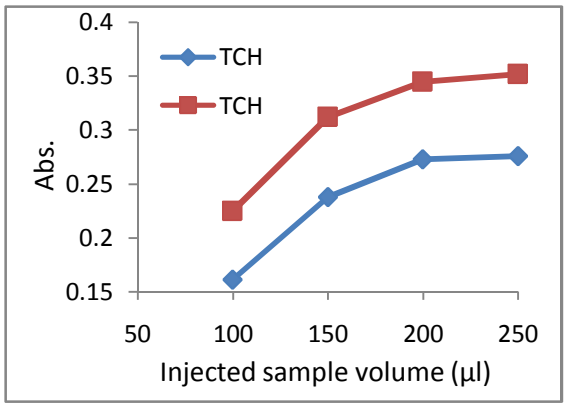


Figure 8-Effect of the total flow rate ($mLmin^{-1}$)**Figure 9-**Effect of the injection volume (µl)

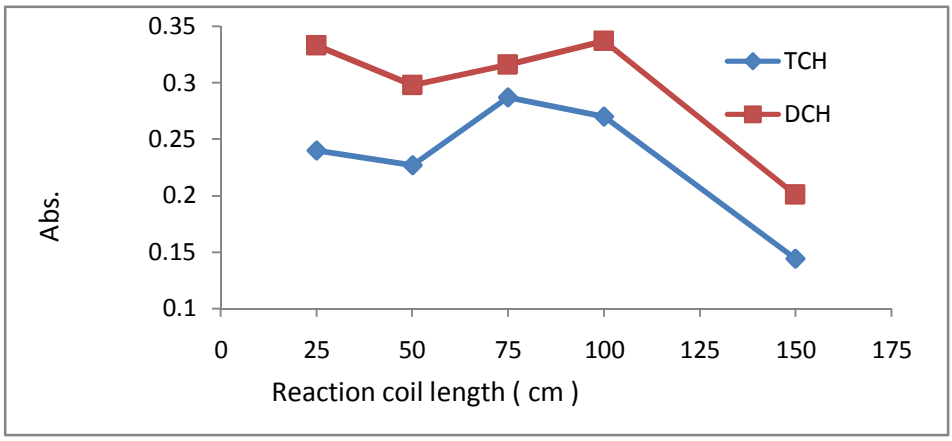


Figure 10- Effect of the length of the reaction coil (cm)

Table 2- The accuracy and precision of the proposed methods (Batch and FI)

Drug	Batch method					Flow injection method				
	Conc.µg.mL^{-1}		E% *	Rec.% *	RSD% *	Conc.µg.mL^{-1}		E%*	Rec.% *	RSD% *
	Present	Found				present	found			
TCH	8	7.87	-1.54	98.45	1.76	32	32.29	0.92	100.93	1.51
	16	16.00	0.02	100.02	2.36	80	79.61	-0.48	99.51	0.38
	24	23.95	-0.18	99.81	1.62	120	120.95	0.79	100.79	0.83
DCH	8	8.27	3.46	103.46	3.09	50	51.04	2.08	102.08	0.84
	16	16.41	2.58	100.58	1.49	80	81.5	1.87	101.87	0.94
	20	20.26	1.31	101.31	1.04	100	100.32	2.32	102.32	0.61

* Average of five determinations

Analyticalapplications:

Analytical application of the proposed methods were applied successfully to the analysis of some pharmaceutical preparations containing TCH and DCH.The results obtained are summarized in Table 3 which is in compration with those obtained by the official standard methods [28]. Finally,statistical analysis [29],showed there is no significant different in precision and accuracy betweenthe proposed methods and the official methods.

Table3-Applications of the proposed and official methods to the determinations of someTCH and DCH in capsule forms

Pharmaceuticals	Batch			Flow injection analysis			Official method	
	Conc. μgmL^{-1}	Rec. %*	RSD %*	Conc. μgmL^{-1}	Rec.% *	RSD %*	Rec.% *	RSD% *
Samacycline (TCH) Capsule 250mg (SDI,Iraq)	16 20	100.01 100.45	0.41 0.787	50 100	101.80 98.75	0.651 0.664	100.744	1.422
Apocycline (TCH) Capsule 250 mg (ajenta,india)	16 20	100.37 100.1	1.518 1.046	50 100	99.67 97.02	1.254	97.529	3.122
Tetracycline(TCH) Capsule.250 mg (MEHECO., china)	16 20	98.93 100.40	0.348 3.33	50 100	97.30 98.75	1.606 0.581	98.69	2.044
Medomycin (DCH) Capsule 100mg (Kyprus)	16 20	100.91 99.8	1.09 1.724	50 100	101.75 98.28	1.518 0.844	101.583	1.364
Doxycycline(DCH) Capsule 100mg (actvas , UK)	16 20	98.87 100.34	2.71 2.138	50 100	99.321 100.70	0.977 0.912	102.286	0.776
Tabocine (DCH) capsule100mg (Tabok,K.S.A)	16 20	98.56 101.7	1.85 1.242	50 100	100.05 99.80	1.428 0.987	99.627	2.181

* Average of five determinations

Conclusion:

The application of diazotization–coupling reaction of diazotized metoclopramide in sodium hydroxide medium to the spectrophotometeric determinations of the tetracycline hydrochloride and doxycylinehyclate in pharmaceutical preparations was described by batch and FIA systems. Although the batch system has the advantages of higher sensitivity and lower limit of detection over the FIA system, the FIA system has several advantages over the batch system simplicity,reproducibility time saving, low reagent consumption need of small sample volume, large dynamic range and high sample

throughput (120 sample h^{-1} for TCH) and (80 sample h^{-1} for DCH) are important features of the FIA system.

The proposed methods offer a good linearity and precision and can be applied to the analysis of awide concentration range of TCH and DCH in real samples with satisfactory results.

The proposed methodsare simple and inexpensive since it requires simple instrumentation.

References:

1.Drollery, E. (ED), **1999 ,**"*Therapeutic Drugs*", 2nd Ed.Charchilllivingstone, Edinburgh.

2.Joseh,patrical and Helena R., **2009**,"Flow–injection spectrophotometric Determination of tetracycline and Doxycycline in pharmaceutical formulations using chloramines –T as oxidizing agents ", *QuimicaNova*, 32, PP: 1764–1769.

3.Adres, F., AL-momani and Samer J.K., **2008**, "Flow–injection spectrophotometric and LC Determination of Doxucycline, oxytetracycline and chlorotetracycline in Biological fluids and pharmaceutical preparations",*Journal of Flow injection Analysis*, 25, PP: 29– 34.

4.Elisha, D. T. Mahommad and Deepth G.K., **2011**, "Spectrophotometric determination of tetracycline using P –N, N–Dimethylphenelenediamineand sodium metaperiodate", *RusssanJournalChem*istry, 4, PP: 539-546.

5.Elisha, D.T., MahammadT.andGarikipatik D., **2011**, "Spectrophotometric determination of tetracycline using 4–aminophenazone and potasiumiodate", *RussanJournalChemistry*, 4, PP: 896 – 900.

6.Labza, and Dovota , **1978,** "Chlorotetracycline determination in alkaline solution", *ActaPoloniaPharmaceutica*,35, PP:327– 335.

7.Shtykov, S.N., Smirnova T.D. and Bylinkin Y.G.,**2005**, "Simple colorimetric method for the determination of doxycycline hydrochloride ", *Journal of AnalyticalChemistry,* 60,PP: 24 – 28.

8.Ahlam, J.A., Hadi H. J.and Abbas S.H., **2013**, "Determination of tetracycline in pharmaceutical preparation by molecular and atomic absorption spectrophotometric and high performance liquid chromatography via complex formation with An (III) and Hg (II)ions in solutions ", *International Journal of AnalyticalChemistry*, Article ID 305124,II pages.

9.Chonj, X. and Tanigawak., **1984,** " liquid chromatographic method for determination tetracyclines ", *JournalAssocity off AnalyticalChemistry*, 67, PP: 1135–1137.

10.Furusawa, N., **2013,** "A 100% water mobile phase HPLC – PDA Analysis of tetracuclineAntibiotics",*American chemical science Journal*,4 , PP:500 – 506.

11.Pena, A., carmona A., Barbosa A., Lino C., Silveira I. and Castillo B., **1998**, " Determination of tetracycline and its major degradation products liquid chromatoghraphy with fluorescence dictation, *Journal of Pharmaceutical and Biomedical Analysis*, 81, PP: 839 – 845.

12.Paragada, J. R., Kanakapura B., Kalsong T., kanakapuraB.V.andHosakere D.R., **2010**, "Development and validation of RP-HPLC method for the determination of doxycycline hyclate in spiked human arine and pharmaceuticals" , *Journal of pre – clinical and clinical research*, 4, PP:101–107.

13.Ai Jia, Xang X., Jianying H., Mari A. and shoichi K., **2009**, " Simultaneaous determination of tetracyclines and their degradation products in environmental waters by liquid chromatography – electrosparay tandem mass spectrometry", *Journal. of Chemical Analysis*,1216 ,PP:4655–4662.

14.LIH, X.,Zhong J., He X. W.and Li J., **2004**, "Specctrofluorimetric investigation of the acid – base and complexationbehaviovor of tetracycline and oxytetracycline " , *Chinese Journal of Chemistry,* 22, PP:177–183.

15.Jian, C. and Luoh , **2004**, "Spectrofluorimetric determination of human serum albumin using tetracycline – europium complex " , *Anaytical. Letters*, 3, PP: 1129–1137.

16.Chang,W.B.Xhao Y.B. and Lu L., **1992**, "Spectropflourmetric determination of tetracycline and anhydro-tetracycline in serum and urine ", *Analyst*, 117, PP:1377-1388.
17.Hirschy, L.M., Dose E.V.andWineforduer J.D., **1983**, "Lanthanide–sensitized luminescence for the detection of tetracycllines*" AnalyticaChimica Acta*,147 ,PP: 311–316.
18.Abdulrahman, A.A. and Townshend A., **1988**, "Determination of tetracycline by flow injection with chemliuminscence detection ", *AnalyticaChimicaActa*, 205,PP: 261–265.
19.Han, H.Y., Hez H. and Zeng Y.E., **1999**, "Chemiluminscence determination of tetracyclines using a $(Ru\ (pby)_3)^{+2}$ and potassium permanganate system ", *Analtyical Science*, 15, PP: 467–470.
20.Norouzi, P., Ganjati M.R. and Daneshgar P., **2007**, "FFT–Adsorption voltametrictechanque for pico–level determination of tetracycline in capsules at an Au microelectrode in flowing solutions , *Turk Journal Chemistry*, 31, PP:279 – 291.
21.Gaiping, G, Faqiong Z., **2004**, "Voltammetric determination of tetracycline by using multi–wall carbon nanotube–ionic liquid film coated glassy carbon electrode",*InternationalJournal ofElectrochemicalScience*, 4, P: 1365-1372.
22.Edgar, N., Paola A., Veronica A., Macuricio B. and Virginia G., **2012**, "Amperometric and voltametric determination of oxytetracycline in trout salmoid muscle using multi – wall carbon nanotube , Ionic liquid Gold nanoparticle film electrodes , *International Journal of Electrochemical Science*, 7, PP: 11745–11757.
23.Karlicek, R. and Solich P., **1994**, "Flow injection spectrophotometric determination of tetracycline antibiotics ", *AnalyticaChimica Acta*,285, PP: 9–12.
24.Wnagfuengkanagul, N.,siangpoh W. and Chailapakul O., **2004**, "A flow injection method for the analysis of tetracycline antibiotics in pharmaceutical formulations using electrochemical detection at anodized boron-doped diamond thin film electrode ", *Talanta*,64,PP:1183-1188.
25.Liawrangraths, S.,Liawruangrath B.,Watanesk S. and Runengitagoon W.J., **2006**,"Fow injection spectrophotometric determination of tetracycline in pharmaceutical preparation by complexation with aluminum (III) ",*AnalyticalScience*, 22, PP: 15-19.
26.Cristina,M.C.,Jose L.F.C.,ConceicaoM.,Montenegro B.S.M. and Reis S., **1998**, "Tetracycline,oxytetracycline and chlorotetracycline determination by flow injection potentiometery " ,*Journalof Pharmaceutical and Biomedical Analysis*,18, PP: 527-533.
27.Al-Abiachi, M.Q.,Al-Ghabsha T.S. and Salih E.S.,**2001**,"Application of promethazine hydrochloride as achromogenic reagent for the spectrophotometric determination of anline and its substituents",*Microchemistry Journal*,41,PP: 58-64.
28."*British pharmacopeia*", H.M.,**2009**, Stationary office, London.
29.Sanders, D.H.,Murph A.F.,**1976**,"*Statistics*",MCGraw-Hill,New York.