

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

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## **ABSTRACT**

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 1-5  $\mu\text{g mL}^{-1}$  for method B and method C, respectively, with corresponding molar absorptivity values of  $2.62 \times 10^5$  and  $6.97 \times 10^4 \text{ L mol}^{-1} \text{ cm}^{-1}$ . 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.

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## INTRODUCTION

Doxycycline hyclate (DCH), (4S,4aR,5S,5aR,6R,12aS)-4-(dimethylamino)-3,5,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide monohydrochloride, compound with ethyl alcohol (2:1), monohydrate, (fig 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 for the determination of DCH in pharmaceutical dosage forms including fluorimetry [5], phosphorimetry [6], liquid chromatography [7-11], thin layer chromatography [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<sub>2</sub>O<sub>2</sub> [24] and multivariate calibration method [25] have also been reported by different workers.

The chromatographic techniques are the 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 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 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* 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.

### *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 5mM  $KBrO_3$ -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  $1000 \mu\text{g mL}^{-1}$   $\text{KBrO}_3$  solution containing a large excess of  $\text{KBr}$  was prepared by dissolving 100 mg of  $\text{KBrO}_3$  and 1 g of  $\text{KBr}$  in water and diluting to the mark in a 100 mL calibrated flask. This was diluted stepwise to get  $10 \mu\text{g mL}^{-1}$  and  $30 \mu\text{g mL}^{-1}$  bromate solutions for use in method B and method C, respectively.

A 0.03 M sodium thiosulphate ( $\text{Na}_2\text{S}_2\text{O}_3$ ) (S.d. Fine Chem Ltd, Mumbai, India) solution was prepared in water and standardized [27]. Hydrochloric acid ( $\text{HCl}$ ) (S.d. Fine Chem, Mumbai, India, Sp gr 1.18) (2M and 5M), potassium iodide ( $\text{KI}$ ) (Merck, India) (10%), methyl Orange (S.d. Fine Chem, India, dye content 85%) ( $50 \mu\text{g mL}^{-1}$ ), indigo carmine (S.d. Fine Chem, Mumbai, India, dye content 90%) ( $200 \mu\text{g mL}^{-1}$ ) 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 (Micro labs Ltd, Mumbai, India) used in the investigation were purchased from local commercial sources.

#### *Doxycycline hyclate standard solution*

A  $1 \text{ mg mL}^{-1}$  standard drug solution was prepared by dissolving 250 mg of pharmaceutical grade DCH in water, the volume was made upto 250 mL in a calibrated flask with water and was used in titrimetry. This solution was then diluted with water to get  $5 \mu\text{g mL}^{-1}$  and  $10 \mu\text{g mL}^{-1}$  solutions for use in method B and method 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 upto 10 mL with water. The solution was acidified by adding 5 mL of 2 M  $\text{HCl}$ . Ten mL of bromate-bromide solution (5 mM w.r.t  $\text{KBrO}_3$ ) 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 formula:

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

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

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

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

### *Spectrophotometry*

#### *Method B*

Different aliquots (0.0-2.5 mL) of  $5 \mu\text{g mL}^{-1}$  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 were added 1 mL each of bromate-bromide solution ( $10 \mu\text{g mL}^{-1}$  w. r. t.  $\text{KBrO}_3$ ) and 5 M hydrochloric acid. The content was mixed well and let stand for 20 min with occasional shaking. Then 1 mL of  $50 \mu\text{g mL}^{-1}$  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.

#### *Method C*

Varying aliquots of standard DCH solution (0.0-5.0 mL;  $10 \mu\text{g mL}^{-1}$ ) 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 ( $30 \mu\text{g mL}^{-1}$  w.r.t.  $\text{KBrO}_3$ ) were added. After mixing the content, the flasks were allowed to stand for 10 min with occasional shaking. Then, 1 mL of  $200 \mu\text{g mL}^{-1}$  indigo carmine solution was added to each flask and diluted to the mark with water. The absorbance was measured at 610 nm against a reagent blank after 10 min.

In method B and method C, a calibration graph was prepared by plotting absorbance versus concentration of 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 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. 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 method B and method C, the tablet solution ( $1000 \mu\text{g mL}^{-1}$  in DCH) was diluted appropriately with water to get 5 and  $10 \mu\text{g mL}^{-1}$  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 solution made as described under "analysis of dosage forms". A convenient aliquot of solution was subjected to analysis by titrimetry (method A) and spectrophotometry (method B and method 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 ( $1000 \mu\text{g mL}^{-1}$  in DCH) was appropriately diluted to get 5 and  $10 \mu\text{g mL}^{-1}$  solutions, and appropriate aliquots were subjected to analysis by method B and method C, separately.

## RESULTS AND DISCUSSION

The determination of DCH is based on 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 (fig 2) or blue colour of indigo carmine at 610 nm (fig 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 two moles of bromate (equivalent to 6 moles of bromine) are consumed in the reaction, two moles of bromine are believed to have been used up for the oxidation of the phenoli-OH groups at 5<sup>th</sup> and 12<sup>th</sup> positions of tetracene, two for the bromination at 7<sup>th</sup> and 8<sup>th</sup> positions, one mole 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 fig 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<sub>3</sub>). 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<sub>2</sub>SO<sub>4</sub>, H<sub>3</sub>PO<sub>4</sub> and CH<sub>3</sub>COOH). 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 quantitative bromination of DCH in the range investigated.

### *Spectrophotometry*

Many dyes are irreversibly destroyed to colourless 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 colourless 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 (fig 2) or 610 nm (fig 3). In either method, the absorbance increased linearly with increasing concentration of DCH (fig 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  $\lambda_{\max}$  with increasing concentration of DCH (fig 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  $5 \mu\text{g mL}^{-1}$  and  $20 \mu\text{g mL}^{-1}$  for methyl orange and indigo carmine, respectively. A bromate concentration of  $1.0 \mu\text{g mL}^{-1}$  was found to irreversibly destroy the red colour of  $5 \mu\text{g mL}^{-1}$  methyl orange whereas  $4.5 \mu\text{g mL}^{-1}$  oxidant was required to bleach the blue colour due to  $20 \mu\text{g mL}^{-1}$  indigo carmine in acid medium. Hence, different concentrations of DCH were reacted with 1.0 mL of  $10 \mu\text{g mL}^{-1}$  bromate in method B and 1.5 mL of  $30 \mu\text{g mL}^{-1}$  oxidant in method C in the presence large excess of bromide and in acid medium followed by the determination of the residual bromine as described under the respective procedures.

None of the acids ( $\text{H}_2\text{SO}_4$ ,  $\text{H}_3\text{PO}_4$  and  $\text{CH}_3\text{COOH}$ ) 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 method 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 method C, respectively, was found sufficient to cause bromination of 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 method 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 hours under these optimized conditions

### ***Method Validation***

#### *Analytical parameters of spectrophotometric methods*

A linear correlation was found between absorbance at  $\lambda_{\text{max}}$  and concentration of DCH in the ranges given in Table 2. The graphs are described by the regression equation:

$$Y = a + bX$$

(where Y = absorbance of 1-cm layer of solution; a = intercept; b = slope and X = concentration in  $\mu\text{g mL}^{-1}$ ). 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 method 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) calculated according to ICH guidelines [36] using the formulae:

$LOD = 3.3 S/b$  and  $LOQ = 10 S/b$ , (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  $\epsilon$  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  $\leq 2.66\%$  (intra-day) and  $\leq 2.98\%$  (inter-day) indicating high precision of the methods. Accuracy was evaluated as percentage relative error (RE) between the measured mean concentrations and taken concentrations for DCH. Bias {bias % = [(Concentration found - known concentration) x 100 / known concentration]} was calculated at each concentration and these results are also presented in Table 3. Percent relative error (%RE) values of  $\leq 3\%$  demonstrate the high accuracy of the proposed methods.

#### *Selectivity*

In all the three methods, results of placebo blank and synthetic mixture analyses revealed that the inactive ingredients used in the preparation had no role in the assay of 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  $98.56 \pm 0.98$ . 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; method B (1.0  $\mu\text{g mL}^{-1}$ ) and method C (3.0  $\mu\text{g mL}^{-1}$ ) : 0.8, 1.0 and 1.2 mL] and contact time (method A and method 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 ( $\leq 2.68\%$ ). 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 method 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 consisted that 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 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 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.

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

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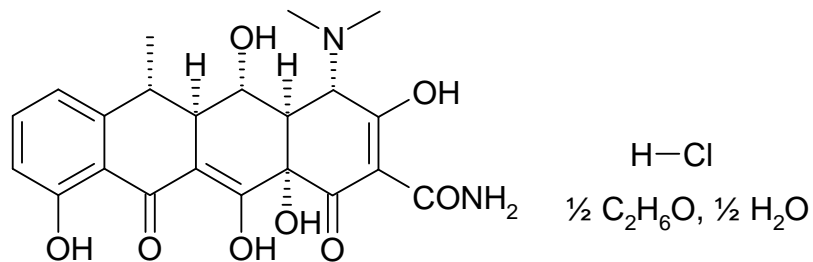
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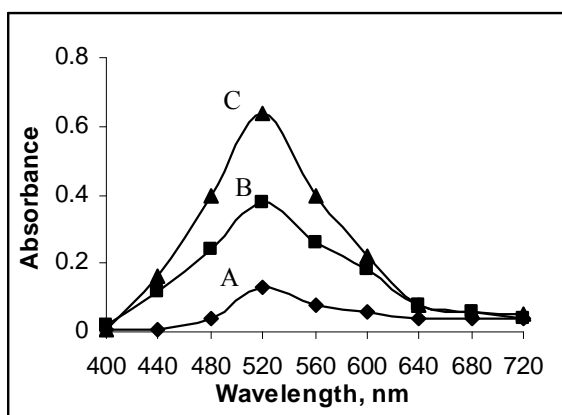
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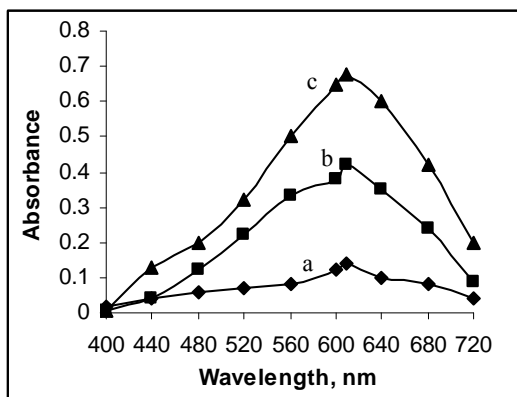
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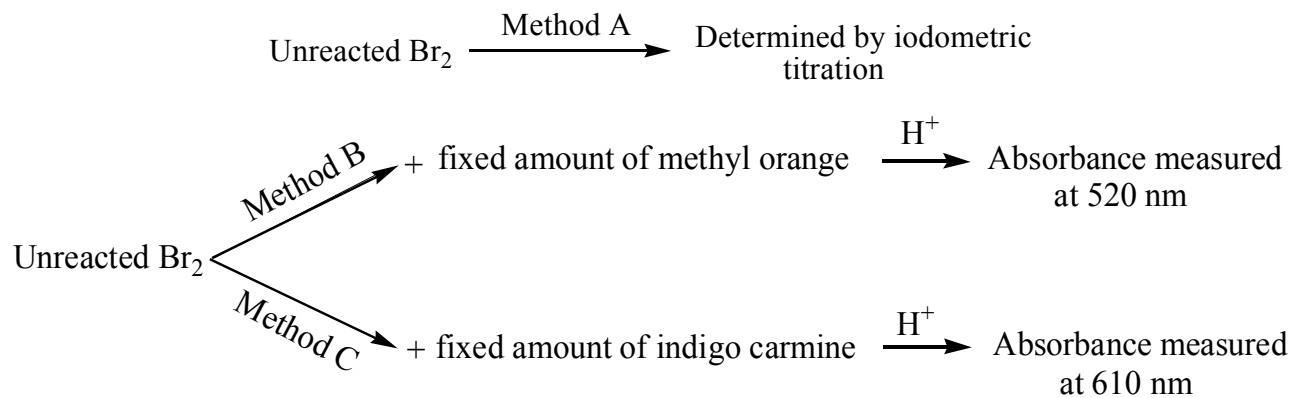
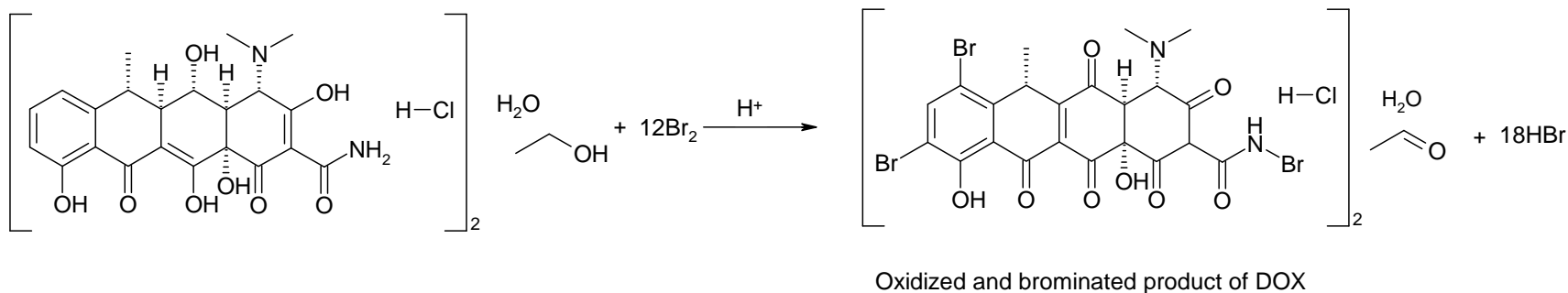
**Figure 1.** Molecular structure of DCH.



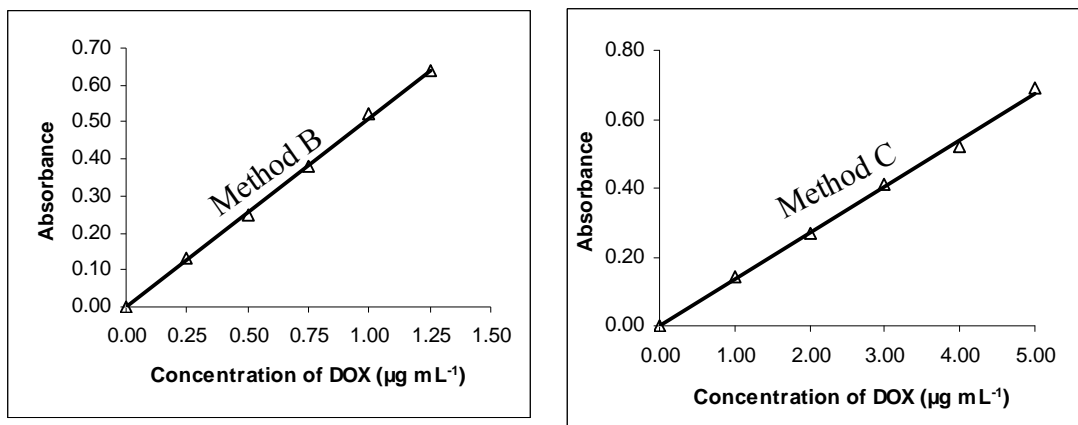
**Figure 2.** Absorption spectra of methyl orange in the presence of A:  $0.25 \mu\text{g mL}^{-1}$  DCH; B:  $0.75 \mu\text{g mL}^{-1}$  DCH and C:  $1.25 \mu\text{g mL}^{-1}$  DCH.



**Figure 3.** Absorption spectra of indigo carmine in the presence of a:  $1.0 \mu\text{g mL}^{-1}$  DCH; b:  $3.0 \mu\text{g mL}^{-1}$  DCH and c:  $5.0 \mu\text{g mL}^{-1}$  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.



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

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

Sl. No.	Reagent/s used	Methodology	$\lambda_{\max}$ (nm)	Linear range	LOQ ( $\mu\text{g mL}^{-1}$ )	Remarks	Ref.
1	a) Copper carbonate	Complex colour measured	395	10.0-80.0 $\text{mg mL}^{-1}$	-	FIA assembly required and least sensitive	18
	b) Chloramine-T	Oxidation of drug in alkaline medium and red coloured product measured	525	$5.37 \times 10^{-5}$ to $7.16 \times 10^{-4}$ $\text{mol L}^{-1}$	-	FIA assembly required and least sensitive	19
	c) 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 $\mu\text{g mL}^{-1}$	-	pH dependent and narrow linear range	21
3	Sodium cobaltnitrite and acetic acid	Colour forming reaction	243	0.01-0.03 $\text{mg mL}^{-1}$	-	Heating required. Less sensitive	22
4	Uranyl acetate-DMF medium	1:1 Complex formation reaction	405	0-135 $\mu\text{g mL}^{-1}$	-	Requires organic solvent, less sensitive	23
5	Cu(II)/H <sub>2</sub> O <sub>2</sub> -alkaline medium	Degradation study	510	2.97-17.78 $\mu\text{g mL}^{-1}$	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 $\mu\text{g mL}^{-1}$	-	Require special equipment, Use of organic solvent, pH dependant	25
7	a) KBrO <sub>3</sub> -KBr /HCl and methyl orange	<b>Bromination of drug and determination of unreacted Br<sub>2</sub> with methyl orange</b>	520	0.25-1.25 $\mu\text{g mL}^{-1}$	0.07	<b>Highly sensitive, non-stringent optimum conditions used, simple instrument employed.</b>	<b>Present work</b>
	b) KBrO <sub>3</sub> -KBr /HCl and indigo carmine	<b>Bromination of drug and determination of unreacted Br<sub>2</sub> with indigo carmine</b>	610	0.5-5.0 $\mu\text{g mL}^{-1}$	0.27		

Table 2. Sensitivity and regression parameters for spectrophotometric methods.

Parameter	Method B	Method C
$\lambda_{\max}$ , nm	520	610
Linear range, $\mu\text{g mL}^{-1}$	0.125-1.25	0.5-5.0
Molar absorptivity( $\epsilon$ ), $\text{L mol}^{-1} \text{cm}^{-1}$	$2.62 \times 10^5$	$6.97 \times 10^4$
Sandell sensitivity*, $\mu\text{g cm}^{-2}$	0.002	0.010
Limit of detection (LOD), $\mu\text{g mL}^{-1}$	0.02	0.091
Limit of quantification (LOQ), $\mu\text{g mL}^{-1}$	0.07	0.27
Regression equation, $Y^{**}$		
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 ( $S_a^2$ )	$9.96 \times 10^{-3}$	0.01
Regression coefficient (r)	0.9997	1.000

\*Limit of determination as the weight in  $\mu\text{g}$  per mL of solution, which corresponds to an absorbance of  $A = 0.001$  measured in a cuvette of cross-sectional area  $1 \text{ cm}^2$  and  $l = 1 \text{ cm}$ .

\*\* $Y=a+bX$ , Where Y is the absorbance, X is concentration in  $\mu\text{g mL}^{-1}$ , a is intercept, b is slope.

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

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

\*The values are in mg for method A and  $\mu\text{g mL}^{-1}$  for method B and method C.

%RE. Percent relative error, %RSD. relative standard deviation and 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 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* (Percent of label claim $\pm$ SD)			
		Reference method	Proposed methods		
			Method A	Method B	Method C
DOX-T 100	100	98.69 $\pm$ 0.98	99.12 $\pm$ 1.58	98.14 $\pm$ 1.24	97.86 $\pm$ 1.34
			<b>t</b> = 0.53	<b>t</b> = 0.78	<b>t</b> = 1.13
			<b>F</b> = 2.60	<b>F</b> = 1.60	<b>F</b> = 1.87
DOXY 100	100	101.3 $\pm$ 1.14	102.5 $\pm$ 1.44	100.4 $\pm$ 2.44	101.8 $\pm$ 2.04
			<b>t</b> = 1.47	<b>t</b> = 0.79	<b>t</b> = 0.50
			<b>F</b> = 1.59	<b>F</b> = 4.58	<b>F</b> = 3.20

\*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.

Table 5. Results of recovery study using 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*)	DCH in tablet extract, µg mL <sup>-1</sup>	Pure DCH added, µg mL <sup>-1</sup>	Total DCH found, µg mL <sup>-1</sup>	Pure DCH recovered (Percent±SD*)	DCH in tablet extract, µg mL <sup>-1</sup>	Pure DCH added, µg mL <sup>-1</sup>	Total DCH found, µg mL <sup>-1</sup>	Pure DCH recovered (Percent±SD*)
DOX 100	2.97	1.5	4.44	98.24±1.09	0.49	0.25	0.74	100.1±1.76	1.96	1.0	2.93	97.38±2.15
	2.97	3.0	5.98	100.3±1.26	0.49	0.50	1.01	104.7±2.36	1.96	2.0	3.88	95.87±1.95
	2.97	4.5	7.37	97.84±1.42	0.49	0.75	1.26	102.6±1.85	1.96	3.0	4.95	99.62±2.38
DOXY 100	3.08	1.5	4.60	101.5±1.26	0.50	0.25	0.75	100.1±2.15	2.04	1.0	3.05	100.5±1.78
	3.08	3.0	6.17	103.1±1.38	0.50	0.50	1.03	106.3±1.92	2.04	2.0	4.09	102.3±2.08
	3.08	4.5	7.84	105.7±1.44	0.50	0.75	1.28	103.7±2.26	2.04	3.0	5.13	103.1±1.56

\*Mean value of three determinations.