

Development and Validation of Extractive Spectrophotometric Method for Estimation of Hydroxychloroquine Sulphate by using Smartphone Application

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Abstract



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A simple, sensitive, accurate, precise and reproducible extractive spectrophotometric method was developed for estimation of Hydroxychloroquine Sulphate (HCQS) in bulk and pharmaceutical dosage form. The method is based on formation of ion pair complex of Hydroxychloroquine Sulphate with Bromocresol green dye in acidic medium. This complex extracted in chloroform and measured quantitatively at 421nm for estimation of Hydroxychloroquine Sulphate present in the sample. In this method estimation was also done by using Smartphone Application known as PhotoMetrix pro. Beer Lambert's law obeyed over a range of 2-10 µg/ml for HCQS by UV spectrophotometric ($r^2=0.9988$) and by SmartPhone Image analysis ($r^2=0.999$) method. The developed method was successfully applied for estimation of Hydroxychloroquine Sulphate in three commercial product tablet dosage form without interference from common additives. The assay was found to be 100.09%, 100.8%, 98.6% by UV and 99.2%, 101.46%, 101.1% using PhotoMetrix for three formulation containing Hydroxychloroquine Sulphate. The developed method was also validated according to ICH Q2 guideline for linearity, range, accuracy, and precision. Comparison for both method UV and PhotoMetrix done by performing t-test, result shows that there is no significant difference between both method so PhotoMetrix can be used as alternative to UV for routine analysis of Hydroxychloroquine Sulphate.

Keywords: Hydroxychloroquine Sulphate, Bromocresol green, Potassium Hydrogen Phthalate buffer, UV spectrophotometric, PhotoMetrix.

INTRODUCTION

Hydroxychloroquine Sulphate (HCQS) is a derivative of Chloroquine, which is a class of drug named as 4-amino quinoline. HCQS was first synthesized in 1946 by hydroxylation of Chloroquine and found to be 40% less toxic than Chloroquine¹. Chemically it is 2-((4-((7-chloroquinoline-4-yl) amino) pentyl) (ethyl)amino) ethanol sulfate. Structure of HCQS is given as in

Figure 1². It is mainly used for Prevention and treatment of malaria, but its anti-inflammatory activity has been used for treatment of rheumatoid arthritis and systemic lupus erythematosus. Absorption of this drug increase pH of vacuoles of parasite which interfere with vesicle function and development and asexual reproduction of the parasite and also inhibit growth of parasite by interfering with conversion of toxic heme which is produced by digestion of hemoglobin to non-toxic hemozoin. HCQS interfere with lysosomal activity, interact with membrane stability and alter signaling pathway that results inhibition of cytokine production which makes it useful in rheumatoid arthritis³. Also, it is noted that HCQS is a less toxic derivative of CQ, effective in inhibiting SARS-COV-2 infection in-vitro⁴.

Literature survey has revealed methods for estimation HCQS are UV spectrophotometric⁵, Liquid chromatography –Mass spectrometry⁶, RP-HPLC for estimation in bulk⁷, HPLC method for estimation of HCQ and its metabolites in blood⁸, stability

indicating HPLC⁹, liquid chromatography – tandem mass spectrometry¹⁰, HPLC with fluorescence detector¹¹, fast ultra-high HPLC-fluorescent method for estimation in biological fluid¹², Ion pairing HPLC, Gas chromatography-Mass spectrometry(GC-MS) in biological fluid¹³, Thermal and non-thermal techniques for compatibility study of HCQS with excipients¹⁴.

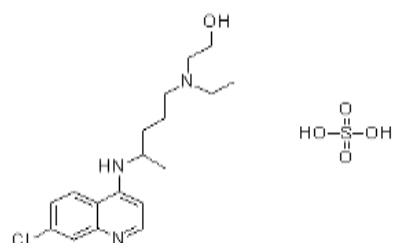


Figure 1: Structure of Hydroxychloroquine Sulphate (HCQS)

Various analytical methods are reported for estimation of HCQS in pharmaceutical dosage form and biological samples.

A colorimetric analysis is a simple spectrophotometric analysis used to figure out how much of a colored component is present in a solution. Colored compounds absorb light in the visible spectrum, according to Beer-Lambert's Law the amount of light

absorbed is proportional to the concentration of the component in the solution ¹⁵.

In this study a simple, precise, accurate and sensitive Extractive spectrophotometric method for estimation of HCQS by using Bromocresol green (BCG) dye which is followed by extraction of complex in organic solvent. Also, the developed method was extended to estimation by SmartPhone application. For this purpose, Mobile application called Photometrix, which employs techniques of simple linear correlation for univariate analysis was used. This application is available free in Google Play Store. The method is based on measurement of signal intensities relative to concentration of sample.

MATERIALS AND METHODS

Apparatus and software

Shimadzu UV-1700 double beam spectrophotometer connected to a computer having Shimadzu UV-Probe 2.10 software installed was used for all the spectrophotometric measurements. The absorbance spectra of the reference and test solution were carried out in 1cm quartz cells over the range of 400-800 nm. The samples are weighted on an electronic balance (Ax120) by Shimadzu. Smart Phone having PhotoMetrix application was used to capture images.

Chemicals and Reagents

Hydroxychloroquine sulphate (API) was supplied by Mangalam Drugs and organic Ltd as a gift sample. Hydroxychloroquine tablet (formulation 1,2,3) was purchased from local medical store. Bromocresol Green dye (Sulab) of AR grade, sodium Hydroxide pellets (Rankem) of AR grade and potassium hydrogen phthalate (Loba chemicals) of AR grade was used for the experiment.

Preparation of Bromocresol green dye (BCG)

Bromocresol green dye was made as per procedure given in IP ¹⁶. For this 50 mg BCG was added to 100 ml volumetric flask, to that 0.72 ml 0.1M NaOH was added. Followed by addition of 20 ml methanol and after that volume was made up to mark with double distilled water.

Preparation of Phthalate Buffer (pH = 2.5)

Buffer was prepared according to procedure given in IP ¹⁶. 50 ml 0.2 M Potassium dihydrogen Phthalate was added in 200 ml volumetric flask. To that 42.4 ml 0.2M HCl was added was added and volume was made up to 200 ml with double distilled water.

Diluent

As HCQS is freely soluble in water, double distilled water was used as a diluent throughout the experiment.

All reagents used in experiments were analytical grade.

Preparation of standard stock solution

10 mg HCQS was weighed accurately and transferred to 10 ml volumetric flask and dissolved in double distilled water and volume was made up to 10 ml with double distilled water. From this 1 ml taken to 10 ml volumetric flask and volume was made up to 10 ml double distilled water to get concentration of 100 PPM. From this aliquot of 0.2, 0.4, 0.6, 0.8, 1 were taken to get final concentration of 2, 4, 6, 8, 10 ppm.

Preparation of sample solution

20 tablets of formulation 1 (200 mg), 2 (300 mg), 3 (400 mg) were accurately weighed and powdered separately. An amount of powder equivalent to label claim of each tablet was weighed and transferred to 100 ml volumetric flask. To that double distilled water added to dissolve powder and sonicated for 10 minutes. Then solution was made up to the mark and filtered.

Further dilution was made to get final concentration of 100 ppm of Hydroxychloroquine Sulphate.

Selection of wavelength for HCQS

For preliminary test, to 3 ml of stock solution 2.5 ml phthalate buffer of pH = 3 and 1 ml of BCG dye were added. This solution was transferred to separating funnel in which 5 ml chloroform was added. After shaking chloroform layer turns to yellow from transparent. Absorbance of organic layer was taken in the region of 400 to 800 nm. The maximum absorbance was found at 421 nm.

Optimization of colorimetric method

Volume of Dye:

To 10 ml of standard working solution, 5 ml of phthalate buffer of pH = 3 and different volumes of dye (0.8 to 1.8 ml) were added in separating funnel. 10 ml chloroform was added and shaking for 1 minute. Followed by separation and measurement of organic layer at 421 nm against blank. Blank was prepared as same manner instead of HCQS solution double distilled water was used in procedure. The result showed that highest absorbance was obtained in 1.6 ml of volume of dye. (Figure 2)

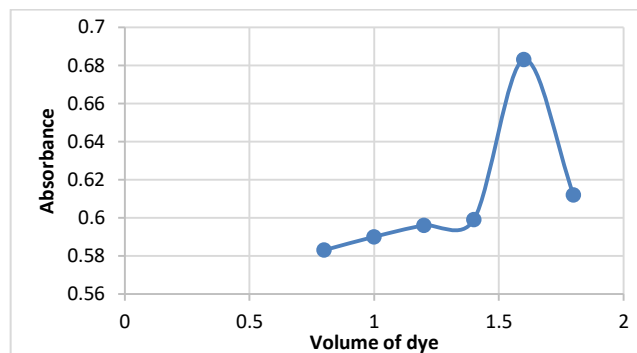


Figure 2: Optimization of Volume of Dye

pH of Buffer:

To 10 ml of standard working solution, 5 ml of different buffer pH ranging from 2 to 3.5 were added, followed by addition of 1.6 ml of BCG dye. To that 10 ml chloroform was added and shaking for one minute and separating chloroform layer scanned in the 400 to 800 nm. Result shows maximum absorbance found at 2.5 pH of buffer (Figure 3).

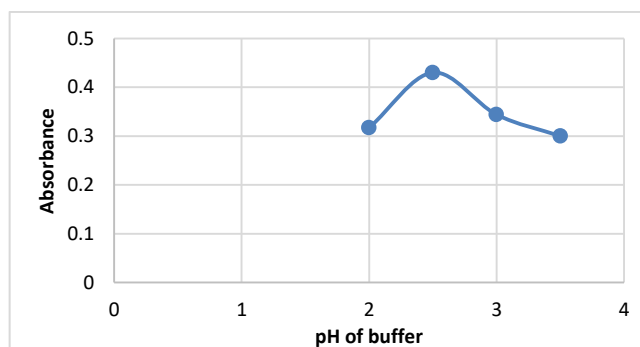


Figure 3: Optimization of pH of buffer

Optimization of reaction time:

For optimization of reaction time experiment is performed for 1 to 5 minutes. It was found that reaction time has not much significant effect on the result of experiment. So, 1 minute was taken as an optimum shaking time and it was found that the addition of the dye solutions resulted in an immediate full color development. Color of the complex is stable for more than 24 hours.

The Table 1 describes optimized value for all three parameters.

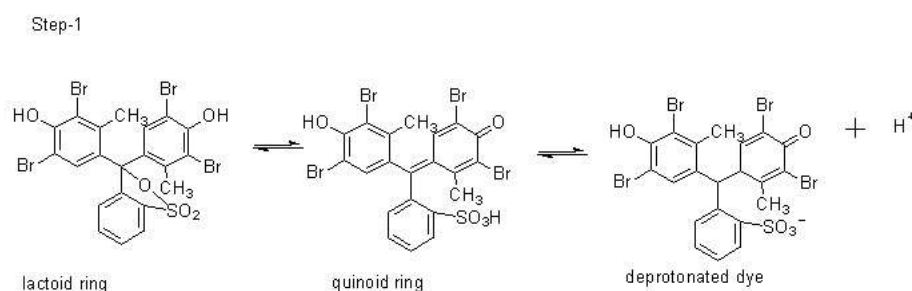
Table 1: value describes optimized condition for colorimetric method

Parameter	Optimized value
Volume of dye	1.6 ml
pH of buffer	2.5
Reaction time	1 minute

Reaction mechanism

Bromocresol green is an anionic dye containing sulphonephthalein group. Color of BCG dye is due to opening of lactoid ring and formation of subsequent quinoid group. Although tautomers are present at equilibrium state but due to acidic nature of sulphonic group, quinoid group (deprotonated) is predominate. Hydroxychloroquine sulphate (HCQS) contains tertiary amino group which is protonated in acidic medium So protonated HCQS forms ion pair complex with deprotonated BCG dye which is quantitatively extracted in chloroform layer. Probable reaction mechanism is illustrated in Figure 4 ¹⁷⁻²⁰.

Step-1 Formation of quinoid ring and deprotonation of dye



Step-2 Formation of Ion-pair complex.

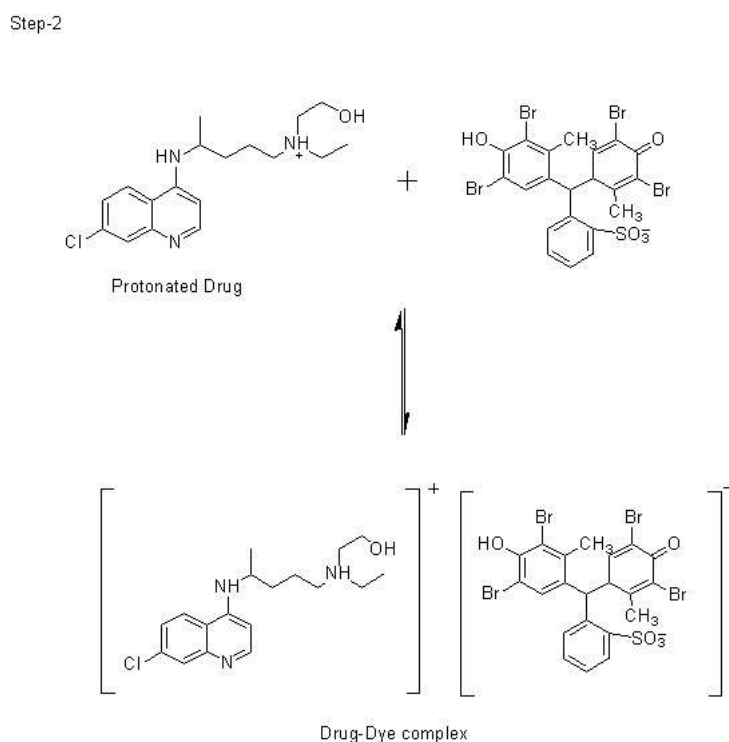


Figure 4: Reaction mechanism

Stoichiometry relationship.

The molar ratio of HCQS to BCG dye in the complex was determined by applying Job's method of continuous variations. In this method, solutions of drug and dye with identical molar concentrations were mixed in varying volume ratios in such a way that the total volume of each mixture was the same. The absorbance of each solution was measured and plotted against the mole fraction of the drug, $[drug]/[drug]+[dye]$ (Figure 5).

In both cases, the plot reached a maximum value at a mole fraction of 0.5 which indicated the formation of 1:1 (HCQS: BCG) complex (Figure 5). Based on this, it was confirmed that only one nitrogen atom in the drug is protonated and through the electrostatic attraction ion pair complex is formed with the negatively charged dye. The formation constant was also estimated and found to be 6.63×10^6 for complex with BCG. ^{17,19}

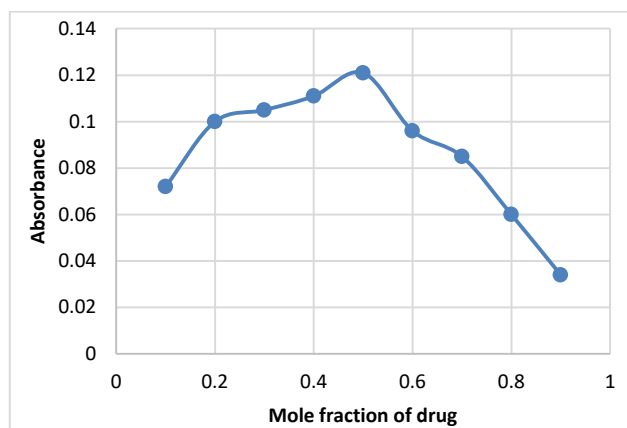


Figure 5: Plot of mole fraction of drug and absorbance

Preparation of calibration graph:

Aliquots were taken from 100 ppm to make 2 to 10 $\mu\text{g}/\text{ml}$ solution of HCQS. Volume was made up to mark with double distilled water. They were transferred to separating funnel, to that 5 ml of phthalate buffer of pH 2.5 and 1.6 ml of BCG dye was added. To that 10 ml chloroform was added followed by shaking for one minute and separation of chloroform layer. Absorbance of chloroform layer measured at a 421 nm against blank. Calibration graph was plotted for absorbance of organic layer against their respective concentration.

Analysis of marketed formulation

From the prepared sample solution of formulation aliquots were taken to prepare 5 $\mu\text{g}/\text{ml}$ solution and treated as same manner given for working standard of HCQS and absorbance was measured at 421 nm. The absorbance of sample was calculated by standard curve method.

Estimation of HCQS using Smart phone application:

Experimental setup:

A self-designed white box was built in the lab to improve accuracy and precision of the measurements. On the upper side of box, the LED bulb was fitted to provide light source. Inner side of box covered by white paper to provide full reflection of the light. Front side made in manner that it can be open to insert cuvette inside the box and in middle of that small square shaped hole was made to allow the camera to capture image of the object placed inside the box. For holding a cuvette, a small cuvette holder was prepared from thermocol and was fixed in the middle of the box.

The experimental setup is illustrated in Figure 6, Figure 7 & Figure 8.

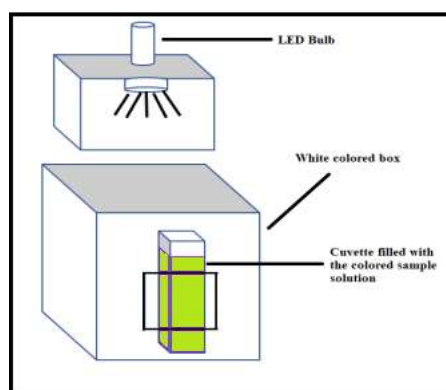


Figure 6: Illustration of experimental setup for image acquisition

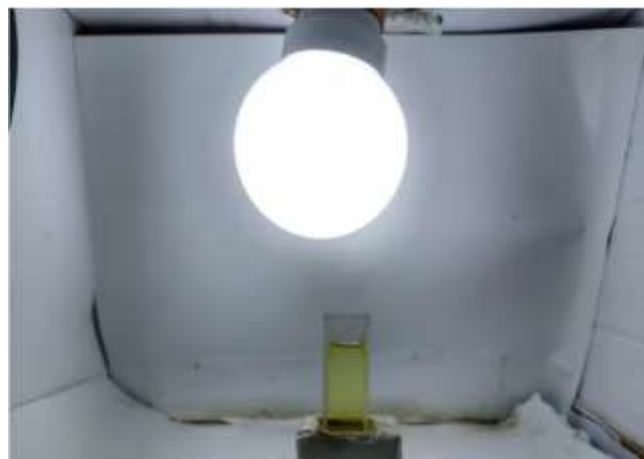


Figure 7: cuvette placed in arranged setup

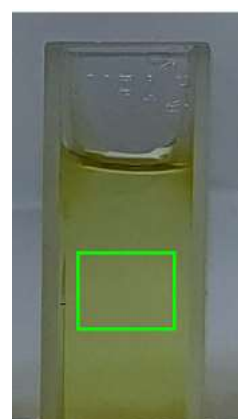


Figure 8: Image captured by mobile phone camera in arranged setup

Preparation of calibration graph by smart phone application:

Working standard solution of 2-10 $\mu\text{g}/\text{ml}$ of HCQS was prepared by taking suitable aliquots from standard stock solution and transferred in 10 ml volumetric flask. Volume was made up to 10 ml with double distilled water. These solutions were transferred in previously cleaned and dried separating funnel and treated as same manner described earlier. Once chloroform layer of all solution separated, images were captured one by one in PhotoMetrix Pro application. Steps to plot calibration graph in application is illustrated in the figure. In application use univariate analysis in which vector RGB was selected, in that calibration option selected. Once you select calibration, app will ask you for number of samples. Here for calibration number of sample was taken is 6 (1 was blank and 5 were working standard). Then first blank solution was filled in cuvette and inserted in the box and image was captured for blank. In the same manner one by one images of all standard working solution were captured in an increasing concentration by providing concentration of sample in the application. Calibration graph and regression equation was provided by application is self.

Once regression equation was obtained, concentration of sample solution from marketed formulation was estimated. For this purpose, instead of calibration, sampling option in univariate analysis was selected and the image of sample solution prepared from marketed formulation was captured in manner similar to standard solution. After saving the data concentration of sample was given by application from generated calibration graph (Figure 9).

Steps for performing calibration by PhotoMetrix

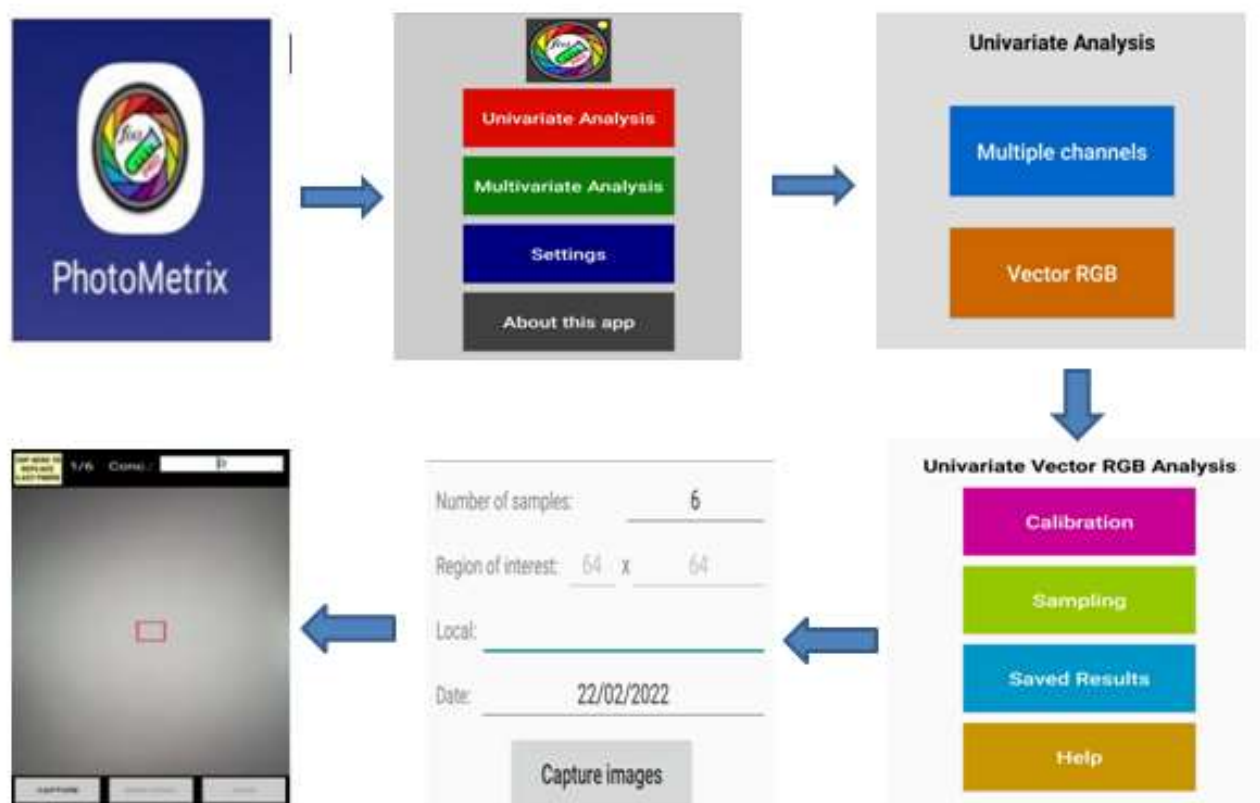


Figure 9: Graphical presentation of PhotoMetrix PRO application and steps to generate calibration graph by application

RESULT AND DISCUSSION

MEHOD VALIDATION:

1. Linearity:

Beer's law obeying in the range of 2-10 µg/ml of HCQS at 421 nm. Linearity graph for HCQS was shown in Figure 10. A calibration graph was plotted between concentration Vs absorbance. The plot was found to be linear as shown in Figure 11.

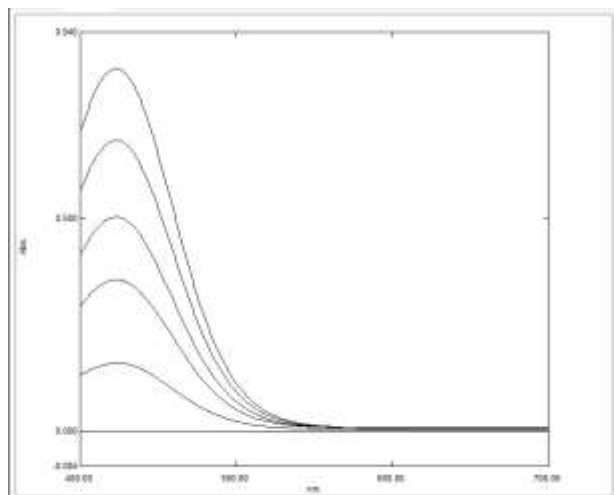


Figure 10: overlay spectra of HCQS (2-10 µg/ml)

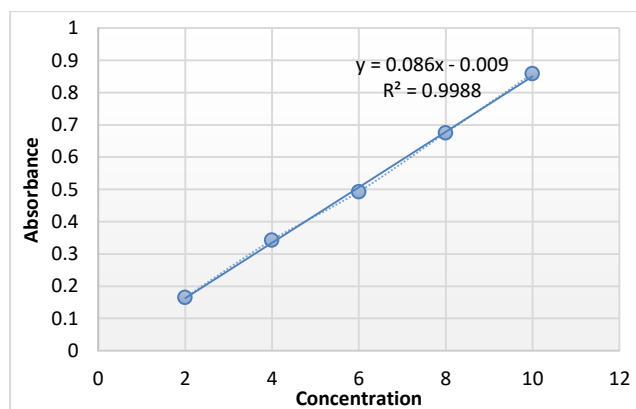


Figure 11: calibration graph of HCQS

2. Precision

The precision of an analytical method expresses the closeness of agreement between a series of measurement, which are obtained by multiple sampling of the same homogenous sample under the given condition of the method. Here the intra-day and inter-day precision was determined. For that concentration of linearity range were taken and analyzed three times on the same day for intra-day precision and for inter-day for inter-day precision at the same concentration level. The %RSD was calculated which are found to be less than 2% as shown in the Table 2.

Intra-day and inter-day precision of HCQS**Table 2: Precision data of HCQS**

	Conc.	Mean± S.D (n=3)	%RSD
Intra-day	2	0.15766±0.003055	1.94
	4	0.34866±0.005131	1.47
	6	0.51466±0.004041	0.79
	8	0.66933±0.007234	1.08
	10	0.78633±0.005859	0.75
Inter-day	2	0.162±0.0026457	1.63
	4	0.34733±0.00585	1.69
	6	0.49166±0.01006	1.24
	8	0.67566±0.00901	1.33
	10	0.85533±0.00351	0.41

3. Accuracy

The accuracy of the method was determined by recovery experiments. A known quantity of pure drug was added to pre-analyzed sample formulation at 80%, 100% and 120%

levels. The recovery studies were carried out and %recovery and % RSD of percentage recovery were calculated which are given in Table 3.

Table 3: Accuracy data for HCQS

Drug	Conc. From formulation(µg/ml)	%Spiked	Standard conc. Added (µg/ml)	Conc. Recovered (µg/ml)	% recovery ± S.D (n=3)	%RSD
HCQS	4	80	3.2	3.24	100.93±1.22202	1.21
	4	100	4	4.01	100.16±1.66667	0.97
	4	120	4.8	4.83	100.5±1.45258	1.45

4. Sensitivity

The limits of detection (LOD) and limit of quantification (LOQ) were calculated using the formulae:

$$\text{LOD} = 3.3 \times \Omega / S$$

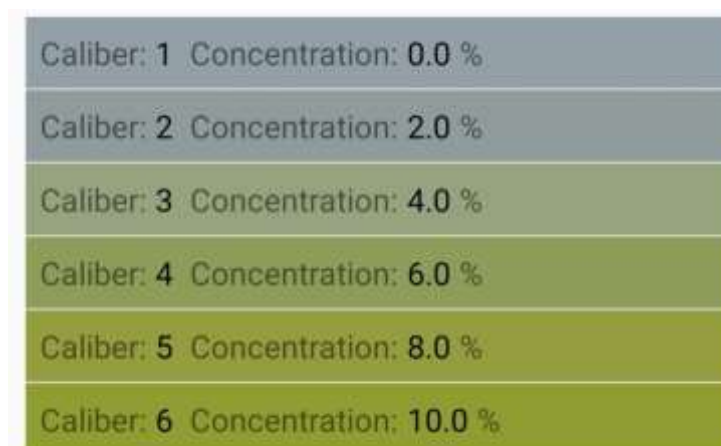
$$\text{LOQ} = 10 \times \Omega / S$$

Where Ω = standard deviation of intercept and S = mean of slope

Values for LOD and LOQ for both methods shown in Table 4.

Estimation of HCQS using Smartphone application:

The linearity of the standard HCQS was taken in the range of 2-10 µg/ml. Gradient given by using an application for the linearity range of HCQS is given as in Figure 12. The calibration curve and regression equation were generated by an equation is shown in Figure 13.

**Figure 12: Gradient of Linearity range of drug given by PhotoMetrix**

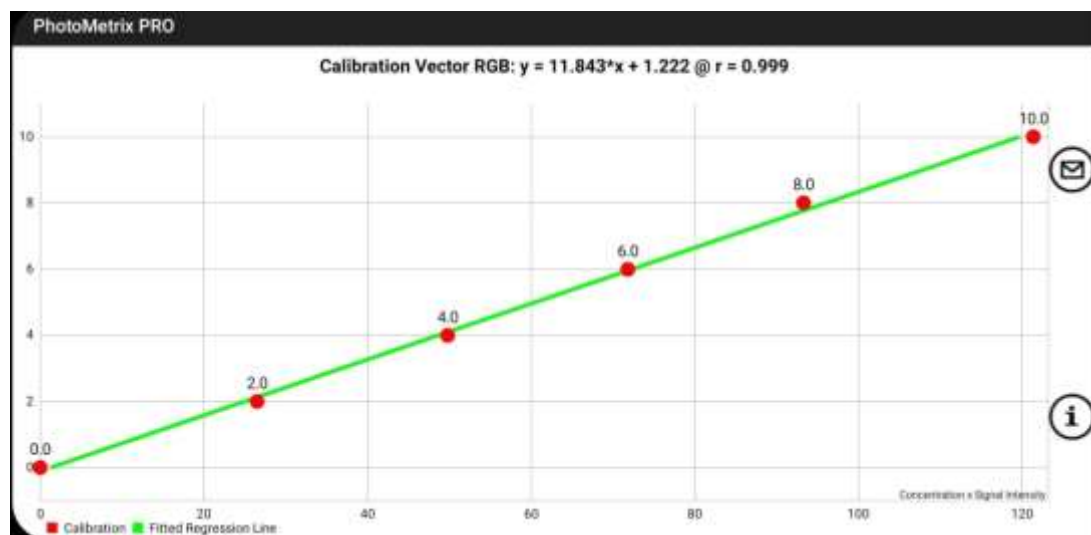


Figure 13: calibration graph given by PhotoMetrix PRO

Table 4: Statistical data for the regression equation of the proposed method for estimation of HCQS using Bromocresol green dye

Parameter	UV	Photometrix
Linearity range (µg/ml)	2-10	2-10
Regression equation	$0.086X - 0.009$	$11.843X + 1.222$
Slope	0.0857	11.685
Intercept	0.0099	1.248
Correlation coefficient	0.9981	0.999
Limit of detection (µg/ml)	0.165	0.105
Limit of quantification (µg/ml)	0.501	0.320

5. Analysis of marketed formulation

The assay of Formulations was found to be 100.09 %, 100.8%, 98.6% by UV and 99.2%, 101.46%, 101.1% by PhotoMetrix which is within the acceptance criteria (98 – 102%). Result for assay is given in table 5.

Table 5: Assay of the marketed formulation by both UV and PhotoMetrix

Marketed Formulation	Method	Conc.(µg/ml)	Conc. Found (µg/ml)	% Recovery±S.D (n=5)	%RSD
1. 200mg tablet	UV	5	5.09	100.09±0.85	0.95%
	PhotoMetrix	5	4.96	99.2±1.2	1.21%
2. 300mg tablet	UV	5	5.004	100.8±1.32	1.31%
	PhotoMetrix	5	5.08	101.46±0.30	0.30%
3. 400mg tablet	UV	5	4.93	98.6±0.73	0.75
	PhotoMetrix	5	5.05	101.1±1.27	1.26

Comparison between UV spectrophotometric method and Smartphone Image Analysis by T -Test

Table 6. The calculated t-value was smaller than the critical t-value, therefore showing no statistical difference at a 95%

confidence level between UV spectrophotometric and Smartphone Image Analysis.

Table 6: t-test table for the comparison of UV spectrophotometric and SmartPhone Application method

	Variable 1	Variable 2
Mean	99.77	99.5
Variance	2.00428	1.516
Observation	6	6
Hypothesized mean difference	0	
df	10	
t-stat	0.352493	
P(T<=t) one tail	0.365892	
t critical one tail	1.812461	
P(T<=t) two tail	0.731785	
t critical two tail	2.228139	

CONCLUSION

A simple, precise, accurate and sensitive method was developed for the estimation of HCQS in bulk and in its Tablet dosage form. The method was validated according to the ICH (Q2) guidelines. The method can be used for the routine analysis of the drugs in their pharmaceutical dosage form. This application can be used as an alternative to sophisticated and high-cost devices in quantitative analysis.

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