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PAVAGADA JAGANNATHAMURTHY RAMESH
KANAKAPURA BASAVAIAH MYSORE RANGANATH
DIVYA NAGARAJU RAJENDRAPRASAD
KANAKAPURA BASAVAIAH VINAY

Department of Studies in Chemistry, Manasagangothri, University of Mysore, Mysore, India

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TITRIMETRIC AND SPECTROPHOTOMETRIC DETERMINATION OF DOXYCYCLINE HYCLATE USING BROMATE-BROMIDE, METHYL ORANGE AND INDIGO CARMINE

One titrimetric and two indirect spectrophotometric methods are described for the determination of doxycycline hyclate (DCH) in bulk drug and in its formulations. The methods use bromate-bromide, methyl orange and indigo carmine as reagents. In titrimetry (method A), DCH is treated with a known excess of bromate--bromide mixture in acid medium and the residual bromine is back titrated iodometrically after the reaction between DCH and in situ bromine is ensured to be complete. In spectrophotometric methods, the excess of bromine is estimated by treating with a fixed amount of either methyl orange (method B) or indigo carmine (method C) and measuring the change in absorbance either at 520 or 610 nm. Titrimetric method is applicable over 1-8 mg range and the calculations are based on a 1:2 (DCH:bromate) stoichiometric ratio. In spectrophotometry, the calibration graphs were found to be linear over 0.25-1.25 and 1.0-5.0 μg mL^{-1} for method B and C, respectively, with corresponding molar absorptivity values of 2.62×10^5 and 6.97×10^4 L mol^{-1} cm^{-1}. The accuracy and precision of the assays were determined by computing the intra-day and inter-day variations at three different levels of DCH.

Key words: doxycycline; determination; dyes; spectrophotometry; pharmaceuticals.

Doxycycline hyclate (DCH): (4*S*,4*a*R,5*S*,5*a*R,6*R*,12*a*S)-4-(dimethylamino)--3,5,10,12,12*a*-pentahydroxy-6-methyl-1,11-dioxo--1,4,4*a*,5,5*a*,6,11,12*a*-octahydrotetracene-2-carboxamide monohydrochloride, compound with ethyl alcohol (2:1), monohydrate, (Figure 1) is a broad spectrum antibiotic, with activity against a wide range of gram-positive and gram-negative bacteria. It has been used for the treatment of infectious diseases caused by rickettsiae, mycoplasmas and chlamydiae [1]. DCH is widely used in medicine and veterinary practice. As a result, DCH residue can occur in food products of animal origin [2].

The drug is official in the British Pharmacopoeia (BP) [3] and the United States Pharmacopoeia (USP) [4], which describes HPLC methods for the determination of DCH either in raw material or in pharmaceutical formulations. Several methods have been reported

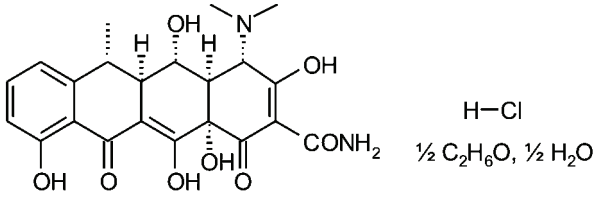

Figure 1. Molecular structure of DCH.

for the determination of DCH in pharmaceutical dosage forms including fluorimetry [5], phosphorimetry [6], liquid chromatography [7-11], thin layer chromategraphy [12], sequential injection chromatography [13], doxycycline opto-sensors [14,15], ion selective electrodes-based potentiometry [16] and capillary electrophoresis [17]. Few visible spectrophotometric methods based on the different reaction mechanisms are found in the literature for the assay of DCH. These include FIA-spectrophotometry with copper carbonate [18], chloramine-T [19] and 4-aminophenazone/potassium hexacyanoferrate(III) [20] and also based on colour reactions with thorium (IV) [21], sodium cobaltnitrite [22] and uranyl acetate [23]. Besides, kinetic spectrophotometry using Cu(II)/H_2O_2 [24] and multivariate calibration method [25] have also been reported by different workers.

Correspondening author: K. Basavaiah, Department of Chemistry, University of Mysore, Manasagangothri, Mysore-570 006, India.
E-mail: basavaiahk@yahoo.co.in
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The chromatographic techniques are most widely used. Although the procedures are specific, most of the described methods are time consuming and require multistage extraction procedures. On the other hand, the reported spectrophotometric methods suffer from one or the other disadvantage such as poor sensitivity, use of organic solvent, scrupulous control of experimental variables and special equipment (Table 1). Titrimetry and spectrophotometry are well established techniques, and owing to their speed, fair selectivity, reduced costs and versatility of application, they can be considered to be advantageous alternatives to sophisticated and expensive techniques normally used in pharmaceutical analysis. A complexometric titration method has been reported for the determination of DCH [26]. The method employs spectrophotometric titration of DCH with Mg^{2+} and Ca^{2+} in aqueous tris-buffer. The titrations were performed at two fixed pH values (pH 7.0 and 8.5) and the consistent sets of UV-Visible absorption and fluorescence spectra were recorded at various times. Since the method utilized buffers, pH is very crucial and a sophisticated instrument to measure fluorescence response is required. By considering these drawbacks, the present work is aimed at developing titrimetric and spectrophotometric methods that would overcome many of the problems encountered in the reported methods. This work describes one titrimetric and two spectrophotometric methods for the determination of DCH in pharmaceuticals based on the bromination reaction using bromate-bromide mixture and by employing two dyes, methyl orange and indigo carmine. The methods were successfully applied to the determination of DCH in two different brands of tablets with good accuracy and precision and without detectable interference by excipients. The accuracy was further ascertained by placebo blank and synthetic mixture analyses and also by recovery experiments *via* the standard-addition procedure and the methods were to be simple, accurate and easy to apply to routine analysis.

EXPERIMENTAL

Apparatus

A Systronics model 106 digital spectrophotometer with 1-cm matched quartz cells was used for all absorbance measurements.

Table 1. Comparison of the performance characteristics of the present spectrophotometric methods with the published methods

No.	Reagent/s used	Methodology	λ_{max} nm	Linear range	*LOQ* μg mL^{-1}	Remarks	Ref.
1	Copper carbonate	Complex colour measured	395	10.0-80.0 mg mL^{-1}	-	FIA assembly required and least sensitive	18
	Chloramine-T	Oxidation of drug in alkaline medium and red coloured product measured	525	From 5.37×10^{-5} to 7.16×10^{-4} mol L^{-1}	-		19
	4-Aminophenazone and potassium hexacyanoferrate(III)	Colour of the dye measured	520	-	-	FIA assembly required and the pH dependent	20
2	Thorium(IV)	Yellow complex measured	398	0.4-3.2 μg mL^{-1}	-	pH dependent and narrow linear range	21
3	Sodium cobaltnitrite and acetic acid	Colour forming reaction	243	0.01-0.03 mg mL^{-1}	-	Heating required. Less sensitive	22
4	Uranyl acetate-DMF medium	1:1 Complex formation reaction	405	0-135 μg mL^{-1}	-	Requires organic solvent, less sensitive	23
5	Cu(II)/H_2O_2-alkaline medium	Degradation study	510	2.97-17.78 μg mL^{-1}	1.89	Use of buffers, scrupulous control of experimental variables and special equipment for kinetic measurement required	24
6	DMF/NaOAc-AcOH buffer (pH 4.5)	Partial least squares multivariate calibration method	277-349	1.7-42 μg mL^{-1}	-	Require special equipment, Use of organic solvent, pH dependant	25
7	$KBrO_3$-KBr/HCl and methyl orange	Bromination of drug and determination of unreacted Br_2 with methyl orange	520	0.25-1.25 μg mL^{-1}	0.07	Highly sensitive, non-stringent optimum conditions used, simple instrument employed.	Present work
	$KBrO_3$-KBr/HCl and indigo carmine	Bromination of drug and determination of unreacted Br_2 with indigo carmine	610	0.5-5.0 μg mL^{-1}	0.27		

Reagents and materials

All the reagents used were of analytical-reagent grade and distilled water was used throughout the investigation.

A bromate-bromide solution equivalent to 5 mM $KBrO_3$-50 mM KBr was prepared by dissolving accurately weighed 418 mg of $KBrO_3$ (S.d. Fine Chem Ltd, Mumbai, India) and 3 g of KBr (Merck, Mumbai, India) in water and diluting to the mark in a 500 mL calibrated flask and this solution was used in titrimetric work. For use in spectrophotometric study, a 1000 μg mL^{-1} $KBrO_3$ solution containing a large excess of KBr was prepared by dissolving 100 mg of $KBrO_3$ and 1 g of KBr in water and diluting to the mark in a 100 mL calibrated flask. This was diluted stepwise to get 10 μg mL^{-1} and 30 μg mL^{-1} bromate solutions for use in method B and C, respectively.

A 0.03 M sodium thiosulphate ($Na_2S_2O_3$) (S.d. Fine Chem Ltd, Mumbai, India) solution was prepared in water and standardized [27]. Hydrochloric acid (S.d. Fine Chem, Mumbai, India, Sp. gr. 1.18) (2 and 5M), potassium iodide (Merck, India) (10%), methyl Orange (S.d. Fine Chem, India, dye content: 85%) (50 μg mL^{-1}), indigo carmine (S.d. Fine Chem, Mumbai, India, dye content: 90%) (200 μg mL^{-1}) and starch indicator (1%) solutions were prepared in distilled water.

Pure DCH (pharmaceutical grade, 99.8% pure) sample was kindly provided by Lotus Pharma Ltd, Bangalore, India, as a gift and used as received. Two brands of tablets, namely, DOX-T 100 (Dr. Reddy's Lab, Hyderabad, India) and Doxy 100 (Microlabs Ltd., Mumbai, India) used in the investigation were purchased from local commercial sources.

Doxycycline hyclate standard solution

A 1 mg mL^{-1} standard drug solution was prepared by dissolving 250 mg of pharmaceutical grade DCH in water; the volume was made up to 250 mL in a calibrated flask with water and was used in titrimetry. This solution was then diluted with water to get 5 and 10 μg mL^{-1} solutions for the use in method B and C, respectively.

General analytical procedures

Titrimetry (method A). An aliquot of pure drug solution containing 1-8 mg of DCH was transferred accurately into a 100 mL Erlenmeyer flask and the total volume was made up to 10 mL with water. The solution was acidified by adding 5 mL of 2 M HCl. Ten mL of bromate-bromide solution (5 mM w.r.t. $KBrO_3$) was transferred to the flask by means of a pipette. The flask was stoppered, the content mixed well and kept aside for 20 min with occasional swirling. The stopper was then washed with 5 mL of water and 5 mL of 10% potassium iodide solution was added to the flask. The liberated iodine was titrated with 0.03 M sodium thiosulphate to a starch end point. A blank titration was run under identical conditions. The stoichiometry was calculated using the formulae:

$$n = \frac{\text{ml of bromate} \times \text{molarity of bromate}}{\text{ml of DCH titrated} \times \text{molarity of DCH}}$$

and the amount of the drug in the measured aliquot was calculated from:

$$\text{mg} = \frac{VMwR}{n}$$

where V = volume of bromate reacted; Mw = relative molecular mass of the drug; R = molar concentration of bromate; n = number of moles of bromate reacting with each mole of the drug.

Spectrophotometry (*method B).* Different aliquots (0.0-2.5 mL) of 5 μg mL^{-1} DCH solution were accurately measured into a series of 10 mL calibrated flasks and the total volume was adjusted to 2.5 ml with water. To each flask 1 mL each of bromate-bromide solution (10 μg mL^{-1} w.r.t. $KBrO_3$) and 5 M hydrochloric acid were added. The content was mixed well and let stand for 20 min with occasional shaking. Then 1 mL of 50 μg mL^{-1} methyl orange solution was added to each flask and diluted to the mark with water. The absorbance of each solution was measured at 520 nm against a reagent blank after 5 min.

Spectrophotometry (*method C).* Varying aliquots of standard DCH solution (0.0-5.0 mL; 10 μg mL^{-1}) were transferred into a series of 10 mL calibrated flasks by means of a micro-burette, and the total volume was brought to 5 mL by adding water. To each flask, 1 mL of 5 M HCl and 1.5 mL of bromate-bromide solution (30 μg mL^{-1} w.r.t. $KBrO_3$) were added. After mixing the content, the flasks were allowed to stand for 10 min with occasional shaking. Then, 1 mL of 200 μg mL^{-1} indigo carmine solution was added to each flask and diluted to the mark with water. After 10 min the absorbance was measured at 610 nm against a reagent blank.

In methods B and C, a calibration graph was prepared by plotting absorbance *versus* the concentration of the drug and the concentration of the unknown was read from the calibration graph or computed from the regression equation derived from the Beer's law data.

Analysis of dosage forms

Twenty tablets, each containing 100 mg of DCH were weighed accurately and pulverized. An amount of the tablet powder equivalent to 100 mg was transferred into a 100 mL volumetric flask. The content was shaken well with about 70 mL of water for 20

min. The mixture was diluted to the mark with water. It was filtered using Whatmann No. 42 filter paper. The first 10 mL portion of the filtrate was discarded and a 5 mL aliquot was subjected to analysis following the procedure described in method A. For methods B and C, the tablet solution (1000 μg mL^{-1} in DCH) was diluted appropriately with water to get 5 and 10 μg mL^{-1} DCH and suitable portions were used in the analysis by following the general spectrophotometric procedures described for pure drug.

Analysis of placebo blank and synthetic mixture

A placebo blank containing talc (250 mg), starch (300 mg), lactose (30 mg), calcium carbonate (50 mg), calcium dihydrogen orthophosphate (20 mg), methyl cellulose (40 mg), sodium alginate (70 mg) and magnesium stearate (100 mg) was extracted with water and the solution made as described under "Analysis of dosage forms". A convenient aliquot of the solution was subjected to analysis by titrimetry (method A) and spectrophotometry (method B and C) according to the recommended procedures.

A synthetic mixture was prepared by adding 100 mg of DCH to the placebo blank prepared above, homogenized, and the solution was prepared as done under "Analysis of dosage forms". The filtrate was collected in a 100-mL flask and a 5 mL aliquot was assayed by method A. The synthetic mixture solution (1000 μg mL^{-1} in DCH) was appropriately diluted to get 5 and 10 μg mL^{-1} solutions, and appropriate aliquots were subjected to analysis by method B and C, separately.

RESULTS AND DISCUSSION

The determination of DCH is based on the oxidation and bromination reaction by bromine generated *in situ* by the action of acid on bromate-bromide mixture. In titrimetry, the reaction is followed by back titration of the residual bromine iodometrically and in spectrophotometry it is followed by change in absorbance of red colour of methyl orange at 520 nm (Figure 2) or blue colour of indigo carmine at 610 nm (Figure 3), the change being caused by the bleaching action of bromine on the dyes. In titrimetry (method A) the stoichiometry was expressed as the number of moles of bromate reacting with each mole of the drug. Since 2 mol of bromate (equivalent to 6 mol of bromine) are consumed in the reaction, 2 mol of bromine are believed to have been used up for the oxidation of the phenolic-OH groups at 5^{th} and 12^{th} positions of tetracene, two for the bromination at 7^{th} and 8^{th} positions, 1 mol each of bromine is likely to have been used up in the bromination of the amide group [28] as well as oxidation of ethanolic moiety. The probable reaction scheme is shown in Figure 4.

Method development

Titrimetry. The quantitative nature of the reaction between DCH and *in situ* generated bromine was checked by treating 1-8 mg of DCH with a measured excess of bromate-bromide mixture in acid medium and determining the surplus bromine iodometrically. In the range studied (1-8 mg), the reaction stoichiometry was found to be 1:2 (DCH:$KBrO_3$). Outside this range, non-stoichiometric results were obtained.

Optimizations of critical response parameters. The reaction stoichiometry was found to be unaffected in the presence of 3-8 mL of 2 M HCl in a total volume of 23-27 mL, and 5 mL was chosen as the optimum volume and better results and consistent stoichiometry were obtained in the preferred HCl medium than the other acid media studied (H_2SO_4, H_3PO_4 and CH_3COOH). The bromination reaction was found to be complete in 15 min and contact time up to 30 min had no effect on the stoichiometry or the results. A 10 mL volume of 5 mM bromate solution in the presence of a large amount of bromide was found adequate for

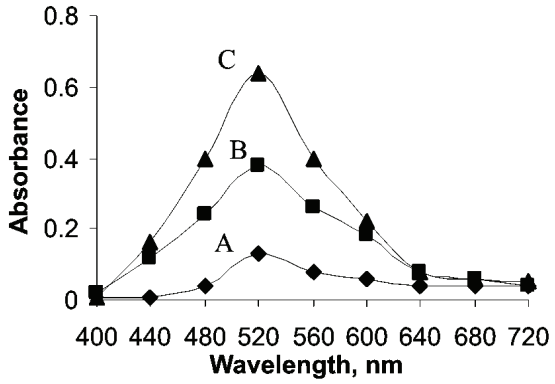

Figure 2. Absorption spectra of methyl orange in the presence of A: 0.25 μg mL^{-1} DCH, B: 0.75 μg mL^{-1} DCH and C: 1.25 μg mL^{-1} DCH.

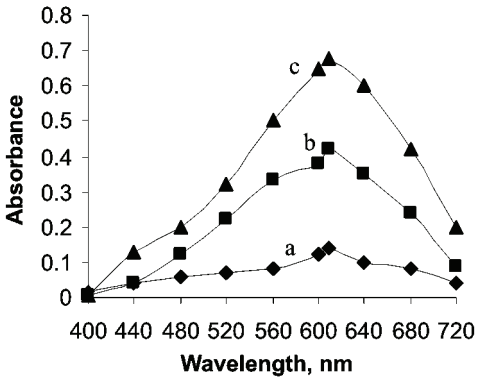

Figure 3. Absorption spectra of indigo carmine in the presence of a: 1.0 μg mL^{-1} DCH, b: 3.0 μg mL^{-1} DCH and c: 5.0 μg mL^{-1} DCH.

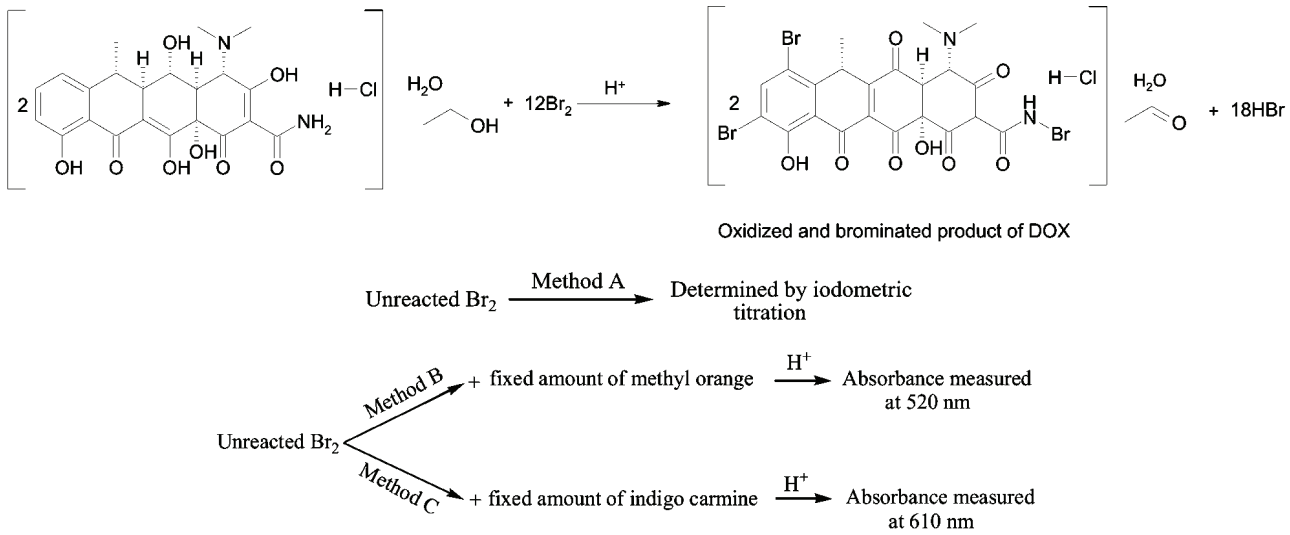

Figure 4. Probable reaction scheme showing the oxidation and bromination of DCH, and determination of in situ generated bromine by titrimetry and spectrophotometric methods.

quantitative bromination of DCH in the range investigated.

Spectrophotometry. Many dyes are irreversibly destroyed to colorless products by oxidizing agents in acid medium [29] and this observation has been exploited for the indirect spectrophotometric determination of some bioactive compounds [30-34]. In the proposed spectrophotometric methods, the ability of bromine to cause bromination of DCH and irreversibly destroy methyl orange and indigo carmine dyes to colorless products in acid medium has been used. Both spectrophotometric methods are based on the bromination of DCH by a measured excess of *in situ* generated bromine and subsequent determination of the unreacted bromine by treating with methyl orange or indigo carmine and measuring the absorbance at 520 nm (Figure 2) or 610 nm (Figure 3). In either method, the absorbance increased linearly with increasing the concentration of DCH (Figure 5).

DCH, when added in increasing concentrations to a fixed concentration of *in situ* generated bromine, consumes the latter proportionately and there will be a concomitant decrease in its concentration. When a fixed concentration of either dye is added to decreasing concentrations of bromine, a concomitant increase in the concentration of dye is obtained. This is observed as a proportional increase in absorbance at the respective λ_{max} with increasing concentration of DCH (Figures 2, 3 and 5).

Preliminary experiments were performed to fix the upper limits of the dye concentrations that could be measured spectrophotometrically, and these were found to be 5 μg mL^{-1} and 20 μg mL^{-1} for methyl orange and indigo carmine, respectively. A bromate concentration of 1.0 μg mL^{-1} was found to irreversibly destroy the red colour of 5 μg mL^{-1} methyl orange whereas 4.5 μg mL^{-1} oxidant was required to bleach the blue colour due to 20 μg mL^{-1} indigo carmine in acid medium. Hence, different concentrations of DCH were reacted with 1.0 mL of 10 μg mL^{-1} bromate in method B and 1.5 mL of 30 μg mL^{-1} oxidant in method C in the presence large excess of bromide and in acid

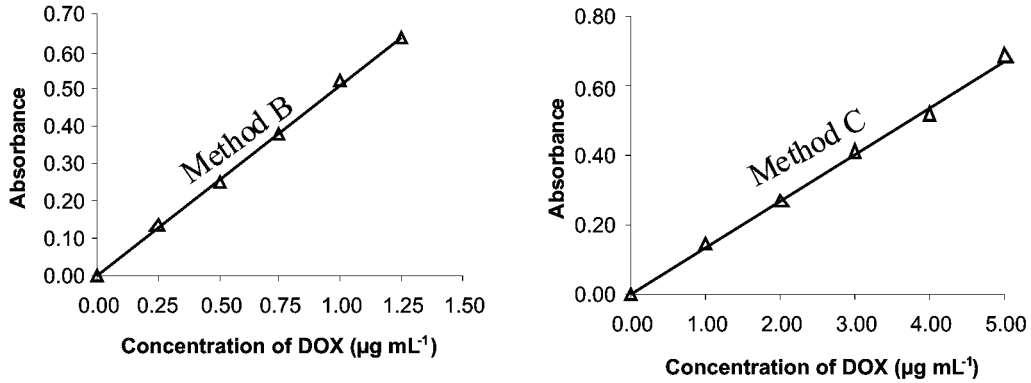

Figure 5. Calibration curves to show the conformity of Beer's law.

medium followed by the determination of the residual bromine as described under the respective procedures.

None of the acids (H_2SO_4, H_3PO_4 and CH_3COOH) showed precise and accurate results than HCl. Therefore, hydrochloric acid was the medium of choice for the bromination of DCH by bromine, as well as the latter's determination employing the dyes. The absorbance of the dyes was not affected in 0.25–1.00 and 0.25-1.5 M hydrochloric acid concentration for method B and C, respectively. However, since 1 mL of 5 M acid in a total volume of about 5.5 and 8.5 mL for method B and C, respectively, was found sufficient to cause bromination of the drug in a reasonable time of 20 and 10 min, respectively, the same concentration (0.5 M overall) was maintained for the determination of unreacted bromine with the dyes. The specified acid concentration for bromination reaction was not critical. The bromination reaction was found to be complete in 20 and 10 min for method B and C, respectively, and contact times up to 60 min had no effect on the absorbance of the dyes. A contact time of 5 min (method B) and 10 min (method C) was necessary for the bleaching of the dye colour by the residual bromine. The absorbance of either dye solution even in the presence of the brominated drug product was found to be stable for more than 48 h under these optimized conditions.

Method validation

Analytical parameters of spectrophotometric methods. A linear correlation was found between absorbance at λ_{max} and concentration of DCH in the ranges given in Table 2. The graphs are described by the regression equation:

$y = a + bx$

where y is absorbance of 1-cm layer of solution, a is intercept, b is slope and x is concentration. The regression analysis of the Beer's law data using the method of least squares was made to evaluate the slope (b), intercept (a) and correlation coefficient (r) for each system and the values are presented in Table 2. A plot of log absorbance and log concentration yielded straight lines with slopes equal to 0.998 and 0.978 for method B and C, respectively, further establishing the linear relation between the two variables. The optical characteristics such as Beer's law limits, molar absorptivity and Sandell sensitivity values [35] of both methods are also given in Table 2. The limits of detection (LOD) and quantitation (LOQ) were calculated according to ICH guidelines [36] using the formula:

$LOD = 3.3S/b$ and $LOQ = 10S/b$

where S is the standard deviation of blank absorbance values and b is the slope of the calibration plot, are also presented in Table 2. The high values of ε and low values of Sandell sensitivity and LOD indicate the high sensitivity of the proposed methods.

Accuracy and precision of the methods

To compute the accuracy and precision, the assays described under "general procedures" were repeated seven times within the day to determine the repeatability (intra-day precision) and five times on five different days to determine the intermediate precision (inter-day precision) of the methods. These assays were performed for three levels of analyte. The results of this study are summarized in Table 3. The percentage relative standard deviation ($\%RSD$) values were ≤ 2.66% (intra-day) and ≤ 2.98% (inter-day) indicating high precision of the methods. The accu-

Table 2. Sensitivity and regression parameters for spectrophotometric methods

Parameter	Method B	Method C
λ_{max} / nm	520	610
Linear range, μg mL^{-1}	0.125-1.25	0.5-5.0
Molar absorptivity (ε), L mol^{-1} cm^{-1}	2.62×10^5	6.97×10^4
Sandell sensitivity[a], μg cm^{-2}	0.002	0.010
Limit of detection (LOD), μg mL^{-1}	0.02	0.091
Limit of quantification (LOQ), μg mL^{-1}	0.07	0.27
Regression equation, y[b]		
Intercept (a)	-0.003	-0.001
Slope (b)	0.516	0.14
Standard deviation of a (S_a)	0.0998	0.10
Standard deviation of b (S_b)	0.1331	0.03
Variance (Sa^2)	9.96×10^{-3}	0.01
Regression coefficient (r)	0.9997	1.000

[a]Limit of determination as the weight in μg per mL of solution, which corresponds to an absorbance of $A = 0.001$ measured in a cuvette of cross-sectional area 1 cm^2 and $l = 1$ cm; [b]$y = a + bx$, where y is the absorbance, x is concentration in μg mL^{-1}, a is intercept and b is slope.

racy was evaluated as percentage relative error (*RE*) between the measured mean concentrations and taken concentrations for DCH. Bias (bias% = (concentration found – known concentration)×100/known concentration) was calculated at each concentration and these results are also presented in Table 3. Percent relative error (*%RE*) values of ≤3% demonstrate the high accuracy of the proposed methods.

Selectivity

In all the three methods, the results of placebo blank and synthetic mixture analyses revealed that the inactive ingredients used in the preparation had no role in the assay of the active ingredient. To study the role of additives added to the synthetic sample, five ml of the resulting solution was assayed (n = 5) by titrimetry which yielded a % recovery of 98.56±0.98. The synthetic mixture analysis by spectrophotometric methods yielded percentage recoveries of 97.56–103.65 with *%RSD* values in the range 1.02–2.53. These results demonstrated the accuracy as well as the precision of the proposed methods and complement the findings of the placebo blank analysis with respect to selectivity.

Robustness and ruggedness

The robustness of the methods was evaluated by making small incremental changes in the volume of acid (method A (6.0 mg DCH): 4.0, 5.0 and 6.0 mL; methods B (1.0 μg mL^{-1}) and C (3.0 μg mL^{-1}): 0.8, 1.0 and 1.2 mL) and contact time (methods A and B: 19, 20 and 21 min; method C: 9.5, 10.5 and 11.5 min) and the effect of the changes was studied on the absorbance of the dye colour. The changes had negligible influence on the results as revealed by small intermediate precision values expressed as *%RSD* (≤2.68%). Method ruggedness was expressed as the RSD of the same procedure applied by four different analysts as well as using three different instruments, (burettes in method A and spectrophotometer in method B and C). The inter-analysts *RSD* were within 2.89% whereas the inter-instruments *RSD* for the same DCH concentrations ranged from 1.99-2.89% suggesting that the developed methods were rugged.

Application to formulations

The proposed methods were applied to the determination of DCH in two representative tablets. The results in Table 4 show that the methods are successful for the determination of DCH and that the excipients in the dosage forms did not interfere. The results obtained (Table 4) were statistically compared with the official BP method [3]. The method involved the determination of DCH by liquid chromatography with UV detection at 350 nm. The results obtained by the proposed methods agreed well with those of the reference method and with the label claim. When the results were statistically compared with those of the reference method by applying the Student's *t*-test for accuracy and *F*-test for precision, the calculated Student's *t*-value and *F*-value [37] at 95% confidence level did not exceed the tabulated values of 2.77 and 6.39, respectively, for four degrees of freedom. Hence, no significant difference exists between the proposed methods and the reference method with respect to accuracy and precision.

Recovery study

To further assess the accuracy of the methods, recovery experiments were performed by applying the standard-addition technique. The recovery was assessed by determining the agreement between the measured standard concentration and added known concentration to the sample. The test was done by spiking the pre-analyzed tablet powder with pure DCH at three different levels (50, 100 and 150% of the content present in the tablet powder (taken) and the total was found by the proposed methods. Each test was

Table 3. Evaluation of intra-day and inter-day accuracy and precision

Method	DCH taken[a]	Intra-day accuracy and precision (n = 7)			Inter-day accuracy and precision (n = 5)		
		DCH found[a] ± *CL*	*%RE*	*%RSD*	DCH found* ± *CL*	*%RE*	*%RSD*
A	2.0	2.03±0.04	1.50	1.88	2.05±0.05	2.50	2.12
	4.0	3.93±0.10	1.75	2.65	4.05±0.14	1.25	2.89
	6.0	6.12±0.14	2.00	2.45	6.10±0.23	1.67	2.98
B	0.50	0.51±0.01	2.00	2.66	0.49±0.10	2.00	1.50
	0.75	0.76±0.01	1.33	1.59	0.76±0.01	1.33	1.44
	1.00	0.99±0.02	1.00	2.48	1.03±0.01	3.00	0.99
C	2.0	2.05±0.04	2.50	2.12	2.04±0.04	2.00	1.53
	3.0	2.96±0.03	1.33	1.26	3.07±0.08	2.33	1.98
	4.0	3.94±0.06	1.50	1.65	4.08±0.11	2.00	2.13

[a]The values are in mg for method A and in μg mL^{-1} for method B and method C. *%RE*: percent relative error, *%RSD*: relative standard deviation, CL: Confidence limits were calculated from: $CL = \pm tS/\sqrt{n}$. The tabulated value of t is 2.45 and 2.77 for six and four degrees of freedom respectively, at the 95% confidence level; S = standard deviation and n = number of measurements

Table 4. Results of the analysis of tablets by the proposed methods and statistical comparison of the results with the reference method

Tablet brand name	Nominal amount, mg/tablet	Found[a] (Percent of label claim±*SD*)			
		Reference method	Proposed methods		
			Method A	Method B	Method C
DOX-T 100	100	98.69±0.98	99.12±1.58	98.14±1.24	97.86±1.34
			t = 0.53	t = 0.78	t = 1.13
			F = 2.60	F = 1.60	F = 1.87
DOXY 100	100	101.3±1.14	102.5±1.44	100.4±2.44	101.8±2.04
			t = 1.47	t = 0.79	t = 0.50
			F = 1.59	F = 4.58	F = 3.20

[a]Average of five determinations; tabulated t value at the 95% confidence level is 2.77; tabulated F value at the 95% confidence level is 6.39

repeated three times. In all the cases, the recovery percentage values ranged between 95.87 and 106.3% with relative standard deviation in the range 1.09-–2.38%. Closeness of the results to 100% showed the fairly good accuracy of the methods. The results are shown in Table 5.

co-formulated substances is far less than that at shorter wavelengths employed in most reported methods, as evident from Table 1.

Table 5. Results of the recovery study using a standard addition method

Tablet studied	Method A				Method B				Method C			
	DCH in tablet extract mg	Pure DCH added mg	Total DCH found mg	Pure DCH recovered (percent± SD[a])	DCH in tablet extract μg mL^{-1}	Pure DCH added μg mL^{-1}	Total DCH found μg mL^{-1}	Pure DCH recovered (percent± SD[a])	DCH in tablet extract μg mL^{-1}	Pure DCH added μg mL^{-1}	Total DCH found μg mL^{-1}	Pure DCH recovered (percent± SD[a])
DOX 100	2.97	1.5	4.44	98.2±1.1	0.49	0.25	0.74	100.1±1.8	1.96	1.0	2.93	97.4±2.2
	2.97	3.0	5.98	100.3±1.7	0.49	0.50	1.01	104.7±2.4	1.96	2.0	3.88	95.9±2.0
	2.97	4.5	7.37	97.8±1.4	0.49	0.75	1.26	102.6±1.8	1.96	3.0	4.95	99.6±2.9
DOXY 100	3.08	1.5	4.60	101.5±1.7	0.50	0.25	0.75	100.1±2.2	2.04	1.0	3.05	100.5±1.8
	3.08	3.0	6.17	103.1±1.9	0.50	0.50	1.03	106.3±1.9	2.04	2.0	4.09	102.3±2.1
	3.08	4.5	7.84	105.7±1.4	0.50	0.75	1.28	103.7±2.7	2.04	3.0	5.13	103.1±1.6

[a]Mean value of three determinations

CONCLUSIONS

Three useful methods for the determination of DCH using bromate-bromide mixture, methyl orange and indigo carmine have been developed and validated according to ICH guidelines. The proposed titrimetric procedure is far simpler than the published method [26] since it is free from critical working conditions and does not use any expensive instrumentation. The proposed spectrophotometric methods do not require any expensive equipment and specialized technicians when compared alongside luminescence spectrophotometry [5,6], chromatography [7-13], potentiometric [16] and electrophoretic [17] methods. The proposed methods are one of the most sensitive ever reported for DCH and are much simpler than the existing spectrophotometric methods with respect to optimum conditions. They rely on the use of simple and inexpensive chemicals. An additional advantage of the methods is that the measurement is made at longer wavelengths where the interference from the

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PAVAGADA
JAGANNATHAMURTHY
RAMESH
KANAKAPURA BASAVAIAH
MYSORE RANGANATH
DIVYA
NAGARAJU
RAJENDRAPRASAD
KANAKAPURA
BASAVAIAH VINAY

Department of Studies in Chemistry, Manasagangothri, University of Mysore, Mysore, India

NAUČNI RAD

TITRIMETRIJSKO I SPEKTROFOTOMETRIJSKO ODREĐIVANJE DOKSICIKLIN HIKLATA KORIŠĆENJEM BROMAT-BROMIDA, METIL ORANŽA I INDIGO KARMINA

U ovom radu su opisane jedna titrimetrijska i dve indirektne spektrofotometrijske metode za određivanje doksiciklin hiklata (DCH) u sirovini i formulacijama. Kao reagense, ove metode koriste bromat-bromid, metil oranž i indigo karmin. U titrimetriji (metoda A), DCH se tretira poznatim viškom smeše bromata I bromida u kiseloj sredini, a rezidualni brom se retitriše jodometrijski nakon što je reakcija između DCH i broma završena. U spektrofotometrijskim metodama višak broma se određuje tretiranjem određenom količinom metil oranža (metoda B) ili indigo-karmina (metoda C) i merenjem promene apsorbance na 520, odnosno 610 nm. Titrimetrijska metoda je primenjiva u opsegu od 1-8 mg, a izračunavanja su zasnovana na stehiometrijskom odnosu DCH i bromata 1:2. U spektrofotometrijskim metodama B i C su kalibracioni dijagrami bili linearni u rasponima 0,25--1,25 i 1-5 μg/ml, respektivno, a odgovarajuće vrednosti molarne apsorptivnosti su bile $2{,}62\times10^5$ *i* $6{,}.97\times10^4$ *L mol*$^{-1}$ *cm*$^{-1}$*. Tačnost i preciznost ovih ispitivanja je određena izračunavanjem intra-dnevnih i inter-dnevnih varijacija za tri različita sadržaja DCH.*

Ključne reči: doksiciklin; određivanje; boje; spektrofotometrija; lekovi.