IJPSR (2015), Vol. 6, Issue 3 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 22 July, 2014; received in revised form, 29 September, 2014; accepted, 01 December, 2014; published 01 March, 2015

INTERACTION OF TETRACYCLINE HYDROCHLORIDE WITH IRON: KINETIC SPECTROPHOTOMETRIC AND CONDUCTOMETRIC INVESTIGATIONS

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Keywords:

Tetracycline HCl, *o*-phenanthroline, bipyridyl, kinetics, spectrophotometry, conductometry

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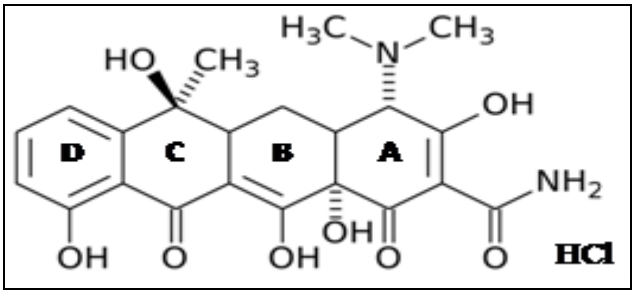
ABSTRACT: The interaction of the antibiotic, tetracycline hydrochloride (TC.HCl) has been investigated employing three simple spectrophotometric and conductometric methods. In methods **(A and B)**, a kinetic spectrophotometric procedure based on the oxidation of (TC.HCl) by Fe^{3+} ion in the presence of 1, 10 - Phenanthroline *(o-phen)* **(A)** and 2, 2′- Bipyridyl *(bipy)* **(B)** was developed. The formation of the ferroin complexes applying both methods was carefully optimized and their absorbance was measured at 509 and 520 nm respectively. The initial rate, rate-constant, fixed-time and fixed-concentration methods were adopted for constructing the calibration curves. In method **(C)**, conductometric titration of (TC. HCl) with $K_3Fe(CN)_6$ was investigated. Mole ratio – molar conductance plots showed that a 1:1complex was formed. Methods **(A and B)** allowed the determination of (TC. HCl) in the range of 1.00–5.00 and1.25–10.00 µg/ml respectively, while method **(C)** was applicable over a wider concentration range0.2-20 mg/ml. The proposed procedures were successfully applied to determine the drug as per se and in its commercial dosage forms with no interference from adjuvants commonly co-formulated with the drug. Inter- and intra-day precision, limits of detection and quantification, and relative standard deviation have been assessed following ICH guidelines for evaluation of analytical procedures, and the results obtained were satisfactory.

INTRODUCTION: Tetracycline hydrochloride (TC.HCl)is chemically recognized as(4S,4aS,5aS, 6S,12aS)- 4-(dimethylamino)-1,4,4a,5,5a,6,11,12a-octahydro-3,6,10,12,12a-pentahydroxy-6-methyl-1, 11 – dioxo – 2 – naphthacene carboxamide, mono hydrochloride **(Scheme 1).** [1, 2] Being broad-spectrum, TC.HCl is widely used for treatment of infections such as UTI, atypical respiratory tract infections, acne, and many others in both humans and animals. TC.HCl acts by inhibiting protein synthesis in the targeted bacteria restraining their growth. [3, 4]



SCHEME 1: TETRACYCLINE HYDROCHLORIDE

By and large, TC and its derivatives are bacteriostatics. [3, 4] This necessitates keeping their minimum inhibitory concentration (MIC) at the designated therapeutic level. Clinical investigations showed that a noticeable reduction in MIC is observed when TCs are concomitantly ingested with multivalent ions from drug or food sources. [5, 6] this effect was noticed whether the TC-multivalent ion associates are soluble or insoluble in water. [7]

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.6(3).1022-32

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.6(3).1022-32

Occupying the second place among antibiotic families in consumption and manufacturing, TCs represent an emerging class of pollutants. [8-10] Being excreted mainly in the un-metabolized form whose biodegradation is negligible, having high water solubility, and being hydrophilic, TCs have the potential to cause serious ecological problems.[8-14] Transformation of TCs employing their metal chelates is one of the adopted processing techniques.[15-17] Metals chelate with TCs in different ratios depending on many factors. Among these factors, nature of the iron salt, pH, and ionic strength are the most significant.[18]

On the other hand, complexation behavior of TCs largely influences their biological action. Talking about chelation with iron in specific, interestingly, and while the efficacy of TC wasn't affected, the efficiency of iron as an enhancer of infection was reduced.[19, 20] Chelation of TCs with different metals have been investigated by several methods, including spectrophotometry, [21-23] fluorimetry,[24-28] NMR spectroscopy,[29, 30] chromatography,[3 1]as well as electro analytical techniques.[32-34]

Herein, we report three new procedures for the determination of TC.HCl. The first set of procedures is based on oxidation of TC.HCl with the help of iron (III) and in presence of either (*o-phen*) or (*bipy*) as ligands.

The reaction products "*Tris*-complex" were determined spectrophotometrically at 509 and 520 nm respectively, and the reaction kinetics was assessed utilizing different strategies. The obtained limits of detection of TC.HCl in its pure samples are encouraging to extend the proposed procedures to determine un-metabolized TCs in effluents of wastewater samples in future applications.[14]In the second approach, an ion associate between TC.HCl and Fe(III) was determined via conductometric titration. Both approaches were employed in the determination of TC.HCl in capsules without interference from commonly used additives.

Experimental:

Apparatus:

A Perkin-Elmer Lambda EZ 210 equipped with two 10 mm matched quartz cells was used for spectrophotometric measurements. HachHQ14D Conductivity / TDS Meter (HI 8033), with an Intelli CAL™ CDC401 standard conductivity probe was used for all conductance measurements.

MATERIALS AND REAGENTS:

All reagents were of analytical pure grade. Water was always doubly distilled. Tetracycline hydrochloride (TC.HCl) was purchased from (Sigma-Aldrich, USA); (M.wt = 480.90 g/mol) and it was used as received. Pharmaceutical formulation (Tetracycline HCl®capsules; Julphar, KSA) labeled to contain 250 mg of TC.HCl, was purchased from local pharmacy stores. Iron(III)-o-phenanthroline was prepared by mixing 0.198 g of 1,10-phenanthroline monohydrate (Aldrich Chem. Co. Miluwakee, USA), 2ml1MHCland0.16 g ferric ammonium sulfate dodecahydrate (Aldrich, Germany) before dilution with doubly distilled water to 100 ml.

Iron(III)–bipyridyl was prepared by mixing 0.16g of 2,2′- bipyridyl (Sigma Chem. Co. Miluwakee, USA) with 2 ml 1 M HCl and 0.16 g ferric ammonium sulfatedodecahydrate, before dilution with double distilled water to 100 ml in a calibrated flask. Potassium ferricyanide (BDH limited, Poole, UK) was prepared as 2 x 10^{-2}M in distilled water. Aqueous stock solutions of TC.HCl of 0.5 $mgml^{-1}$were used for all methods. To establish the molar ration, equimolar concentrations ($1x10^{-2}$) of both TC.HCl and $K_3Fe(CN)_6$ were used.

Procedure for pure pharmaceuticals:

Methods A and B:

Aliquots of standard drug solutions ranging between 10 – 50 and 12.5 – 100 µg/ ml for methods A and B respectively were transferred into 10 ml calibrated flasks. Three milliliters of Fe (III) – *o-phen* (method A) and 2.5 ml of Fe (III) – *bipy* were added (method B) and the mixture was heated in boiling water bath for 45 and 30 min. for methods A and B respectively. Mixtures were cooled down to room temperature and the volume was made to the mark with distilled water. The orange – red complexes were measured at 509 and 520 nm against a reagent blank similarly prepared as described in methods A and B respectively.

Method C: Aliquots of standard drug solutions containing 0.2 – 20 mg of the pure TC.HCl solution

were transferred into 50 ml volumetric flasks and the volume was made with water up to 50 ml. Flask contents were transferred into a conductivity cell. The conductivity probe was immersed and the solution was titrated with 2 x 10^{-2} M of the titrant using a micro burette. The conductance was measured 2 min. subsequent to each addition of the reagent and after thorough stirring. A conductivity (corrected for dilution) *vs.* volume plot for a particular titrant was constructed and the endpoint was determined. The nominal content of the compound under study was calculated using the following expression: Amount of the drug (mg) = VMR / N

Where V = volume (ml) of the titrant consumed in the titration, M = relative molecular mass of the analyte, R = molarity of the titrant, and N = number of moles of the titrant consumed per one mole of the analyte. Determination of mole ratio was done using a fixed concentration of the analyte and varying concentrations of the titrant. Experimental data were fitted to a non-linear predefined fitting model.

Pharmaceutical Formulations:

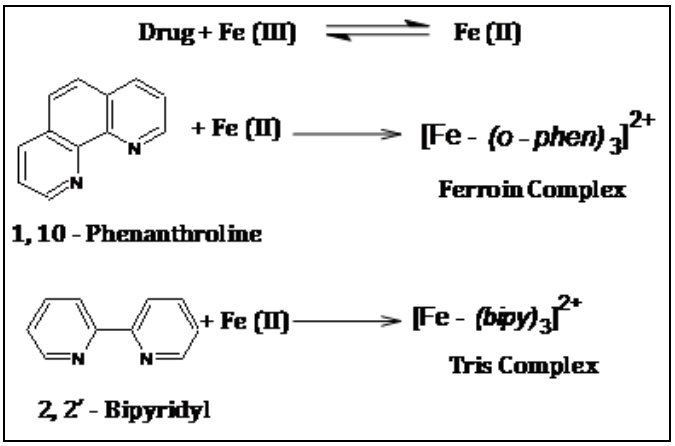
The contents of ten capsules were emptied and mixed. An amount of the powder equivalent to 125 mg of TC.HCl was accurately weighted and transferred into a 250 ml conical flask. The drug was extracted three times with 70 ml of distilled water. After extraction, the flask was washed with few mls of water, then, combined washings and extracts were filtered into a 250 ml volumetric flask. The volume was made to the mark with distilled water. The nominal content of TC.HCl in capsules was determined as described in the procedure section.

RESULTS AND DISCUSSION:

Methods A and B:

Investigations of the chemistry behind the formation of iron-*(o-phen)/(bipy)* complexes have anextended history. Literature survey has shown that formation of such complexes depends chiefly on the synthetic conditions such as pH, counter ion, and molar ratio of iron: *o-phen*. So, andwhile ferroin complexes $[Fe (phen)_3]^{2+}$ are formed when the source is iron(II), the analogous ferrin complex $[Fe (phen)_3]^{3+}$ doesn't exist in solutions made of iron(III) source.[35, 36]

This phenomenon has been utilized in the determination of many compounds of pharmaceutical interest. [37-40] In the current investigation, solutions prepared from Fe(III): *phen* (*bipy*) with a molar ratio of (1:3) were employed. Reduction of Fe(III) into Fe(II) was done using aqueous solution of TC.HCl "Scheme 2". The amount of *Tris* complex produced is directly proportional to [TC.HCl] in the ranges shown under each kinetic procedure. According to previously reported studies, [24] phenolic moiety at D-ring is a possible site for oxidation.



SCHEME 2: REACTION OF TC.HCl WITH *(O-PHEN)* AND *(BIPY)*.

Absorption spectra of the orange-red complex were measured at 509 and 520 nm for methods A and B respectively, **Figure 1**. Reaction conditions were optimized to obtain the maximum absorbance. A volume of 3 and 2.5ml of (*o-phen*) and *(bipy)* respectively were suitable to give optimum results. On the other hand, the reaction was so slow at room temperature and when temperature was increased, the reaction was faster until it reached a maximum in a boiling water bath (100 °C ± 2 °C). Heating for 45 and 30 min. in a boiling water bath gave the utmost absorbance for methods A and B respectively.

The reaction was conducted in different media such as ethanol, methanol, isopropanol, and water. Water was found to be the best solvent, an issue that adds to the advantages of the proposed methods. The formed complex was stable for at least 24 hours, another plus that allowed the

processing of large arrays of samples with expediency.

Investigation of Reaction Kinetics:

The absorbance of the produced chromogen was found to increase as the heating time increases, **Figure 2**. This finding was utilized as a basis for a kinetic inspection of the interaction between TC.HCl and Fe (III). Under the previously outlined conditions, the initial rates of the studied reaction were determined from the slopes of absorption-time curves, **Figure 3**.

The order of the reaction was determined with respect to the analytical reagent in presence of a fixed concentration of TC.HCl. Alternatively, reaction order was determined with respect to variable concentrations of TC.HCl in presence of an excess fixed concentration of the reagent. Plots of reaction rates *vs* initial absorbance in both cases were found to be close to one. Thus, the reaction rate can be described by the following equation:

$$\text{Rate}=K'[\text{reagent}]^m[\text{drug}]^n \quad (1)$$

Under the optimized reaction conditions, concentration of [Fe (III)] was at least 50 times the concentration of TC.HCl, and the concentration of (ligand) was at least 3 times that of [Fe (III)]. Consequently, the rate of reaction was found to be [TC.HCl] dependent, and would be best described by the *pseudo-first order* rate equation:

$$\text{Rate}=K'[\text{drug}]^n \quad (2)$$

Where K' is the first order rate constant, and n is the order of reaction with respect to [TC.HCl]. Several schemes were then tested to obtain TC.HCl concentration from the rate data and taking in consideration Eq. (2). Investigated techniques included initial rate, rate constant, fixed concentration and fixed time methods.[41] Methods were compared and selection was based on applicability, sensitivity, and linearity of the obtained data.

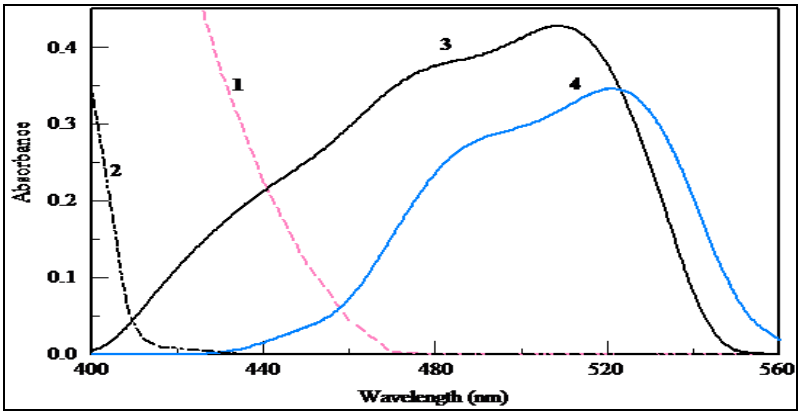


FIGURE 1: ABSORPTION SPECTRA OF 1, 10 – PHENANTHROLINE (1- METHOD A) 2, 2′- BIPYRIDYL (2 – METHOD B) BEFORE ADDITION OF ANALYTE, AND THE *TRIS* COMPLEX AFTER ADDITION OF 2.5 µg/ml (3) AND 3.5 µg/ml (4) OF TC.HCL TO SOLUTIONS 1 AND 2 RESPECTIVELY.

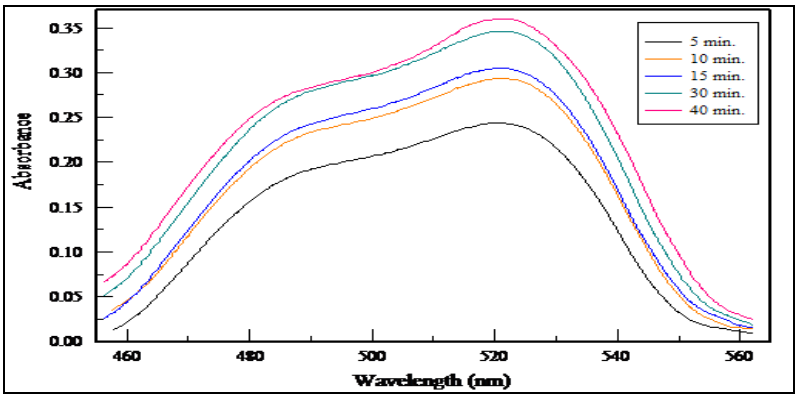


FIGURE 2: REPETITIVE SCANS FOR THE REACTION OF A MIXTURE OF 3.5 µg/ml TC.HCL AND 2.5 ml *(BIPY)* HEATED FOR DIFFERENT TIMES (5-40 MIN.) IN A BOILING WATERBATH. ABSORBANCE IS INCREASING WITH TIME TILL ≈30 MIN., AFTER WHICH NO SIGNIFICANT INCREASE IN ABSORBANCE

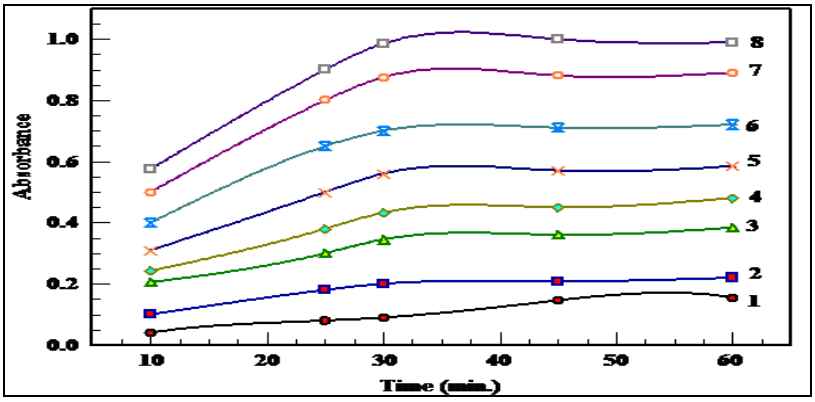


FIGURE 3: TC.HCL AND *(O-PHEN)* SHOWING THE RELIANCE OF THE REACTION ON [TC.HCL]. CONCENTRATIONS OF TC.HCL ARE: (1) 2.079× 10^{-6}; (2) 3.119 × 10^{-6}; (3) 4.159 × 10^{-6}; (4) 5.198 × 10^{-6}; (5) 6.238 X 10^{-6}; (6) 7.278×10^{-6}; (7) 9.357 X 10^{-6}; (8)1.039 x 10^{-5}.

Initial rate method:

As described before, the initial rates for oxidation of TC.HCl would pursue a pseudo-first order behavior, and were found to comply with the following equation:

$$Rate = {}^{\Delta A}/_{\Delta t} = K'[\text{drug}]^n \qquad (3)$$

Where A is the absorbance and *t* is the time. Taking the logarithm of both equation sides would result is the following logarithmic formula:

$$log[Rate] = log\, {}^{\Delta A}/_{\Delta t} = \log K' + n\ log[\text{drug}] \qquad (4)$$

In order to evaluate values of slope, intercept, and coefficients of variation, the method of least squares was considered, and kinetics data were fitted to Eq.4. Regression analysis resulted in the following equations for methods A and B respectively.

$$log\ (Rate)_A = -1.2942 + 0.9986 \log [TC.HCl] \qquad (5\text{-A})$$

$$log\ (Rate)_B = -2.1757 + 0.6994 \log [TC.HCl] \qquad (5\text{-B})$$

Results of regression analysis are summarized in **Table 1**. Values of *n* (≈1) obtained from regression data confirmed that investigated reaction is first order with respect to [TC.HCl].

TABLE 1: ANALYTICAL PARAMETERS OF THE INITIAL RATE TECHNIQUE FOR THE DETERMINATION OF TC.HCl EMPLOYING METHODS A AND B.

Method	Linear range, M	Least square equation: $log[Rate] = log\,{}^{\Delta A}/_{\Delta t} = \log K' + n\ log[\text{drug}]$		
		Intercept, (*log K'*)	Slope, (*n*)	Correlation coefficient, (*r*)
A	2.079×10^{-6} - 1.039 x10^{-5}	- 1.2942	0.9986	0.9791
B	2.599 x 10^{-6}- 1.039 x10^{-5}	- 2.1757	0.6994	0.9975

Rate constant method:

Plots of log absorbance versus time for TC.HCl concentration in the range of3.119×10^{-6} – 1.039×10^{-5} (method A) and 2.599×10^{-6} - 1.039×10^{-5}M (method B) were drawn and all were rectilinear. Pseudo-first order rate constant (*K'*) corresponding to different TC.HCl concentrations (*C*) were calculated from the slopes multiplied by −2.303 and are presented in **Table 2**. Regression of (*C*) versus *K'* gave the following equations:

$$K' = 10.537C - 0.0004 (r^2 = 0.8158) \qquad (6\text{-A})$$

$$K' = 151.42C - 0.0018 (r^2 = 0.736) \qquad (6\text{-B})$$

Values of r^2 showed a poor linearity. This can be attributed to the high temperature at which the reaction was conducted.

Fixed-concentration method:

Reaction rates were determined for different concentrations in the ranges shown in **Table 3**. A chosen value of the absorbance was predetermined

and the time was measured in seconds. The reciprocal of time (i.e. *1/t*) was plotted versus the initial concentration of the studied drug. The following equations for calibration graphs were obtained by linear regression:

1/t= 228.55C - 0.0008 (r^2 = 0.9802) (7-A)
1/t= 1335.8C - 0.0093 (r^2= 0.9868) (7-B)

TABLE 2: VALUES OF *K′*CALCULATED FROMS LOPE OF LOG *A*vs. *t* GRAPHS MULTIPLIED BY−2.303 FOR DIFFERENT CONCENTRATIONS OF TC.HCl AT CONSTANT CONCENTRATION OF REAGENT.

Method A		Method B	
***-2.303 K′* x 10^{-4}, S^{-1}**	**[TC.HCl], M**	***-2.303 K′* x 10^{-4}, S^{-1}**	**[TC.HCl], M**
-3.488	3.119 x10^{-6}	-16.29	2.599 x 10^{-6}
-2.944	5.198 x10^{-6}	-4.442	6.238 x10^{-6}
-2.901	6.238 x10^{-6}	-4.544	7.278 x10^{-6}
-2.733	7.278 x10^{-6}	-4.277	8.317 x10^{-6}
-2.693	9.357 x10^{-6}	-4.481	9.357 x10^{-6}
-2.631	1.039 x10^{-5}	-4.078	1.039 x10^{-5}

TABLE 3: VALUES OF RECIPROCAL OF TIME TAKEN AT FIXED ABSORBANCE FOR DIFFERENT RATES OF VARIABLE CONCENTRATIONS OF TC.HCl

Method A		Method B	
***1/t* x 10^{-4}, S^{-1}**	**[TC.HCl], M**	***1/t* x 10^{-4}, S^{-1}**	**[TC.HCl], M**
6.67	6.238 x10^{-6}	5.56	7.278 x10^{-6}
9.06	7.278 x10^{-6}	16.67	8.317 x10^{-6}
12.82	9.357 x10^{-6}	33.33	9.357 x10^{-6}
16. 67	1.039 x10^{-5}		

Fixed time method:

In this technique, reaction rates were determined for different concentrations of TC.HCl and after heating for different times, Table 4. The heating time that resulted in the best absorbance was chosen and fixed for the rest of the study. As shown in Table 4, 45 and 30 min. were selected as the most suitable reaction times for methods A and B respectively.

Method C:

In the current treatise, formation of a soluble complex between TC.HCl and Fe (III) has been used for quantitative conductometric determination of TC.HCl. While chelation of TCs with iron (III) has been widely investigated, [23-34] no conductometric procedure; to the best of our knowledge, has been reported in literature for determination of TC.HCl. The proposed procedure was simple and straightforward. Specific conductivity values corrected for dilution were plotted as a function of volume of titrant. [42] The obtained graphs showed two straight lines with different slopes. The point of intersection between the two straight lines was recognized as the equivalence point of titration.

TABLE 4: CALIBRATION EQUATIONS FOR TC.HCl AT DIFFERENT FIXED TIMES OVER THE RANGE OF 2.079×10^{-6} – 1.039×10^{-5} (METHOD A) AND 2.599×10^{-6} - 1.039×10^{-5} M (METHOD B) AND IN PRESENCE OF CONSTANT CONCENTRATION OF REAGENTS.

Method	Time (min.)	Calibration equation	Coefficient of determination (R^2)
A	10	A = 0.1326C - 0.0847	0.994
	25	A = 0.2075C - 0.1227	0.9951
	30	A = 0.2245C - 0.1218	0.9964
	45	A = 0.218C - 0.0863	0.9958
	60	A = 0.2133C - 0.0604	0.9944
B	5	A = 0.1157C - 0.1593	0.9892
	10	A = 0.1393C - 0.1948	0.9936
	15	A = 0.1429C - 0.191	0.9909
	30	A = 0.1698C - 0.2452	0.9944
	40	A = 0.1677C - 0.2298	0.9954

Molar Ratio: The molar-ratio for the interaction of Fe^{3+} and TC.HCl was established employing various procedures. In the first approach, equimolar concentrations of both Fe^{3+} and TC.HCl were usedand both reactants were alternatively used as a titrant. Initially, andusing $K_3Fe(CN)_6$ as titrant,a

curve break was obtained indicating a molar ratio of 1:1. Alternatively, Fe^{3+} was titrated with TC.HCl, and the curve showed only one inflection at a molar ratio of 1:1. Molar conductance (Λ_m) of the drug solution was monitored as a function of mole ratio ([D]/[R]). Obtained data were fitted using a non-linear least squares fitting model predefined by PSI Plot software, **Figure 4**. As shown in **Figure 4**, molar conductance starts to level off at the point where the [D]/[R] ratio is equal to unity (applying first approach). Such a behavior further confirms the formation of 1:1 complex. In the second approach, concentration of $Fe^{3+}$was varied to be 5, 10, and 20 times that of TC.HCl. In all cases, the mole ratio was found to be 1:2 (D: R).

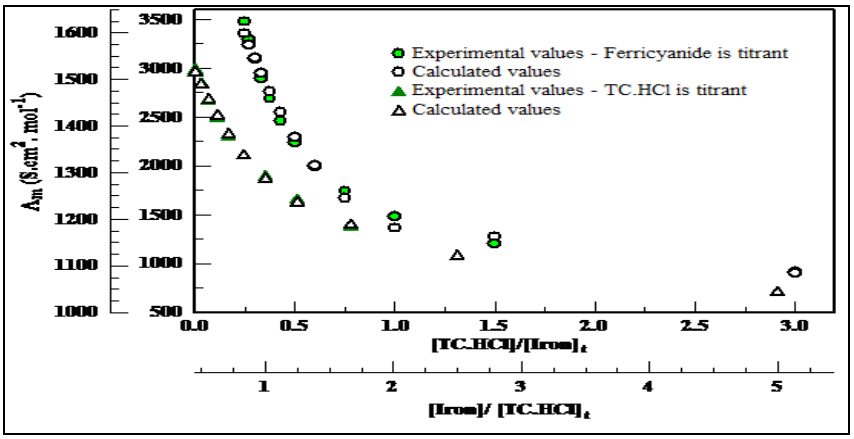


FIGURE 4: MOLAR CONDUCTANCE – MOLE RATIO PLOTS FOR THE COMPLEX OF $K_3Fe(CN)_6$ WITH TC.HCl IN PURE WATER. EACH REACTANT WAS USED AS TITRANT ALTERNATIVELY. EXPERIMENTAL VALUES ARE REPRESENTED BY CLOSE GEOMETRIES, WHILE CALCULATED VALUES ARE REPRESENTED WITH OPEN ONES. CALCULATED VALUES ARE OBTAINED BY FITTING THE EXPERIMENTAL VALUES USING NON-LINEAR LEAST SQUARES FITTING ALGORITHM.

Validation of the Proposed Analytical Procedures:

Appraisal of the kinetic procedures:

A comparison between the proposed kinetic procedures in terms of linear range, detection limits, and standard analytical error is shown in **Table 5**. As shown in this table, fixed concentration approach for both methods A and B showed the narrowest concentration range for quantification. Rate constant approach achieved a poor linearity as previously shown by equations (6A-B). Fixed-time method, however, was more practical in application in terms of reproducibility as revealed by the calculated analytical error and confidence limits for slope, intercept, and regression, especially when compared to the initial rate procedure. The lowest LOD was achieved employing the initial rate approach. However, and since LOD is not a component of method validation as per ICH guidelines, [43] fixed-time method at 45 and 30 min is recommended for determination of TC.HCl using methods A and B respectively.

TABLE 5: OPTICAL AND REGRESSION CHARACTERISTICS FOR THE DETERMINATION OF TC.HCl USING THE PROPOSED KINETIC PROCEDURES.

Parameter	Method A				Method B			
	Initial rate method	Rate constant method	Fixed concentration method	Fixed time method	Initial rate method	Rate constant method	Fixed concentration method	Fixed time method
Linear range (µgml^{-1})*	1.00-5.00	1.50-5.00	3.00-5.00	1.00-5.00	1.25-5.00	1.25-5.00	3.50-4.50	1.25-7.50
S_b	8.46x10^{-2}	5.21x10^{-6}	4.78x10^{-5}	5.81x10^{-3}	2.01x10^{-2}	9.43x10^{-5}	3.21x10^{-4}	3.65x10^{-3}
± tS_b	5.86x10^{-2}	4.17x10^{-6}	4.68x10^{-5}	4.01x10^{-3}	1.39x10^{-2}	7.54x10^{-5}	3.63x10^{-4}	2.16x10^{-3}
S_a	0.338	1.84x10^{-5}	1.95 x 10^{-4}	1.84x10^{-2}	8.72x10^{-2}	3.53x10^{-4}	1.29x10^{-3}	1.86x10^{-2}
± tS_a	0.234	1.47x10^{-5}	1.90 x 10^{-4}	1.27x10^{-2}	6.04x10^{-2}	2.82x10^{-4}	1.46x10^{-3}	1.10x10^{-2}
$S_{y/x}$	5.25x10^{-2}	1.5x10^{-5}	7.56 x 10^{-5}	2.16x10^{-2}	1.52x10^{-2}	2.8x10^{-4}	2.27x10^{-4}	2.97x10^{-2}
LOD (µgml^{-1})	0.158	2.05	0.477	0.297	0.065	2.66	0.244	0.541
LOQ (µgml^{-1})	0.527	6.85	1.59	0.992	0.217	8.88	0.816	1.805

* Regression equation: $A=b\,C+a$, where ***A*** is the absorbance, ***C*** is concentration in µgml-1, ***a*** is intercept, ***b*** is slope.

S_b = SD of slope ± tS_b = confidence limit for slope S_a = SD of intercept± tS_a= confidence limit for intercept $S_{y/x}$= SD of the regression, LOD = limit of detection LOQ = limit of quantification.

Accuracy and precision:

The accuracy and precision of the proposed procedures (methods A-C) was determined for both pure samples and formulations. Assessment was conducted by measuring 3-4 different concentrations for 3-5 times within the same day employing the fixed-time approach for methods A and B, and the conventional procedure for method C. Alternatively, daily accuracy and precision were calculated on five subsequent days (methods A and B) and on three days (method C). All data were calculated at 95% confidence limits, and are shown in **Tables 6-7**. Data shown are considered reasonable.

Similarly, the accuracy of methods A and B was assessed using the standard addition technique and via calculation of recovery, **Table 8**. No interference from excipients and additives was observed. All data were compared to a reference method.[44] **Table 9** shows that calculated *t*- and *F*-values are less than the theoretical ones, [45] confirming the absence of significant difference between the compared methods.

TABLE 6: INTRA AND INTER-DAY ASSAY: EVALUATION OF ACCURACY AND PRECISION OF THE PROPOSED ANALYTICAL PROCEDURES FOR DETERMINATION OF TC.HCl IN BULK POWDER.

Method	Amount taken, μgml^{-1}	Intra-day			Inter-day		
		Amount found ± SD*	RSD (%)	Er (%)	Amount found ± SD*	RSD (%)	Er (%)
A	3.00	3.01± 0.027	0.90	0.33	2.97± 0.072	2.40	1.00
	4.50	4.43± 0.097	2.16	1.56	4.48± 0.081	1.80	0.44
	5.00	4.98± 0.079	1.58	0.40	4.93± 0.118	2.36	1.40
B	3.00	2.91± 0.112	3.82	3.00	2.90 ± 0.113	3.85	3.33
	4.00	3.98± 0.046	1.16	0.50	4.02 ± 0.075	1.88	0.50
	5.00	4.92± 0.051	1.03	1.60	5.01 ± 0.117	2.35	0.20
Method	**Amount taken, $mgml^{-1}$**	**Intra-day**			**Inter-day**		
		Amount found ±SD**	**RSD (%)**	**Er (%)**	**Amount found ± SD****	**RSD (%)**	**Er (%)**
C	0.50	0.51 ± 0.005	0.991	2.00	0.505 ± 0.015	2.96	1.00
	5.00	5.05 ± 0.076	1.52	1.00	5.10 ± 0.100	1.96	2.00
	10.00	10.09 ± 0.084	0.836	0.90	10.21 ± 0.105	1.04	2.10
	20.00	20.19 ± 0.244	1.215	0.95	20.45 ± 0.226	1.12	2.25

*Average of 5 determinations.

**Average of 3 determinations.

TABLE 7: INTRA AND INTER-DAY ASSAY: ASSESSMENT OF ACCURACY AND PRECISION OF THE PROPOSED ANALYTICAL PROCEDURES FOR DETERMINATION OF TC.HCl IN CAPSULES.

Method	Amount taken, μgml^{-1}	Intra-day			Inter-day		
		Amount found ± SD	RSD (%)	Er (%)	Amount found ± SD	RSD (%)	Er (%)
A	2.00	2.04 ± 0.033	1.65	2.00	1.98 ± 0.062	3.13	1.00
	2.50	2.52 ± 0.077	3.05	0.80	2.57 ± 0.095	3.74	2.80
	4.00	4.02 ± 0.110	2.72	0.50	4.07 ± 0.091	2.23	1.75
B	2.00	1.93 ± 0.036	1.84	3.50	1.97 ± 0.036	1.83	1.50
	3.00	2.90 ± 0.075	2.60	3.33	2.92 ± 0.113	3.82	2.67
	6.00	5.92 ± 0.071	1.21	1.33	5.92 ± 0.113	1.89	1.33
Method	**Amount taken, $mgml^{-1}$**	**Intra-day**			**Inter-day**		
		Amount found ±SD**	**RSD (%)**	**Er (%)**	**Amount found ± SD****	**RSD (%)**	**Er (%)**
C	5.00	5.05 ± 0.076	1.52	1.00	5.17 ± 0.113	2.18	3.40
	10.00	10.22 ± 0.054	0.54	2.20	10.21 ± 0.295	2.89	2.10
	12.00	12.19 ± 0.134	1.12	1.58	11.65 ± 0.125	1.08	2.92

*Average of 5 determinations.

**Average of 3 determinations.

TABLE 8: STANDARD ADDITION METHOD FOR DETERMINATION OF TC.HCl IN PHARMACEUTICAL FORMULATIONS EMPLOYING METHODS A AND B.

Method A			Method B		
Taken (μgml^{-1})	Added(μgml^{-1})	Recovery%	Taken(μgml^{-1})	Added(μgml^{-1})	Recovery%
1.75	-	98.86	2.00	-	98.50
	1.25	100.67		1.25	98.77
	1.50	101.11		1.50	100.29
	1.75	101.76		2.00	99.87
	2.00	98.67		2.50	100.16
	2.25	98.21		3.00	98.80
	2.50	101.07		3.50	99.09
	2.75	100.55		4.00	97.71
	3.00	100.00		4.50	100.00
Mean* ± SD		100.10 ± 1.245			99.24 ± 0.884
N		9			9
RSD		1.244			0.891
Variance		1.550			0.781

*Mean of 3 determinations.

TABLE 9: STATISTICAL DATA FOR THE DETERMINATION OF TC.HCl USING METHODS (A–C) COMPARED WITH REFERENCE METHOD*.[44]

Parameter	Comparison Method[44]	MethodA	MethodB	MethodC
Mean** ±S.D.	100.54 ± 1.154	100.08 ± 1.617	98.81 ± 1.450	100.91 ± 0.985
N	5	5	5	4
RSD	1.148	1.616	1.467	0.976
V	1.332	2.615	2.103	0.970
t-test***	-	0.599 (2.306)	2.087 (2.306)	0.519 (2.365)
F-test***	-	1.96 (6.388)	1.578 (6.388)	1.361(6.591)

* Oxidation with ammonium molybdate in acidic medium.

**Mean of 3 determinations.

***Values in parentheses indicate theoretical values oft and Fat *P*=0.05.

Robustness and ruggedness:

Robustness was investigated by assessing the significance of minor changes in method variables such as reagent concentration, volume, and reaction time on method performance. In each test, one factor was varied per time, keeping the rest constant. It was observed that none of these variables had a note worthy effect on the proposed method, indicating the consistency of the proposed procedures and the suitability for routine applications. Ruggedness was evaluated using the same experimental parameters for determining the drug using two different instruments at two different laboratories and at different times. Results obtained were found to be reproducible, as RSD did not exceed 2%, **Table 10**.

TABLE 10: EVALUATION OF THE RUGGEDNESS OF THE PROPOSED SPECTROPHOTOMETRIC METHODS FOR DETERMINATION OF TC.HCl USING THE PROPOSED SPECTROPHOTOMETRIC METHODS.

Method	Perkin-Elmer Lambda EZ 210	Agilent 8453 – UV-Vis Diiode-array
A	100.08 ± 1.617	100.21 ± 1.095
B	98.81 ± 1.450	99.98 ± 0.985

CONCLUSION: Three simple and non-time consuming procedures have been proposed for the determination of TC.HCl. All of the proposed methods were based on interaction of TC.HCl with iron and didn't involve sophisticated treatment of analyte or tedious extraction. The spectrophotometric procedures were followed kinetically employing four different approaches.

Among the proposed procedures, initial rate and fixed-time approaches were more suitable for determining TC.HCl in pure form and pharmaceuticals in terms of sensitivity and reproducibility. The conductometric procedure was less sensitive compared to the spectrophotometric one; however, it was applicable over a wider concentration range. In general, the proposed procedures were successfully applicable for determination of TC.HCl in dosage forms without interference from additives commonly formulated with the drug. Moreover, the proposed procedures were validated and results shown were satisfactory.

ACKNOWLEDGMENT: The author is grateful for the financial support provided by Qatar University (Grant No. QUSG-CAS-CHD12/13-10). The author wishes to express her gratitude to Ms. Duaa W. Al-Sadek, Department of Biomedical Sciences, College of Arts and Sciences, Qatar University for her help in completing this work.

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How to cite this article:
Elazazy MS: Interaction of Tetracycline Hydrochloride with Iron: Kinetic Spectrophotometric and Conductometric investigations. Int J Pharm Sci Res 2015; 6(3): 1022-32.doi: 10.13040/IJPSR.0975-8232.6(3).1022-32.