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Research Article

NOVEL TURBIDIMETRIC-CONTINUOUS FLOW INJECTION ANALYSIS METHOD FOR THE DETERMINATION OF TETRACYCLINE HCl IN PHARMACEUTICAL FORMULATION USING HOMEMADE LINEAR ARRAY AYAH 5SX1-T-1D-CFI ANALYSER

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ABSTRACT

A new, simple and sensitive turbidimetric-flow injection method has been developed for the determination of Tetracycline HCl in pure and pharmaceutical formulation. The method was based upon the formation of yellow precipitate for the Tetracycline-Phosphomolybdic acid as ion pair complex in aqueous medium. Turbidity was measured by homemade Linear Array Ayah 5SX1-T-1D-CFI analyser via the attenuation of incident light from the surfaces of precipitated particles at 0-180° angle. Optimum concentrations of chemical reactants, physical instrumental conditions have been investigated. Linear dynamic range for the attenuation of incident light versus Tetracycline HCl concentration was 0.25-25 mmol.L^{-1} while correlation coefficient (r) was 0.9986 and percentage linearity (%r^2) C.O.D was 99.74%. Limit of Detection (S/N=3) 96.18 pg/sample from the stepwise dilution of minimum concentration for the lowest concentration in the linear dynamic range of the calibration graph. The R.S.D% (n=6) at 4, 12 mmol.L^{-1} Tetracycline is less than 2 % using 100 µL sample volume. The method was applied successfully for the determination of Tetracycline HCl in pharmaceutical formulations. A comparison was made between the developed method with the official method via the use of paired t-test. It shows that there were no significant differences between either methods. Therefore the newly developed method (Tetracycline HCl-Phosphomolybdic acid) can be adopted as an alternative method for determination of Tetracycline HCl.

Keywords: Tetracycline HCl, Turbidimetric method, Flow injection analysis.

INTRODUCTION

Tetracycline (TC) is a broad spectrum antibiotic showing activity against Gram-positive/Gram-negative bacteria[1,2]. Tetracycline having a fused, partially aromatic, 4-ring structure with a wide variety of functional groups[3,4] as shown in Figure (1). Tetracycline chemically known 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.

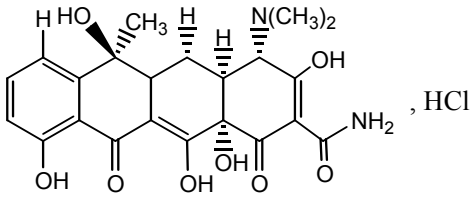


Fig. 1: Structure of Tetracycline

Tetracycline has been used not only in human medicine for the treatment of infectious diseases but also as additives in animal feed to promote

growth[5]. It is used to treat bacterial infections, including pneumonia and other respiratory tract infections; acne; infections of skin, genital and urinary systems; and the infection that causes stomach ulcers (Helicobacter pylori)[6].

TC characterized as Yellow, crystalline powder and freely soluble in water, slightly soluble in ethanol (96 percent), practically insoluble in acetone[7]. TC has a molecular formula of $C_{22}H_{24}N_2O_8$.HCl and molecular weight is 480.9 g.mol^{-1}.

Several procedures can be found in literature for analytical determination of Tetracycline in various matrices for example pharmaceutical formulations, biological samples. Some of the most commonly used methods include atomic absorption spectrophotometry[8], derivative spectrophotometry[9-11], fluorimetry[12-15], liquid chromatography[16-18], HPLC[19-20], voltammetric[21] and flow injection analysis[22-27] .

This paper describes a simple and rapid turbidmetric flow injection method for determination of Tetracycline HCl in pharmaceutical preparation. The method uses phosphomolybdic acid as a precipitating reagent in aqueous medium to form a yellow precipitate as ion pair complex. The precipitate is measured by the attenuation of incident light from the surfaces of precipitated particles at 0-180° angle using Linear Array Ayah 5SX1-T-1D-CFI Analyser.

MATERIALS AND METHODS

Chemicals

All chemicals used were of analytical reagent grade and distilled water throughout this work. Tetracycline HCl (The state company for drugs industry & medical appliances-Samaara, Iraq (SDI)) ($C_{22}H_{24}N_2O_8$.HCl, 480.9 g.mol^{-1}), (50 mmol.L^{-1}) was prepared by dissolving 1.2022 g/50 mL distilled water. A stock solution of phosphomolybdic acid (PMA) (Hopkin & Williams) ($H_3PMo_{12}O_{40}$, 1825.25 g.mol^{-1}), (0.1 mol.L^{-1}) was prepared by dissolving 18.2525 g in 100 mL distilled water. A 1 mol.L^{-1} Hydrochloric acid (BDH) solution was prepared by diluting 88.25 mL of 35% HCl (1.18 g.mL^{-1}) with distilled water in 1L calibrated flask, Standardized with Na_2CO_3 solution. A stock solution of sodium hydroxide (BDH) (NaOH, 40 g.mol^{-1}), (0.1 mol.L^{-1}) was prepared by dissolving 0.4 g in 100 mL distilled water.

Sample Preparation

The adopted procedure was through the selection of thirteen capsules randomly from different strips and packets (SDI Company and Aganta Company). The capsules were weighted, and collected in a container, then followed by weighing an amount equivalent to 0.3290g (SDI) and 0.2869g (Aganta) active ingredient to prepare the concentration of 10 mmol.L^{-1} for 250mg drug dose. The powder was dissolved in deionized water followed by filtration to remove any undissolved residue affecting the response. The filtrate was completed to 50 mL with distilled water.

A series of solution were prepared of each pharmaceutical drug (10 mmol.L^{-1}) by transferring 4 mL to each of the six volumetric flask (10 mL), followed by the addition of gradual volumes of standard TC (0, 0.4, 1.2, 2.0, 2.8, 3.6) mL of 50 mmol.L^{-1} to obtain (0, 2, 6, 10, 14, 18) mmol.L^{-1} flask no.1 is the sample. The measurements were conducted by both methods and the results were mathematically treated (for standard addition method).

Apparatus and Manifold

The flow system shown in Figure (2) was used for the determination of Tetracycline HCl. A four channels, variable speed peristaltic pump (Ismatec, Switzerland), the 6-port injection valve (Rheodyne, U.S.A) with a sample loop (0.5 mm i.d., Teflon, variable length) used for sample injection. The instrument response was measured by Linear Array Ayah 5SX1-T-1D-CFI analyser[29] (homemade) which uses five super white Light Emitting Diode (LED) for irradiation of the flow cell at 2 mm path length. One solar cell used as a detector for collecting signals output via sample segment passes through 55 mm length of flow cell. The output signals was recorded by x-t potentiometric recorder (KOMPENSO GRAPH C-1032) Siemens (Germany) and Digital AVO-meter (auto range) (0.00-2000 mV) (China). Peak height was measured for each signal.

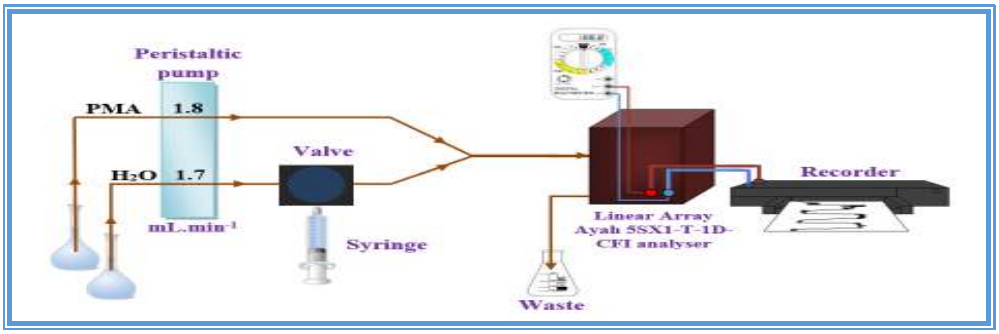


Fig. 2: Schematic diagram of manifold flow injection analysis system used for determination of Tetracycline HCl

Methodology

Using the FI manifold shown in Figure (2). The manifold reaction system was composed of two lines: first line represent the carrier stream (distilled water) leading to the injection valve, a 100 µL sample or standard solution containing Tetracycline was injected on carrier stream at flow rate 1.7 mL.min^{-1}. The second line is for phosphomolybdic acid (4 mmol.L^{-1}) at 1.8 mL.min^{-1} flow rate. Both lines mixes together at a Y-junction made from methyl methacrylate polymer.

The attenuation of incident light peak of the resulting yellow precipitate is followed using Linear Array Ayah-5SX1-T-1D-CFI anlayser and the variation of response was monitored using super white Light Emitting Diode (LED).

A preliminary experimental results revealed that the reaction of TC with PMA in aqueous medium produce a yellow precipitate. The profile peak (attenuation of incident light) of preliminary experiment shown in Figure (3).

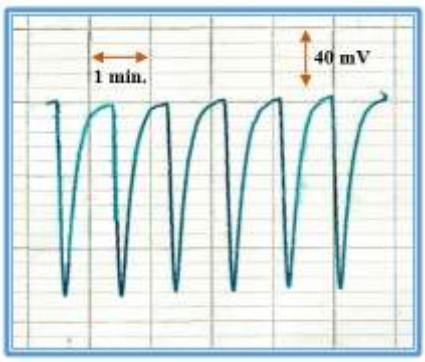
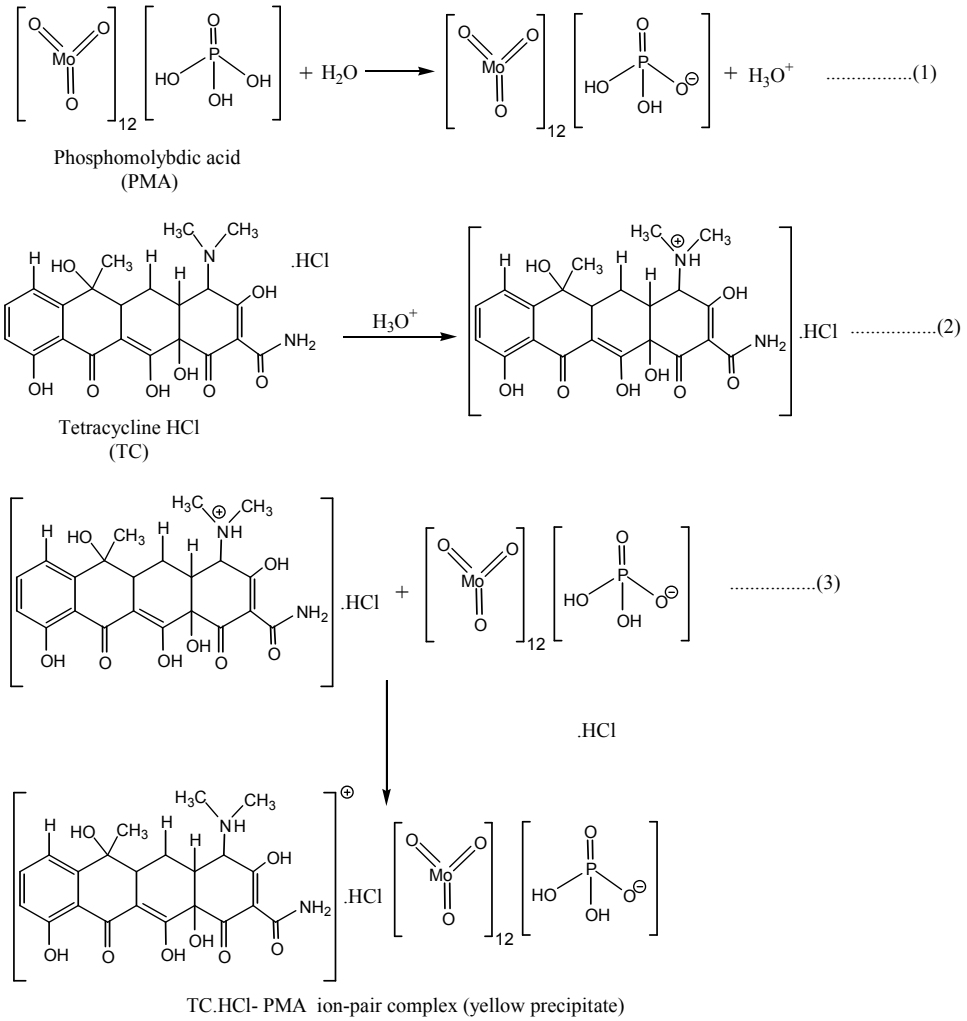


Fig. 3: Profile of preliminary experiment for determination Tetracycline HCl

[PMA]= 10 mmol.L^{-1}, [TC]= 5 mmol.L^{-1}, sample volume= 100 µL and 1200 mV intensity of incident light

Mechanism of Reaction

The proposed suggested mechanism for ion pair complex formation depending on the reference[28], and it is shown in Scheme no.1.



Scheme 1: Proposed mechanism of complex formation

RESULTS AND DISCUSSION

Study of the Optimum Parameters

The chemical parameters (mainly such as concentration of reagents used for the precipitation reaction and pH of the reaction medium) as well as physical parameters (flow rate, intensity of incident light, sample volume, purge time) were investigated.

Optimization of Chemical Parameter

1- **Phosphomolybdic Acid (PMA) Concentration**

A series of PMA solutions were prepared ranging (1-8) mmol.L^{-1} to establish the optimum concentration that can be used. A preliminary concentration was used for TC (5 mmol.L^{-1}) and 60 µL sample volume and 1200 mV intensity of incident light, each measurement was repeated for three successive times. The study was conducted that 4 mmol.L^{-1} of PMA was necessary to achieve the maximum attenuation of incident light as shown in Table (1) and Figure (4-A). It can be shows that an increase in PMA might be cause an increase in particles density due to accumulation effect of precipitate particles up to 4 mmol.L^{-1}, following this concentration there was a stability of the attenuation of incident light represent to the same growth of particle with increase PMA concentration as shown in Figure (4-B). Therefore, 4 mmol.L^{-1} PMA concentration was chosen as the optimum concentration that used for further experiment.

2. Effect of Acid or Basic Media on TC-PMA Precipitation System

The effect of acid and base of the reaction medium on the sensitivity in general was studied by using optimum concentration of PMA solution 4 mmol.L^{-1}. Series of diluted solutions (0-70) mmol.L^{-1} from hydrochloric acid and sodium hydroxide respectively was used in this study. A preliminary physical condition was used i.e. 60 µL as sample volume, 1200 mV as the intensity of incident light.

Figure (5) show a stability of the obtained response with increase of HCl concentration while decrease in response height when increase NaOH concentration. Therefore, it was concluded that distilled water can be used equally compared to acid used. Distilled water was preferred as a carrier stream.

Table 1: Effect of PMA concentration on the measurement of attenuation of incident light for TC-PMA precipitate system

Concentration [PMA] mmol.L^{-1}	Attenuation of incident light $\bar{y}i$ (n=3) (mV)	Standard deviation σ_{n-1}	Repeatability %RSD	Confidence interval of the mean $\bar{y}_i \pm t_{\frac{0.05}{2},n-1}\frac{\sigma_{n-1}}{\sqrt{n}}$
1	73.67	2.08	2.826	73.67±5.17
2	105.83	1.76	1.659	105.83±4.36
3	114.17	1.89	1.658	114.17±4.70
4	115.67	1.53	1.321	115.67±3.79
5	115.33	2.08	1.805	115.33±5.17
8	113.67	1.53	1.344	113.67±3.79

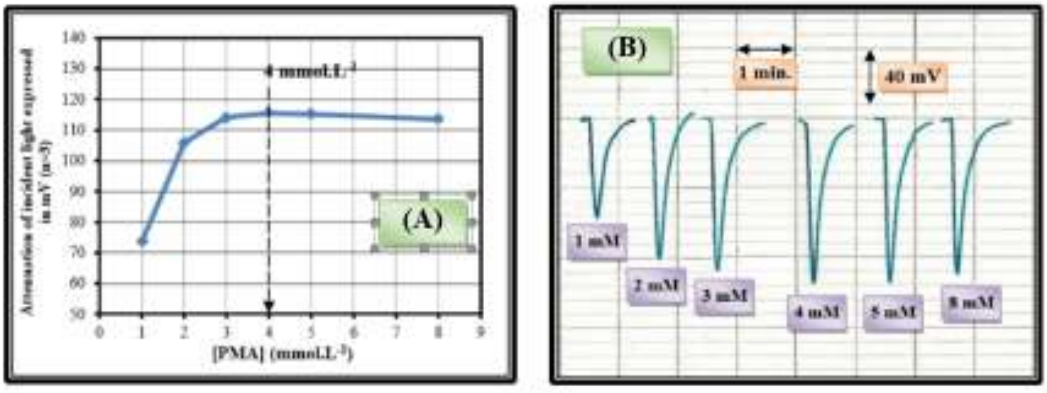


Fig. 4: Effect of variation of PMA concentration on (A): Attenuation of incident light (B): Response profile

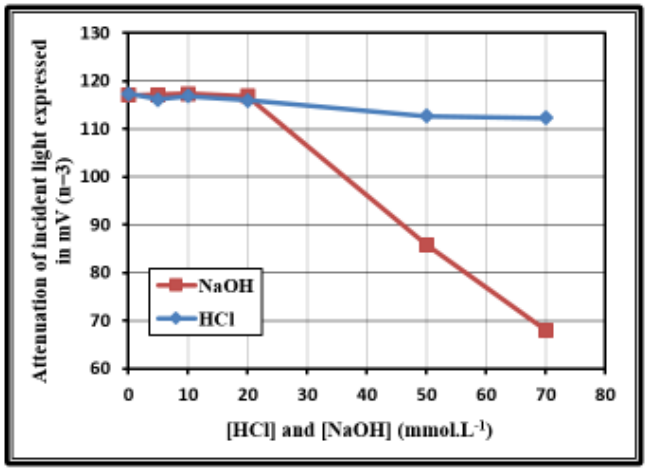


Fig. 5: Effect of the acidic and basic media on attenuation of incident light for TC-PMA precipitation system

Optimization of Physical Parameter

1. Reaction Coil Addition

The effect of reaction coil length was evaluated through the use of a coil length from 10 to 50cm with i.d. 1mm. This range of length comprises a volume of 314 to 1570 µl that was connected after Y-junction directly in flow system in Figure (1). The optimum concentration was used for precipitation system with TC solution (5 mmol.L^{-1}) with 60µL as the injected sample volume, 1200 mV as intensity of incident light.

Figure (6) show clearly that an decrease peak height with increase coil length, increase of the Δt_B, and arrival time of injected sample from injection valve to the measuring flow cell, which might probably attributed to the increase effect of dilution and dispersion of the sample zone and continuous longer time duration of precipitate species in front of detector.

2. Incident Light Intensity

Intensity of light source was studied using the optimum physical and chemical parameters achieved in previous section; with 5 mmol.L^{-1} of TC, 60 µL sample volume. Variable intensity of light source was used 750-2100 mV by changing of light intensity channel in Linear Array Ayah 5Sx1-T-1D-CFI analyser operation where read by AVO-meter. The obtained results tabulated in Table (2) which shows that an increase on the attenuation of incident light with increased intensity of light source. The intensity of 1750 mV was selected as the optimum voltage that can be supplied to give a better transducer energy response. Figure (7) shows the plot of attenuation of incident light vs. change in its intensity light source and peak profile.

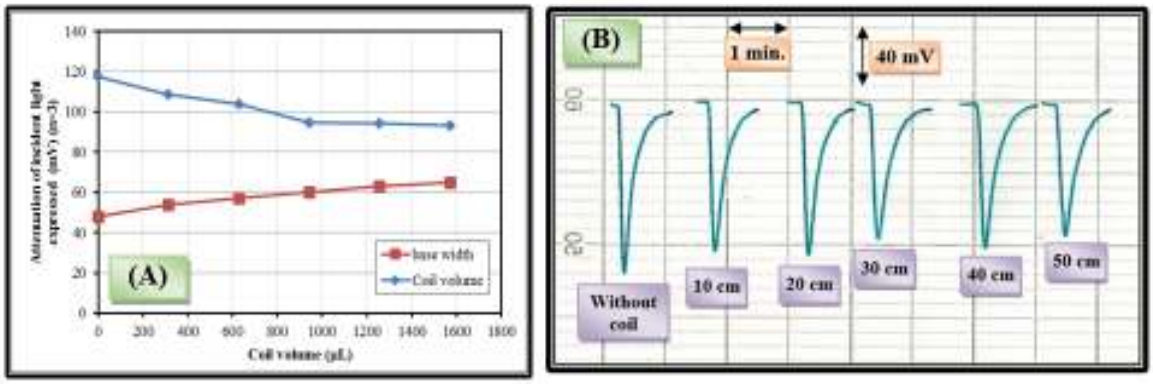


Fig. 6: (A) Effect of variation of reaction coil length on attenuation of incident light for PMA-TC precipitation system. (B): Response profile

Table 2: Effect of intensity of incident light expressed mV on attenuation of incident light expressed in mV

Intensity of incident light (mV)	Attenuation of incident light $\bar{y}_i$ (n=3) (mV)	Standard deviation σ_{n-1}	%RSD	Confidence interval of the mean $\bar{y}_i \pm t_{\frac{0.05}{2},n-1}\frac{\sigma_{n-1}}{\sqrt{n}}$
750	70.17	2.25	3.213	70.17±5.60
1000	90.33	2.08	2.304	90.33±5.17
1250	115.17	1.76	1.525	115.17±4.36
1500	126.00	2.00	1.587	126.00±4.97
1750	133.17	1.26	0.945	133.17±3.13
2000	102.67	2.52	2.451	102.67±6.25
2100	62.33	2.52	4.037	62.33±6.25

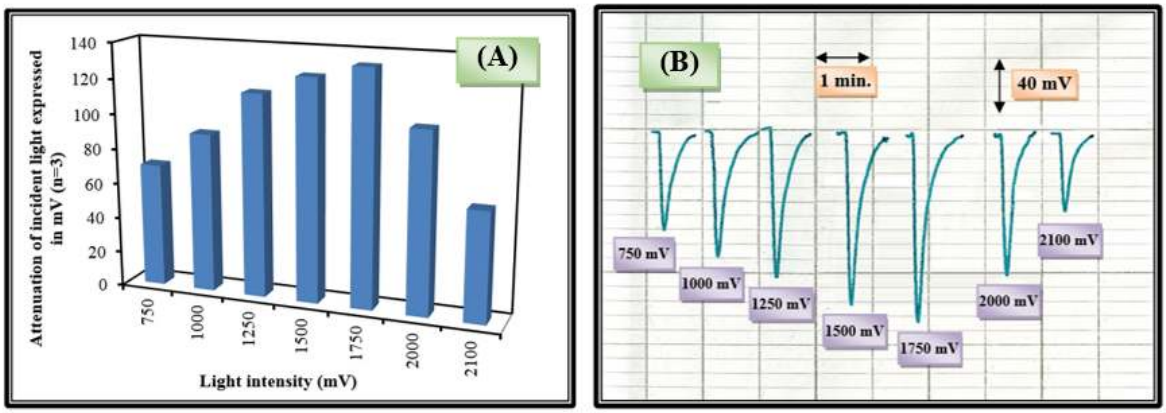


Fig. 7: (A) Variation of incident light vs. light intensity expressed in mV. (B) Output response profile of variation of light intensity

Table (2) and Figure (7) shows the continuation for the increase in attenuation of incident light with increase of intensity of incident light up to 1750 mV then followed by a decrease in attenuation of incident light with increase of intensity of incident light. The decrease in attenuation of incident light above 1750 mV was attributed to the effect of transparency of fine particulate with the increase of incident power of radiation. This effect can be prominent when very fine particle is formed.

3. Flow Rate

Using optimum concentration of the reactant: PMA (4 mmol.L^{-1}) and preliminary concentration of TC (5 mmol.L^{-1}) for the optimization of flow rate that ranged from 0.7-3.1 mL.min^{-1} for carrier stream and 0.7-3.0 mL.min^{-1} for PMA with 60 μL of sample volume. The results are tabulated in Table (3). It was noticed that at low flow rates, there were an increase in peak base width (Δt_B) as shown in Figure (8), this mostly attributed to the dispersion and dilution which causes to an extended length of sample segment of the precipitate product. While at higher flow rate (i.e: > 1.75, 1.7 mL.min^{-1} for the carrier stream and PMA respectively), although which in turn causes in obtaining regular response and sharp maxima, but it is not very high due to its departure at relatively higher speed, they not allowing enough time to detect and manipulate the precipitate of sample reaction product in the measuring cell. Therefore, the best flow rate for the completion of the reaction were 1.75, 1.7 mL.min^{-1} for carrier stream and PMA line respectively.

Table 3: Effect of the variation of flow rate on the measuring of attenuation of incident light

Pump speed indication approximate	Flow rate (mL.min-1)		Attenuation of incident light $\bar{y}i$ (n=3) (mV)	σ_{n-1}	RSD%	Confidence interval of the average response $\bar{y}_i \pm t_{\frac{0.05}{2},n-1}\frac{\sigma_{n-1}}{\sqrt{n}}$	Δt_B peak base width (sec)	t* (sec)
	Carrier stream	PMA line						
10	0.7	0.7	174.33	2.52	1.444	174.33±6.25	120	30
20	1.2	1.2	147.33	2.08	1.413	147.33±5.17	60	15
30	1.75	1.7	138.33	1.53	1.104	138.33±3.79	42	12
40	2.45	2.4	132.50	1.80	1.361	132.50±4.48	36	9
50	3.1	3	117.00	1.73	1.480	117.00±4.30	30	6

t*(sec): arrival time of sample segment to the measuring cell.

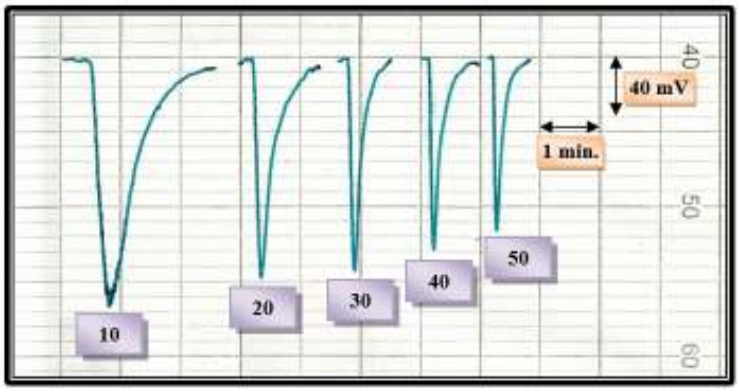


Fig. 8: Response profile of variation of flow rate on Tetracycline HCl precipitation system

4. Sample Volume

Using precipitation system PMA(4 mmol.L^{-1})-TC(5 mmol.L^{-1}) system with the optimum flow rate and light intensity. A variable volume (20-120) µL were injected using open valve mode i.e. allowance for continuous purge of sample from the sample loop in the injection valve. The plot of change in sample volume versus attenuation of incident light is shown in Figure (9-A). It was noticed that an increasing of sample volume up to 100 µL lead to a significant increase in response height and more perceptible than low volume as shown in Figure (9-B).

While when using larger volume i.e more than 100 µL, it gave a stability of higher response and it was characterized with the width Δt_B and response maxima which was might be attributed to the continuous relatively longer time duration of precipitate particles segment in front of the detector and increase of particle size causing a slow movement of precipitate particles therefore; 100 µl was chosen as an optimum sample volume.

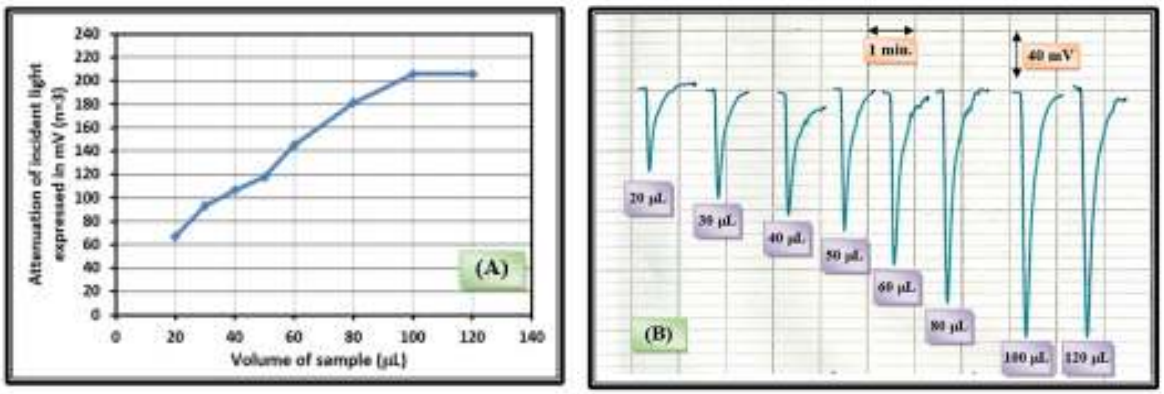


Fig. 9: Effect of variation of sample volume on the (A) Transducer Energy output of Ayah-5SX1-T-1D-CFI analyser (B): response profile

5. Purge Time

Allowed permissible time for the sample to be injected via the carrier stream was studied. The effect on the response and it is sensitivity was followed using the optimum physical and chemical parameters achieved in previous sections. 3-20 seconds were used in this study in addition to allowed the injection valve in the open mode. It can be seen from the Figure (10) that there is an increase in the response with increasing the allowed permissible time. Therefore, open valve (open time) more than 20 sec was chosen as optimum purge time to complete purge of the sample from sample loop in the next studies.

Calibration Curve for Variation of Tetracycline HCl Concentration

A series of solutions for TC ranging from 0.25 to 30 mmol.L^{-1} were prepared for the purpose of using them for the preparation of scatter plot diagram followed by the choice of calibration graph. A straight line graph (Figure (11)) ranging from 0.25 to 25 mmol.L^{-1} of TC was obtained. All results of the linear regression analysis[30-32] was summarized in Table (4) which shows the correlation coefficient, linearity percentage, straight line equation and the calculated t-value at 95% confidence level of 65.16 which larger than tabulated t-value indicating clearly that the linearity against non-linearity is accepted.

Analysis of variance was carried out as shown in Table (5) which indicated that $F_{tab.} = F^{\nu_1}_{\nu_2} = F^{1}_{12} = 4.75 << F_{Stat.} = 161.560$ therefore, it can be concluded that there is a significant relation between the concentrations of TC and the response obtained.

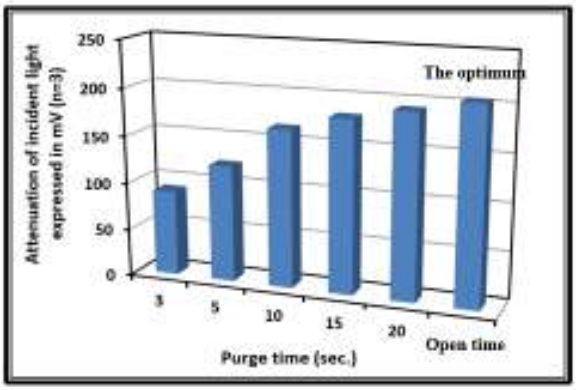


Fig. 10: Effect of variation of purge time on attenuation of incident light using optimum conditions

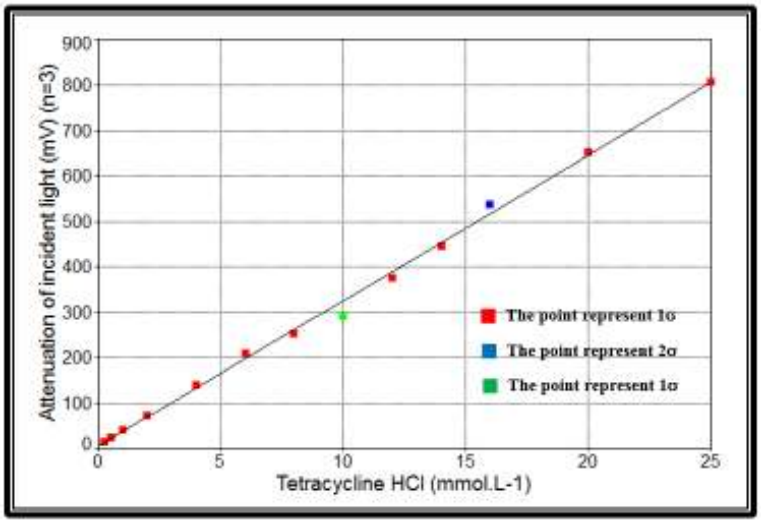


Fig. 11: Linear calibration graph for the energy transducer response expressed in mV with Tetracycline HCl concentration in mmol.L^{-1}

Table 4: A statistical summary of calibration graph for the determination of TC using PMA-TC precipitation system

Measured TC mmol.L^{-1}	TC range for n=13 mmolL^{-1}	y^(mV)=a±S_at+b±S_bt [TC] mmol.L^{-1} at confidence interval 95%, n-2	r, r^2, r^2%	$t_{tab.}$	$t_{cal}=\frac{\lvert r\rvert\sqrt{n-2}}{\sqrt{1-r^2}}$
				at 95%, n-2	
0.25-30	0.25-25	6.21±12.84+31.95±1.078 [TC] mmol.L^{-1}	0.9986 0.9974 99.74	2.201 <<< 65.16	

Table 5: ANOVA for linear equation results

Source	Sum of squares	D_f	Mean square	$F_{stat.}=S_1^2/S_2^2$
Regr. $(\hat{y}_i-\bar{y})$	773601	ν_1=1	773601	161.560
Error $(y_i-\hat{y}_i)$	1938.729	ν_2=12	4788.297	
Total $(y_i-\bar{y})$	775604.9	13		

Limit of Detection (L.O.D)

Three different approaches were used, gradual dilution of lowest concentration in the calibration graph, or detection based on the numerical value of slope and from the linear regression plot. Table (6) tabulated all these calculation value of detection limit for 100 μL sample volume; in addition to limit of quantitative.

The Repeatability

The repeatability of proposed method was studied via measurements of %RSD at a selected concentration of Tetracycline HCl (4 and 12 mmol.L^{-1}). All results was tabulated in Table (7). The response profile of six successive injected sample measurements for above concentration shown in Figure (12).

Table 6: Limit of detection for Tetracycline HCl at optimum parameter

Gradual dilution for the minimum concentration in calibration graph	Based on the value of slope $x=\frac{3S_B}{slope}$	Linear equation $\hat{y}$ (mV) = y_B+3S_B	Limit of Quantification L.O.Q $\hat{y}$ (mV) = y_B+10S_B
2×10^{-9} M/sample **96.18 pg/sample**	2.82×10^{-6} M/sample 0.135 μg/sample	1.26×10^{-3} M/sample 60.93 μg/sample	4.22×10^{-3} M/sample 203.11 μg/sample

x= value of L.O.D. based on slope, S_B = standard deviation of blank solution
y_B= average response for the blank solution (equivalent to intercept in straight line equation)

Table 7: Repeatability of energy transducer for TC at selected concentration

[TC] mmol.L^{-1}	Number of measuring (n)	Average of attenuation of incident light $\bar{y}i$ (mV)	Standard deviation σ_{n-1}	Repeatability %RSD	Confidence interval of the mean $\bar{y}_i \pm t_{\frac{0.05}{2},n-1}\frac{\sigma_{n-1}}{\sqrt{n}}$
4	7	140.57	2.44	1.74	281.14±2.57
12	6	375.33	3.26	0.87	375.33±3.42

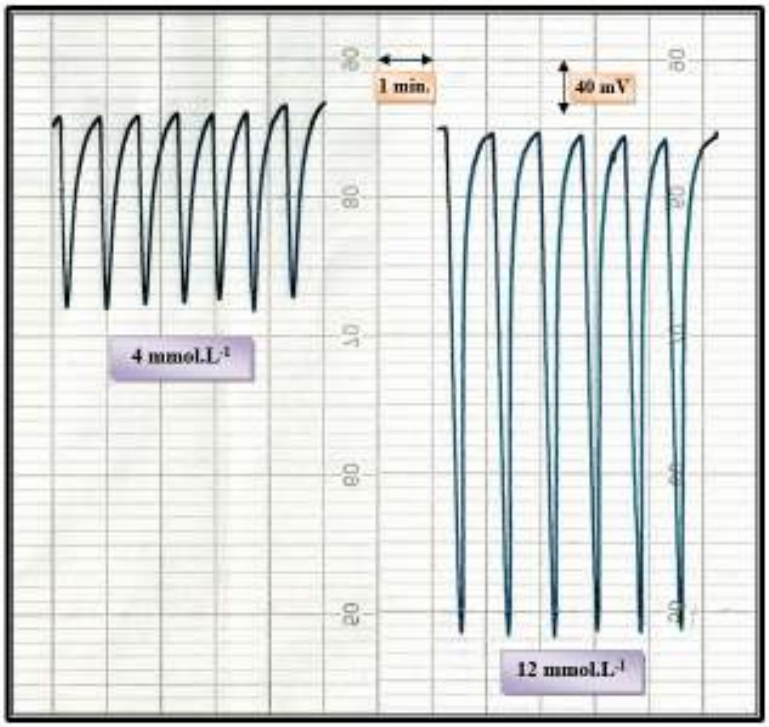


Fig. 12: Response time profile of six successive repeatable measurements of Tetracycline HCl concentrations (4 and 12 mmol.L^{-1}) in Linear Array Ayah-5SX1-T-1D-CFI analyser

Analytical Application

The Turbidimetric-flow injection via attenuation of incident light expressed as ($T_{0\text{-}180^\circ}$) method using Ayah 5Sx1-T-1D-CFI analyser achieved in this work used for the analysis of TC in two different samples of pharmaceutical preparations (Tetracycline-SDI, Tetracycline-Aganta). Each sample containing 250 mg of Tetracycline HCl (active ingredient) and was compared with official method.

Table (8) shows the names of the company supplier, drug dose and results at confidence interval 95%, paired t-test was used as shown in Table (9). The obtained results indication clearly that there was no significant difference between developed method FIA with official method [7] at 95% confidence interval as the calculated t-test value is less than tabulated t-test value.

Table 8: Results for the determination of Tetracycline HCl in pharmaceutical preparations

Commercial name Content Country	Confidence interval for average volume at 95% $\bar{w} \pm 1.96 \frac{\sigma_{n-1}}{\sqrt{n}}$ (mg)	Weight of sample (g) that equivalence to (250 mg) of active ingredient to obtain 0.01mol.L^{-1} (g)	Theoretical content of active ingredient at 95% n=∞ (mg)	found content of active ingredient at 95 n=∞ (mg)	% Recovery
Tetracycline 250 mg SDI-Iraq	0.3290±0.001544	0.3290	250±0.231	264.50±6.01	105.79
Tetracycline 250 mg Aganata-India	0.2869±0.003610	0.2869	250±1.776	243.45±3.01	97.38

Table 9: Results of paired t-test for the comparison between practical content of Turbidimetric-FIA method with quoted value

Sample no.	Practical content of Tetracycline (mg)		$\bar{x}$	$\bar{x}$-μ	σ_{n-1}	Paired t-test $t_{cal.} = \frac{(\bar{x} - \mu)\sqrt{n}}{\sigma_{n-1}}$	$t_{tab.}$ at 95% confidence interval n-1
	New method	Quoted value					
1	270.51	250	264.50	14.50	6.01	4.17 < 4.303	
	264.50	250					
	258.48	250					
2	240.45	250	243.46	-6.54	3.01	|-3.77| < 4.303	
	246.46	250					
	243.46	250					

CONCLUSION

The proposed Turbidimetric flow-injection method is simple, rapid, inexpensive and sensitive for the analysis of Tetracycline HCl in pure and pharmaceutical formulation. The method is based on precipitation of TC by PMA in aqueous medium to yield yellow precipitate as ion pair complex. The precipitate is measured via the attenuation of incident light at 0-180° using linear array of five super white light emitting diode as a source and one solar cell as a detector. The proposed method uses cheaper instruments and reagents with those spectrophotometry, fluorimetry, HPLC and other turbidimetric-FIA method with different precipitating agents. The %R.S.D was < 2% and good agreements were observed for all samples, which is an indication of satisfactory accuracy of the proposed method. The standard additions method was used to avoid matrix effects.

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