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APPLICATION OF SMARTPHONE AND DEVELOPMENT OF COLOUR KIT FOR THE ESTIMATION OF DOXYCYCLINE IN PHARMACEUTICAL DOSAGE FORMS AND ITS COMPARISON WITH A VALIDATED DIFFERENCE SPECTROSCOPIC METHOD BY UNCERTAINTY

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ABSTRACT: In the proposed research paper, a simple, accurate, and precise colorimetric method has been developed for the estimation of doxycycline in bulk and pharmaceutical dosage forms. The proposed method utilizes a smartphone camera and an application instead of the UV spectrophotometer for the measurement of color intensity. This colorimetric method was found to be linear in the range of 10µg/ml to 50µg/ml concentration, with an r^2 value of 0.9968. These results have been then compared with a highly selective difference spectroscopic method developed and validated by us using the principles of uncertainty. When we report the result of a measurement, it becomes obligatory that some quantitative indications of the quality of the results should also be given, which will aid in the assessment of the reliability of the results and, in turn, the method which was used to obtain those results. Without such an indication, the measurement results may not be compared, either amongst themselves or with the reference standard values. Hence, the uncertainty appraisal becomes essential to express the dispersion of the values that could be reasonably attributed to the results. The proposed difference spectroscopic method obeys Beer-Lambert's Law in the concentration range of 10 - 30µg/ml with an r^2 value of 0.999.

INTRODUCTION: Doxycycline Hydrochloride is chemically known as (4*S*,4a*R*,5*S*,5a*R*,6*R*,12a*S*)-4-dimethylamino-1,4,4a,5,5a,6,11,12a-octahydro-3,5, 10,12, 12a pentahydroxy-6-methyl-1,11-dioxonaphthacene-2-carboxamide hydrochloride hemiethanolate hemihydrate[1], as shown in **Fig. 1**. Doxycycline is a broad spectrum antimicrobial substance of the tetracycline class, which inhibits the synthesis of bacterial proteins by binding to the 30S ribosomal subunit, which is only found in bacteria.

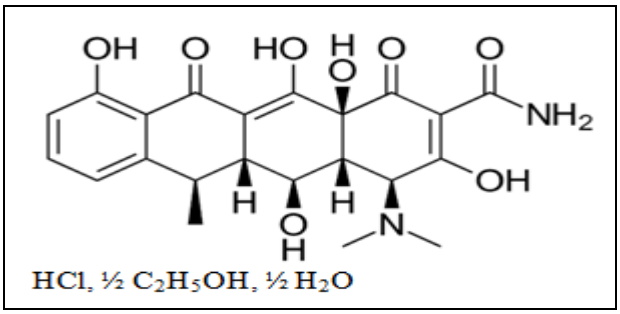

FIG. 1: STRUCTURE OF DOXYCYCLINE HYDROCHLORIDE[2]

After literature review, it was known that numerous methods have already been described for the estimation of Doxycyclines such as visible spectrophotometric methods [3], stability indicating UV spectrophotometric method [4], and RP-HPLC methods [5]. For the determination of doxycycline in human plasma, advanced techniques such as LC-

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MS were also found [6]. The use of a mobile phone instead of the UV spectrophotometer for the detection of the color intensity would be highly beneficial as well as cost-effective.

Here the solutions of sample and calibration curve is made according to the method given below, and a picture is taken of the same, which is then uploaded to a mobile application. The green color intensity, which is provided by the application, is inversely proportional to the concentration of Doxycycline. The green color intensity was chosen here because it has the highest correlation coefficient amongst the various color intensities.

The presented difference spectroscopic method is an attempt to develop a method for the accurate, precise, and selective estimation of Doxycycline in pharmaceutical dosage forms. The principle behind difference spectrophotometric assay is that the measured value is the difference in the absorbance (ΔA) between equimolar solutions of the analyte (Doxycycline), which exhibits different spectral characters in both acidic and basic mediums [7]. By keeping an acidic solution in the reference cell and a basic solution of equimolar concentration in the sample cell, the absorption spectra show maxima and minima at 232 nm and 264 nm, respectively for Doxycycline. The difference in this absorbance at the two wavelengths is measured. This method leads to the enhanced selectivity and accuracy of spectrophotometric analysis of samples containing absorbing interferents.

Simple Colourimetric Method and Colour Kit Application:

Materials and Method:

Apparatus and Applications: The samples were weighed on an electronic balance (A×120) by Shimadzu. A picture of the sample was taken using the camera of a smartphone and uploaded to the mobile application.

Chemicals and Reagents: Ferric Ammonium Sulphate (FAS) solution (5 g of Ferric ammonium sulphate was dissolved in 100 ml of 0.05M H_2SO_4, which shall be stored in a refrigerator to show stability over one month period), 0.01N H_2SO_4 and Distilled Water.

Preparation of Standard Stock Solution: For the preparation of the standard stock solution, 10 mg of Doxycycline was accurately weighed and transferred into a previously calibrated 10 ml volumetric flask. The final volume was made up to the mark using 0.01M H_2SO_4 to obtain the standard stock solution of 1000 µg/ml concentration.

Preparation of Calibration Curve by Application of Smartphone: From the standard stock solution prepared above, aliquots of 0.1 ml, 0.2 ml, 0.3 ml, 0.4 ml, 0.5 ml are taken and transferred to a series of previously calibrated 10ml volumetric flasks. 1 ml of Ferric Ammonium sulphate is added and allowed to stand for 5 min. The volume is then made up to the mark using distilled water to produce concentrations of 10 µg/ml to 50 µg/ml. Against a white background, a clear picture of these solutions is taken using the camera of a smartphone, and the picture is uploaded to the RGB application. This application will provide us with various color intensities. The green color intensity is noted here since it was found to have the maximum correlation coefficient with the concentration of doxycycline. This color intensity is then plotted against the concentration to get the calibration curve.

Preparation of Sample Solution: A quantity equivalent to 100 mg of Doxycycline was weighed accurately and transferred to a 100 ml volumetric flask. This was dissolved in 0.01M H_2SO_4 and made up to the mark using the same. Take any volume (x) in between 0.1 ml to 0.5 ml from this solution was taken into a 10 ml volumetric flask, add 1ml of Ferric Ammonium Sulphate and allow to stand for 5 min. Volume was made up to the mark using distilled water. A picture of this solution is then taken and uploaded to the application and the green color intensity is noted, and the amount of Doxycycline may be directly quantified using the equation (1).

mg of Doxycycline = Green intensity obtained from application-y intercept / Slope × volume taken(x) …… (1)

Optimization Procedure: The effect of changes in the Molarity of H_2SO_4 in the range of 0.03M to 0.07M was performed, keeping the other parameters constant. It was seen that there was an increase in the assay results from 0.03M to 0.05M after which the effect of molarity had come to a saturation point. Similarly, the effect of increasing the concentration of Ferric Ammonium Sulphate

from 0.1503M to 0.2255M (4g FAS in 100ml to 6g FAS in 100ml) was done. Here, it was seen that there was a linear increase in the results with the increase in the concentration of FAS from 0.1503M to 0.1879M after which there was only a slight increase in the results up to 0.2255M. It can be seen from **Fig. 2A** below that the optimum conditions are achieved when 0.05M H_2SO_4 is used and 0.1879M FAS (5g FAS in 100ml) is used in the estimation of Doxycycline.

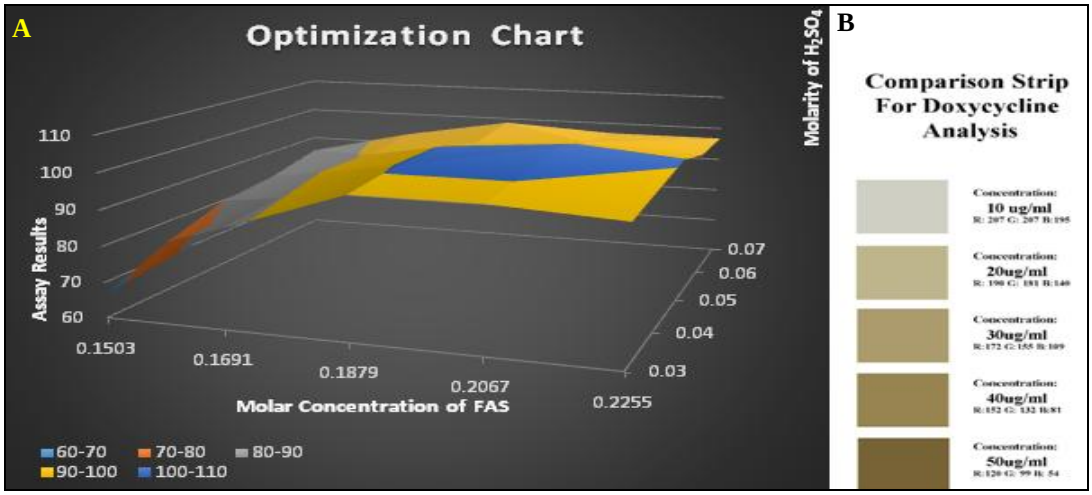

FIG. 2: A. OPTIMIZATION CHART SHOWING THE OPTIMUM CONDITIONS FOR THE COLOURIMETRIC ESTIMATION OF DOXYCYCLINE. B. COMPARISION STRIP

Application of Colour Kit for the simple and quick estimation of Doxycycline: A simple color kit was prepared by us in which a solution of Ferric Ammonium Sulphate has been provided in a dropper bottle. To use this kit, one has to only dissolve the content of a capsule or crush a single tablet of Doxycycline and dissolve the content in 100ml of 0.01M H_2SO_4. From this, the user may accurately measure a quantity of their choice in between 0.1ml to 0.5ml and transfer this into the 10ml volumetric flask. Add 16 drops of FAS from the dropper bottle provided to this volumetric flask and allow it to stand for 5 minutes. Make up the volume to the mark of the volumetric flask using distilled water. The color which is produced may be easily compared with the color strip **Fig. 2B,** which is provided in the color-kit or by using the method above of the smartphone application.

TABLE 1: RUGGEDNESS OF METHOD

S. no.	Parameter	Mean Assay	Standard Deviation	%RSD
1	Lab 1	100.88	0.605	0.603
2	Lab 2	99.67		
3	Smartphone 1	100.88	0.165	0.163
4	Smartphone 2	100.55		

Study of Method Ruggedness: The above method is comparatively new for the analysis of pharmaceutical dosage forms, and hence the method ruggedness study was performed. The ruggedness of the developed method was studied in two labs as well as with the use of two different smartphones. The % RSD of both these parameters was found to be less than 2, as shown in **Table 1**.

Difference Spectroscopic Method:

Materials and Method:

Apparatus and Software/Applications: Shimadzu UV-1700 double beam spectrophotometer connected to a computer with Shimadzu UV-Probe 2.10 software installed was used for all the spectrophotometric measurements. The samples were weighed on an electronic balance (A×120) by Shimadzu.

Chemicals and Reagents: Distilled water was used as the initial solvent, whereas further dilutions were made using 0.1N HCl and 0.1N NaOH.

Preparation of Standard Stock Solution: For the preparation of the standard stock solution 10mg of Doxycycline was accurately weighed and transferred into a previously calibrated 10ml volumetric flask. Distilled water was added to the volumetric flask to dissolve the standard drug and sonicated for 5 min. The final volume was made up to the mark with distilled water to obtain the standard stock solution of 1000 µg/ml concentration.

Preparation of Working Standard Solution: From the standard stock solution of Doxycycline, a working standard solution is prepared by transferring 2.5 ml of the standard stock solution in a previously calibrated 25ml volumetric flask and making up the volume with distilled water to achieve a concentration of 100 µg/ml.

Preparation of Calibration Curve of Standard Doxycycline: From the standard working solution of 100 µg/ml concentration, aliquots of 1 ml, 1.5 ml, 2 ml, 2.5 ml, and 3 ml were withdrawn and transferred to a series of previously calibrated 10 ml volumetric flasks (Series A). The volume of this series was made up to the mark using 0.1N HCl to produce concentrations of 10 µg/ml, 15 µg/ml, 20 µg/ml, 25 µg/ml and 30 µg/ml respectively. Similarly, from the working standard aliquots of 1 ml, 1.5 ml, 2 ml, 2.5 ml, 3 ml were taken again and transferred to another series of previously calibrated 10 ml volumetric flasks (Series B). The volume of this series was made up to the mark using 0.1N NaOH to achieve the same concentrations of 10 µg/ml, 15 µg/ml, 20 µg/ml, 25 µg/ml and 30 µg/ml respectively. The Difference absorbance spectrum of the above concentrations are taken by placing the acidic series of solutions (Series A) in the reference cell of the UV Spectrophotometer and the series of basic solutions (Series B) of equimolar concentration in the sample cell. The absorbance spectrum formed this way will show a maxima peak at 232 nm and a minima peak at 265 nm wavelengths, which are recorded for each concentration. The difference in the absorbance (ΔA) between the two wavelengths is noted and plotted against the concentration to obtain the calibration curve. The regression equation may thus be generated to ensure the linearity of the method.

Preparation of Sample Solution: A quantity equivalent to 100 mg of Doxycycline was weighed accurately and transferred to a 100 ml volumetric flask. This was dissolved using sufficient distilled water and then sonicated for 5 min. The volume was made up to the mark using distilled water to get a concentration of 1000 µg/ml. 1 ml of the above solution was further diluted up to 10 ml (100 µg/ml). 2 ml of this solution was diluted up to 10 ml (20 µg/ml) and the absorbance was taken at both the wavelengths of maxima and minima. The difference in the absorbance (ΔA) is directly proportional to the concentration of Doxycycline, which can be obtained from the calibration curve.

Method Validation:

Total Error Approach: In general, any measurement has imperfections, which lead to some amount of error in the ultimate result. These errors may be broadly divided into two components, namely random error, and systematic error. Random errors are unpredictable and are caused by unknown factors, whereas the systematic errors are quantifiable and sometimes be corrected by a correction factor [8]. Both these errors cannot be nullified but may be reduced by increasing the number of observations of the same result. Regardless of taking into consideration these errors, some degree of uncertainty still remains that the measured value is completely true. Hence, a neoteric approach to method validation has been taken upon here to validate the developed difference spectroscopic method.

Estimation of Uncertainty: According to the Guide to the Expression of uncertainty in measurement the definition of Uncertainty is, "A parameter which is associated with the result of a measurement that characterizes the dispersion of the values that could reasonably attributed to the measurand". [9] The overall uncertainty may be calculated by taking into account the uncertainty of the individual sources. These sources are expressed best in the form of a cause-effect diagram, as shown below in **Fig. 3.** The parameters which are taken into consideration are the volume of the volumetric flasks used (V_{10}), the concentration of the analyte (C_{10}), the precision of the method, mass (m_{sample}), and recovery of the method (R_m). From these parameters, an equation (2) is depicted below.

$$Doxy_{sample} = C * V_{10}\,10^{-3}/m_{sample} * R_m \quad \ldots (2)$$

Where $Doxy_{sample}$ is the doxycycline quantity in (mol/kg), C is the concentration of the analyte (M), V_{10} is the volume of 10ml volumetric flask, m_{sample} is the sample mass taken (kg), R_m Recovery of the method. After the consideration of all these parameters individually, the effect on the overall uncertainty was studied by calculated the Combined Standard Uncertainty and the Expanded Uncertainty.

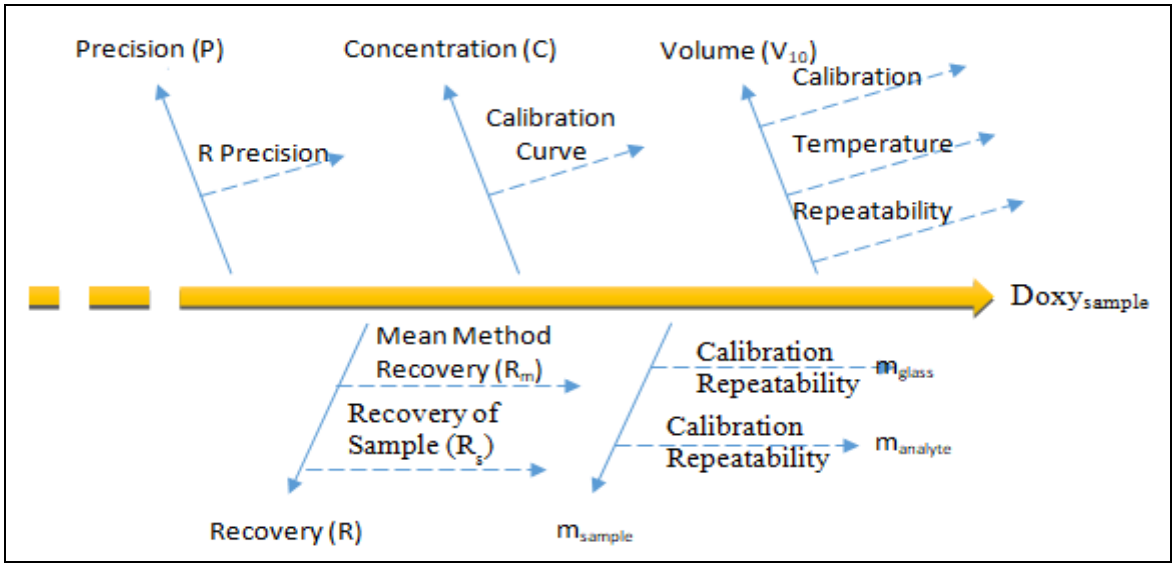

FIG. 3: CAUSE AND EFFECT DIAGRAM IDENTIFYING THE SOURCES OF UNCERTAINTY

Discharge of Solution from Volumetric Flask: The uncertainty due to the discharge of the sample from the volumetric flask is quantified by weighing a 10ml volumetric flask with the standard solution 10 times.

Concentration: The uncertainty in the concentration of the sample is obtained from the calibration curve using the equation (3) shown below.

$$U_{(c)} = \frac{s_r}{b}\sqrt{\frac{1}{n}+\frac{1}{p}+\frac{(c-\bar{c})^2}{s_{xx}}} \quad \ldots (3)$$

Where,

$$S_r = \sqrt{\sum_{j=1}^{n}\frac{[Y_j-(bxi+a)]}{n-2}} \quad \ldots (4)$$

and

$$S_{xx} = \sum(c_i-\bar{c})^2 \quad \ldots (5)$$

Here S_r is the standard deviation of residuals, n is the number of measurements used in the calibration curve, b is the slope of the calibration curve, p is the number of measurements used to obtain the concentration of the unknown sample, C is the concentration of the sample, $\bar{C}$ is the average of the standard concentration, Y_j is the analytical signal response obtained from measurements, j depicts the index of a number of measurements made in order to obtain the calibration standard, i is the index fro number measurements for calibration, a is calibration curve intercept. The Doxycycline sample solution was measured ten times, and the concentration was back-calculated from the regression equation.

Precision: The overall precision uncertainty measurement of the method was estimated by taking into consideration the data of repeatability and variability, which was carried out during the validation of the method.

Recovery of Method (R_m): Uncertainty parameter related to the recovery of the method is calculated using the equation (6) given below.

$$U(R_m) = R_m * \sqrt{\left(\frac{{S_{obs}}^2}{n*{C_{obs}}^2}\right)+\left(\frac{U(C_{spike})}{C_{spike}}\right)^2} \quad \ldots (6)$$

Where,

$$R_m = \frac{C_{obs}}{C_{spike}} \quad \ldots (7)$$

U (C_{spike}) here is the standard uncertainty in the concentration of the spiked samples, and C_{obs} is the number of replicates of the spiked samples, C_{spike} is the nominal concentration of drug in the spiked sample, S_{obs} is the standard deviation results from the replicate analysis of the spiked samples and n is the number of replicates.

Mass of the Sample (m_{sample}): The sample mass was calculated by finding the difference between the weight of the glass with and without the sample.

RESULTS AND DISCUSSION:

Calibration Curve (Response Function): In this developed method, 4 sets of calibration curves were plotted between the difference in the absorbance (ΔA) and concentration of Doxycycline, which follows Beer's Lambert Law in the specified range.

On each of these four calibration curves, regression analysis was performed, and the calibration curve with the best co-efficient of assurance (r^2 value) was selected, as shown in **Fig. 4** and **Fig. 5**.

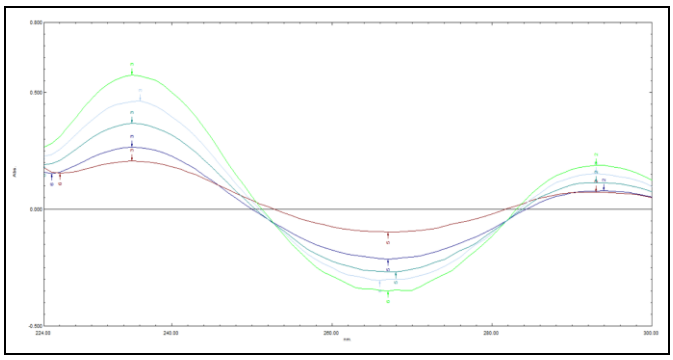

FIG. 4: DIFFERENCE SPECTROSCOPY GRAPH FOR DOXYCYCLINE

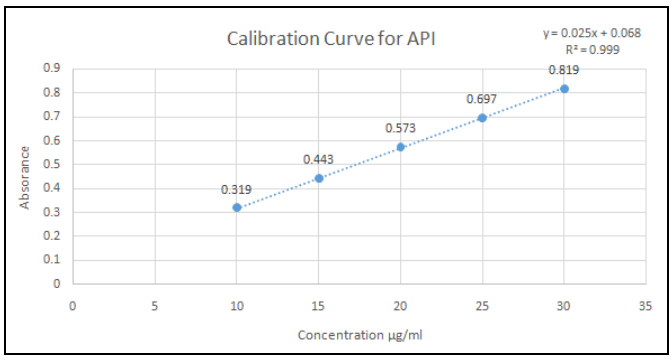

FIG. 5: CALIBRATION CURVE FOR DOXYCYCLINE USING DIFFERENCE SPECTROSCOPY

The equation of this calibration curve was used to calculate the residual values. Residual value may be defined as the deviation in the mean calculated result (from the regression equation), and the actual result found.

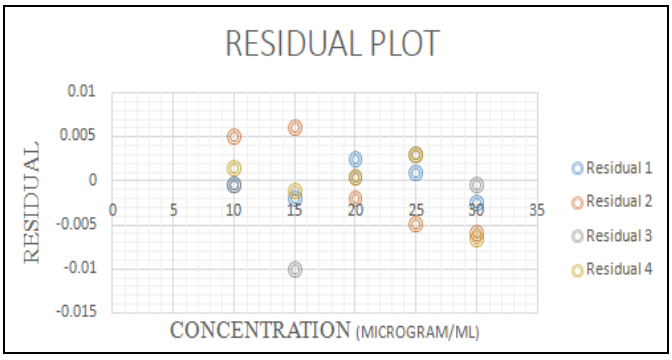

FIG. 6: RESIDUAL PLOT AGAINST CONCENTRATION

These residuals were plotted in the residual plots against the concentration, as presented in **Fig. 6**.

Trueness: According to the ISO, the trueness of any analytical procedure represents the closeness between the average value of the repeated measurements and an accepted reference value [10]. In the developed method, the trueness of the calibration curves was calculated by back-calculation of the concentration using the regression equation. The absolute, as well as the relative bias, were calculated between the average results of a particular concentration obtained by back-calculations and its actual concentration. A 95% confidence interval was also found for the same and is presented in **Table 2**.

TABLE 2: RESULTS OF TRUENESS IN TERMS OF RELATIVE BIAS (%)

S. no.	Concentration (µg/ml)	Mean Back Calculated Concentration	Absolute Bias	Relative Bias	Recovery	95% Confidence Interval of Recovery
1	10	10.045	0.0458	0.4581	100.458	100.458 ± 0.0799
2	15	14.856	-0.1434	-0.9561	99.043	99.043 ± 0.1690
3	20	20.015	0.0159	0.0796	100.079	100.079 ± 0.0552
4	25	25.045	0.0458	0.1832	100.183	100.183 ± 0.07989
5	30	29.836	-0.1633	-0.5444	99.455	99.455 ± 0.10143

Precision: The precision of an analytical method expresses the closeness of agreement between a series of measurements which are obtained by performing multiple samplings of the same homogenous sample under the given conditions of method [9]. In this method, precision was analyzed at two levels: the first being repeatability (precision under the same operating conditions over a short interval of time), and the second is intermediate precision (variations in the results obtained on different days). The results of this precision data are expressed in terms of relative standard deviation (% RSD). Along with this, the 95% upper confidence limits were also determined. Both relative and absolute intermediate precision and repeatability are presented in **Table 3** shows the precision of the difference spectroscopic method. The precision of the method was analyzed in tablets and capsules of doxycycline by taking the assay of the same concentration six times to study intraday precision. Inter-day precision was studied over a period of three days, were-in each day, the sample was analyzed thrice. The results of intraday and inter-day precision are shown in **Table 4**.

TABLE 3: RESULTS OF RELATIVE AND ABSOLUTE INTERMEDIATE PRECISION AND REPEATABILITY

Conc. (µg/ml)	Relative Intermediate Precision and Repeatability				Absolute Intermediate Precision and Repeatability	
	Repeatability (%RSD)	Intermediate Precision (%RSD)	95% Upper Confidence Limit			
			Repeatability (UCL)	Intermediate Precision (UCL)	Repeatability (SD)	Intermediate Precision (SD)
10	0.735	0.7754	0.3257	03278	0.00235	0.00249
15	0.7731	0.2686	0.4478	0.447	0.00339	0.00163
20	0.2859	0.5806	0.575	0.5754	0.00163	0.00329
25	0.2949	0.3592	0.7017	0.7004	0.00205	0.00249
30	0.3048	0.2076	0.8244	0.8224	0.00249	0.00169

TABLE 4: INTRADAY AND INTERDAY PRECISION

Capsule	Intra-day Precision		Inter-day Precision	
	Assay ± SD	%RSD	Assay ± SD	%RSD
	99.017 ± 0.0028	0.5069	98.596 ± 0.0022	0.4021
Tablet	**Intra-day Precision**		**Inter-day Precision**	
	Assay ± SD	%RSD	Assay ± SD	%RSD
	99.050 ± 0.0018	0.3293	98.795 ± 0.0025	0.4581

Accuracy: Accuracy is one of the most important parameters in the validation of the method. The results of the accuracy studies are represented in terms of β-expectation tolerance limits. Recovery studies were conducted using the standard addition method by taking a nominal concentration of 10µg/ml and spiking this solution by 80%, 100%, and 120% with standard API, as shown in **Table 5**. These recovery studies gave us the total error of the test results, which are expressed below in the form of β-expectation tolerance limits. The risk profile was also found, keeping the maximum risk level at 5% and is presented below in **Table 6**.

TABLE 5: RESULTS OF ACCURACY STUDIES

	%Spiked	Average Recovery ± SD (n=3)	%RSD
Tablet	80	99.69 ± 0.00216	0.416
	100	99.61 ± 0.00189	0.331
	120	98.76 ± 0.00326	0.531
Capsule	80	99.32 ± 0.00265	0.507
	100	99.54 ± 0.00249	0.438
	120	98.94 ± 0.00244	0.398

TABLE 6: RESULTS OF ACCURACY OF METHOD AND RISK ANALYSIS

S. no.	% Spiked	B-Expectation Tolerance Interval (µg/ml)	90% Confidence Interval	Relative B-Expectation Tolerance Interval (%)	% Risk
1	80	[17.595, 18.306]	[17.805, 18.096]	[-2.250, 1.704]	0.006314
2	100	[19.660, 20.278]	[19.843, 20.095]	[-1.698, 1.392]	0.004936
3	120	[21.596, 22.247]	[21.788, 22.054]	[-1.835, 1.123]	0.004724

Limits of Detection and Quantification: The results of Limit of Detection and Limit of Quantification depict that this difference spectroscopic method for Doxycycline is sufficiently sensitive to use for analysis. These limits were found using the standard deviation of the y-intercepts and the average of the slopes of three separate calibration curves. The limit of detection was found to be 0.8431µg/ml, and the limit of Quantification was found to be 2.5548 µg/ml.

Robustness: Robustness of the developed difference spectroscopic method is represented below in the form of % RSD. This parameter was analyzed by making small yet deliberate changes in the Molarity of the solvents used for difference spectroscopy. The results are shown below in **Table 7** justify that even if there is a variation of 0.01M concentration of HCl or NaOH, the results have a negligible difference, which is depicted by the % RSD, which is found to be within range.

TABLE 7: RESULTS OF ROBUSTNESS STUDIES

S. no.	Molarity of HCl and NaOH	Nominal Concentration (µg/ml)	Mean Back Calculated Concentration (µg/ml)	Standard Deviation	%RSD
1	0.09M	20	19.697	0.0659	0.333
2	0.1M	20	19.843		
3	0.11M	20	19.830		

Ruggedness: The ruggedness parameter of validation was performed by changing the analysts, which justifies that the method is rugged even if there is a change in the analysts. The results of ruggedness are represented below in **Table 8** and it is evident from the RSD which is less than 2 that the method passes ruggedness.

TABLE 8: RESULTS OF RUGGEDNESS STUDIES

S. no.	Analysts	Nominal Concentration (µg/ml)	Mean Back Calculated Concentration (µg/ml)	Standard Deviation	%RSD
1	Analyst 1	20	20.069	0.111	0.558
2	Analyst 2	20	19.989		
3	Analyst 3	20	19.803		

Uncertainty Measurements: After the identification of the sources which lead to uncertainty of measurements, their individual estimation was done and represented below. All these calculations were done in the SI units to ensure minimal error in the results.

Discharge of the Solution from the Volumetric Flask: The sources of uncertainty when we discuss the discharge of sample solution from the volumetric flask are mainly from the below three factors.

Calibration of the Volumetric Flask: The nominal volume of a volumetric flask is 10ml, and the deviation from this volume is ± 0.005 ml (at 27°). Here the value of standard uncertainty is calculated by the use of the triangular distribution, as shown below.

$$u(V_{10cal}) = \frac{0.005}{\sqrt{6}} = 2.04 \times 10^{-3} ml$$

Repeatability: The measurement uncertainty from repeatedly filling and finding the weight of the volumetric flask the standard uncertainty of the volumetric flask was found to be 1.4×10^{-3}

Temperature: The temperature during calibration of the volumetric flask was 27°, whereas the temperature while preforming the validation procedures varied by ± 5°. This variation may be overcome by taking into consideration the temperature difference and the volume dilation coefficient.

Volume expansion of liquid is taken into consideration here by the calculation given below, where the volume expansion coefficient of water is 2.1×10^{-4} /°C.

$$\Delta V_{10} = V_{10} \times \Upsilon \times \Delta t = 10 \times 2.1 \times 10^{-4} \times 5 = 0.0105 ml$$

Assuming that the temperature variation is expressed as a rectangular distribution, the standard uncertainty for the 10 ml volumetric flask is calculated as below.

$$u(V_{10temp}) = \frac{\Delta V_{10}}{\sqrt{3}} = \frac{0.0105}{\sqrt{3}} = 0.0606 ml$$

Thus, the standard uncertainty due to discharge of solution from 10 ml volumetric flask was calculated as shown below.

$$u(V_{10}) = \sqrt{(u(V_{10cal}))^2 + (u(V_{10rep}))^2 + (u(V_{10temp}))^2} = \sqrt{0.003678} = 0.06064 ml$$

Relative Standard Uncertainty will thus be,

$$\frac{u(V_{10})}{V_{10}} = 6.064 \times 10^{-3}$$

Uncertainty due to Repeatability (Precision): The uncertainty parameter which is added due to the repeatability, is in terms of RSD. So directly the uncertainty due to repeatability is given as:

$$\text{Uncertainty}_{(\text{Repeatability})} = \text{RSD}$$

$$U_{(\text{Rep})} = 0.00314$$

Uncertainty due to Concentration: The data of six calibration curves over a range of five different concentrations were taken in the measurement of uncertainty due to concentration. The sample was analyzed ten times to ensure minimum error. S_r and S_{xx} were found from the equations (3, 4, and 5), and the standard uncertainty was also calculated.

$$U_{(c)} = \frac{s_r}{b}\sqrt{\frac{1}{n} + \frac{1}{p} + \frac{(c-\bar{c})^2}{s_{xx}}}$$

Where, $S_r = \sqrt{\frac{\sum_{j=1}^{n}[Y_j - (bxi+a)]}{n-2}}$

And $S_{xx} = \sum(c_i - \bar{c})^2$

$U_{(c)}$ = 2.905×10^{-11}, and Relative Standard Uncertainty = 1.45×10^{-3}

Uncertainty due to Recovery of the Method: From the results of the validation studies as represented above the uncertainty may be calculated. The recovery of the analyte from the sample and the recovery of the spike may differ, leading to a source of error in the results which must be determined. Thus the analysis of the uncertainty is needed in such cases. The simple recovery may be calculated by the help of equations (6) and (7) shown below, depending on the spiked and the recovered concentration of the standard.

$$R_m = \frac{C_{obs}}{C_{spike}} \&$$

$$U(R_m) = R_m \times \sqrt{\left(\frac{{S_{obs}}^2}{n*{C_{obs}}^2}\right) + \left(\frac{U(C_{spike})}{C_{spike}}\right)^2} =$$

$$0.995 \times \sqrt{1.826^{-5}} = 0.00425$$

The Relative Standard Uncertainty associated with the recovery of the method was found to be 0.00427.

Uncertainty due to Sample Mass (m_{sample}): The uncertainty due to the sample mass can be bifurcated into several sources; sensitivity, linearity and repeatability. The expression of the uncertainty due to mass is done in SI units (kg).

Sensitivity: This parameter may be neglected as the same weighing balance was used during the entirety of the experiment and the range of the weighed masses had minimal difference.

Linearity: From the regression analysis the uncertainty associated with the linearity of Doxycycline is found to be 0.0067g. Using a rectangular distribution to represent the contribution of linearity uncertainty the result is as below.

$$u_{lin} = \frac{0.413 \times 10^{-6}}{\sqrt{3}} = 2.384 \times 10^{-7} kg$$

Repeatability: Similarly, the uncertainty which is associated with the repeatability was found to be 0.0031g.

$$u_{rep} = \frac{0.375 \times 10^{-6}}{\sqrt{3}} = 2.165 \times 10^{-7} kg$$

Calculation of Relative Standard Uncertainty Associated with the Sample Mass: The standard uncertainty due to the sample mass may be calculated by the expression below.

$$u_{(m_{sample})} = \sqrt{2 \times ({U_{lin}}^2 + {U_{rep}}^2)} = 4.554 \times 10^{-7} kg$$

The Standard relative uncertainty is thus found to b 0.004554.

Combined and Extended Standard Uncertainty (CSU& ESU): To estimate the Combined Standard Uncertainty (CSU) of the measurement results, the individual standard uncertainties are added using the "root-sum-of-squares" method. The values of the parameters are summarized in **Table 9**. Using equation (2) the quantity of Doxycycline was found to be 2.009×10^{-6}. These individual standard uncertainty values, when inserted in the below equation (8) give us the Combined Standard Uncertainties.

$$\frac{U(Q_{sample})}{Q_{sample}} = \sqrt{\left(\frac{U_{V_{10}}}{v_{10}}\right)^2 + \left(\frac{U_{C_{10}}}{c_{10}}\right)^2 + \left(\frac{U_{m_{sample}}}{m_{sample}}\right)^2 + \left(\frac{U_{Rm}}{R_m}\right)^2 + \left(\frac{U_{rep}}{rep}\right)^2} \quad \ldots (8)$$

$U_{sample} = 9.3\times10^{-3}$mol/kg

By multiplying the combined standard uncertainty by a coverage factor k=2 at confidence interval of 95% we get the Extended Standard Uncertainty (ESU).

$ESU_{sample} = 1.87\times10^{-2}$mol/kg

TABLE 9: A SUMMARY OF THE CONTRIBUTION OF VARIOUS SOURCES IN THE MEASUREMENT OF UNCERTAINTY

Parameter	Volume (ml) V_{10}	Concentration (M) C_{10}	Sample mass (kg)	Recovery of method	Repeatability
Value	10	2×10^{-8}	1×10^{-4}	99.545×10^{-2}	-
Standard Uncertainty	6.064×10^{-2}	2.9×10^{-11}	4.554×10^{-7}	4.25×10^{-3}	3.140×10^{-3}
Relative Standard Uncertainty	6.064×10^{-3}	1.45×10^{-3}	4.554×10^{-3}	4.27×10^{-3}	3.140×10^{-3}

Comparing the two Analytical Methods using T-Test: For the comparison of the two analytical methods described above, the use of paired t-test is done here to represent that there is no difference in the results between the two analytical methods. An important feature of the paired t-test is that both the analytical methods are indeed related, and with the null hypothesis of equity between the paired samples, the true difference is zero [11].

Here, Method A is the Colourimetric Method, and Method B is the validated Difference Spectroscopic Method. It was found that the null hypothesis holds true, and there is no statistical difference between the two methods as the T_{calc} is less than $T_{tabulated.}$

CONCLUSION: The accurate and precise estimation of the drug content in any given formulation is the key aim of pharmaceutical analysis, and in doing so, ensuring that there is a minimal error in the data is highly recommended. Thus, a quantitative estimation of the quality becomes necessary and is done so by the use of the principles of uncertainty in the given research work.

Here, an attempt was made to make the colorimetric estimation of drug content easier with the aid of smartphones and the use of a color kit for the first time and was deemed to be fruitful. A difference spectroscopic method was also developed and validated by us using the principles of uncertainty and "Total error approach". The two methods were then compared with each other, and it was found that there is no statistical difference in the assay results of the two methods. This novel method will aid in the fast on-sight estimation of drugs in their pharmaceutical dosage forms with the use of the colour kit.

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