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Research Article

Quantification of doxycycline hyclate in different pharmaceutical samples by UV assay

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ABSTRACT

A simple, selective, linear, precise and accurate ultraviolet detection (UV) method has been developed and applied for the determination of doxycycline hyclate in different pharmaceutical samples. Acid-base analysis and titrimetric method were utilized to determine the value of pH and moisture content of purchased pharmaceutical samples. A mixture of methanol and hydrochloric acid (0.01N methanolic HCl) was used to determine the biochemical properties of doxycycline hyclate. UV detector set at 349 nm was used to monitor the effluent. The purified water was used as solvent. In 1% aqueous solution of doxycycline, three samples (4th, 5th and 7th) showed lower pH values of 1.97, 1.98, and 1.99 respectively. Furthermore, the same samples indicated the additional moisture contents of 2.81%, 2.85% and 2.83% respectively while considering the acceptance level (1.4% to 2.8%). The method proved to be linear ($R^2 = 0.993$), precise (RSD = 0.79% for inter-day precision), accurate (Recovery = 100.59%) and selective regarding possible impurities and excipients of the samples. The doxycycline content obtained in the sample analysis was within the range of 84.05% to 85.80%. The optimized and validated method may be successfully employed to perform routine quality control analyses. Investigation of the pH, moisture content and potency of doxycycline hyclate in different samples give a general view of local pharmacies trade and ensure that the method applied here was validated for this kind of analysis.

Keywords: Doxycycline hyclate; Methanolic HCl; pH; Moisture content; Ultraviolet detection; Method validation.

1. INTRODUCTION

Doxycycline is a broad-spectrum antibiotic of the tetracycline class that is used in the treatment of infections caused by bacteria and protozoa [1]. Like other agents of this class it kills bacteria and protozoa by inhibiting protein synthesis [2]. Researchers show that tetracycline antibiotics are second most extensively used antimicrobials world-wide [3]. Tetracyclines are one of the cheapest antibiotics, hence extensively used in countries with limited health care budgets [4].

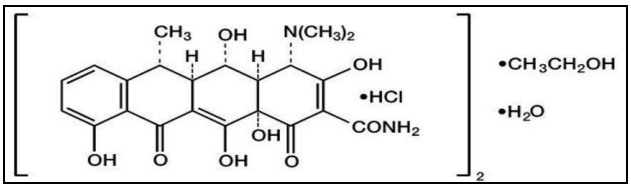

Figure 1. Structure of doxycycline hyclate.

In gram-negative bacteria, transportation of the doxycycline into the cell occurs either by passive diffusion or through an energy-dependent active transport system. The later system is also believed to exist in gram-positive bacteria. Doxycycline is more lipophilic than the other tetracyclines, which allows it to pass easily through the lipid bilayer of bacteria. Doxycycline penetrates the bacterial cell and interferes with the protein biosynthesis, stopping the process of bacterial reproduction. It is the drug of choice in the treatment of sexually transmitted diseases [5]. It is preferred to other tetracyclines in the treatment of specific infections because of its fairly reliable absorption and its long half-life, which permits less frequent dosage. Doxycycline presents itself in three forms: hyclate, monohydrate and hydrochloride.

The molecular formulae of doxycycline hyclate is $C_{22}H_{24}N_2O_8.HCl.½C_2H_6O.½H_2O$. From doxycycline hyclate, it is possible to obtain other forms. The hyclate dissolved in water and neutralized with sodium hydroxide, then becomes doxycycline monohydrate which undergoes doxycycline hydrochloride with the addition of hydrochloric acid (Figs. 1 and 2).

Doxycycline hyclate is much more soluble than doxycycline monohydrate, which is one of the main reasons for it more frequent uses as antibacterial drug. Various methods for quantification of doxycycline *in vitro* and *in vivo* have been reported. These include in vitro experimental model [6], fluorimetry [7], TLC-fluorescence scanning densitometry [8] and HPLC [9-19] for the quantification of doxycycline in biological materials. HPLC was also applied for the determination of doxycycline in pharmaceutical formulations [20].

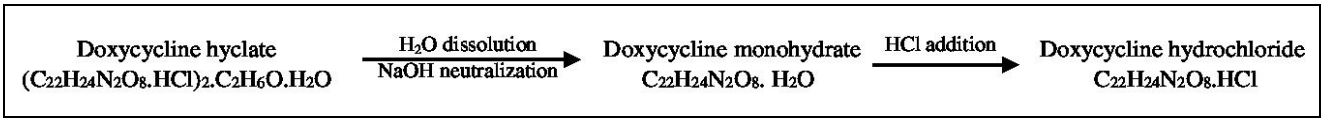

Figure 2. Conversion of doxycycline hyclate into doxycycline monohydrate and doxycycline hydrochloride.

Various chromatographic methods have also been reported for the determination of doxycycline in human tissues [21] and foods [22, 23]. Sequential injection chromatography (SIC) for pharmaceutical preparations [24] and derivative spectrophotometry for the determination of doxycycline in pharmaceuticals, urine and honey have additionally been developed [25]. Many researchers reported that there are a few methods available till date for the determination of doxycycline hyclate in pharmaceutical samples [26-28]. However, the procedure provided by British Pharmacopoeia is widely used for the determination of doxycycline hyclate in pharmaceutical samples till date [29].

The aim of the present study was to establish a standard procedure to quantify doxycycline hyclate in pharmaceutical samples and determine the biochemical properties of doxycycline hyclate. In addition, investigation of the pH, moisture content, purities and potency of this active ingredient in different samples collected from local pharmacies in Bangladesh have been performed. Furthermore, analytical method validation was done to check whether this method could pass the performance characteristics such as specificity, linearity, repeatability, intermediate precision and accuracy. Due to the extensive market value and clinical applications of this drug, product quality, shelf life, other active ingredients and excipients should be analyzed and justified for different brands.

2. MATERIALS AND METHODS

2.1. Reagents and materials

Chemicals such as hydrochloric acid (HCl), Karl Fischer reagent and methanol were purchased from Merck (Darmstadt, Germany). Doxycycline

hyclate reference standard (PromoChem, Teddington, United Kingdom) and HPLC grade solvent were used here for all analytical purposes. Ten doxycycline hyclate capsules in concentration with 100 mg/ml were collected from local pharmacies.

2.2. Instrumentation and methodologies

Balance Sartorius LE225D, pH meter BT-600, Shimadzu UV-1650 visible spectrophotometer, KF Titrando were utilized. UV spectrophotometric method was applied here for the determination of doxycycline hyclate. Several performance characteristics were also analyzed such as pH, water content and impurities. To perform this analysis, following methods were applied:

2.2.1. pH analysis

Doxycycline hyclate is considered as soluble [35] or freely soluble [36] in water. Aqueous solution of doxycycline hyclate, containing 1% doxycycline, has a pH of 2-3 [37, 38]. For pH analysis, 1.0 g test sample transferred in a 100 ml volumetric flask and dissolved [39]. Then volume with water up to the mark and shaken well. Rinsed and clean the electrode with deionized water. Wiped the electrode with tissue paper and immersed it into the sample solution. When the reading stabilized, pH value recorded.

2.2.2. Water content determination

Titrimetric determination of water is based upon the quantitative reaction of water with an anhydrous solution of sulfur dioxide and iodine in the presence of a buffer that reacts with hydrogen ions [33]. The water content should be not less than 1.4% and not more than 2.80% [33, 39]. For this analysis, weighed about 35 to 40 mg of the test sample and transferred it into a Karl Fischer's vassal. Afterwards KF Titrando automatically showed the results on the print out in percentage.

2.2.3. Light Absorbing Impurities Detection

Dissolved 0.25 g in a mixture of 1 volume of 1M HCl and 99 volumes of methanol and diluted to 25.0 ml with the same mixture of solvents [41]. The absorbance of the filtrate determined at 490 nm is not greater than 0.07 (anhydrous and ethanol-free substance) and carried out the measurement within 1 hour of preparing the solution [41].

2.2.4. UV Assay

Standard preparation A (System Suitability) and B (Assay Calculation): According to the Code of Federal Regulations (Title 21, Food and Drugs), 50 mg of doxycycline hyclate was taken in 5 ml volumetric flask and bring to the volume with 0.05N methanolic hydrochloric acid [39]. Here, 50 mg of doxycycline hyclate was transferred in a 50 ml volumetric flask dissolved with 0.01N methanolic HCl and volume up to the mark and sonicated. Then 1ml of this solution added to the 100 ml volumetric flask, volume up to the mark with 0.01N methanolic HCl.

Sample preparation: Same as standard preparation.

Procedure: Concomitantly measured the absorbance of the standard and sample solution in a 1 cm quartz cell at the wavelength of 349 nanometers (nm) [39] with a suitable spectrophotometer using 0.01N methanolic HCl as blank.

Standard Concordance:

$$\frac{\textbf{Average area of SS standard x Weight of C standard x 100}}{\textbf{Average area of C standard x Weight of SS standard}}$$

% of Concordance

Assay:

$$\frac{\textbf{Sample absorbance x Standard weight in mg x potency of standard (\%) x 100}}{\textbf{Standard absorbance x Sample weight mg x (100 – LOD)}}$$

% of doxycycline hyclate on dried basis.

Here, SS standard = System suitability standard and C standard = Calculation standard

2.2.5. Method Validation

2.2.5.1. Specificity

The specificity of the method can be determined with the addition of impurities and degradation products, obtained experimentally or by inducing their formation [40].

Standard preparation: Weighed and transferred accurately about 50 mg of doxycycline hyclate working standard into a 100 ml volumetric flask.

Placebo preparation: Placebo is a substance having no pharmacological effect but administered as a control in testing the efficacy of a biologically active preparation. Weighed and transferred accurately about 108.81 mg of placebo into a 100 ml volumetric flask. For both standard and placebo preparation, 60 ml of diluent added and sonicated for 15 minutes to dissolve. Cooled it and diluted it with same solvent. Diluted 2 ml of this solution to 50 ml with same solvent.

2.2.5.2. Linearity

Linearity was determined by linear regression analysis by the method of least squares. Health Canada (HC) states that the coefficient of determination for active ingredients should be ≥ 0.997, for impurities 0.98 and for biologics 0.95 [46].

Sample preparation: Weighed and transferred accurately about 40 mg, 45 mg, 50 mg, 55 and 60 mg of doxycycline hyclate standard into five 100 ml volumetric flask. Added 60 ml of diluent into each 100 ml volumetric flask and sonicated to dissolve. Cooled it and dilute it with same solvent. Diluted 2 ml of this solution to 50 ml with same solvent. Plotted a graph of concentration versus absorbance (Abs).

2.2.5.3. Repeatability

This parameter determines the repeatability of assay results under the same operating conditions over a short period of time. HC states that the RSD should be 1% for drug substances and 2% for drug products [44].

Preparation of standard: Weighed and transferred accurately about 50 mg of doxycycline hyclate standard into a 100 ml volumetric flask.

Preparation of sample: Weighed accurately about 50 mg of doxycycline hyclate standard and 108.81 mg placebo and transferred into a 100 ml volumetric flask. For both standard and sample preparation, same procedures were performed as mentioned above.

2.2.5.4. Accuracy

Samples were prepared at three concentrations levels over the range of 80 to 120% of the target concentration. Three individually prepared replicates at each concentration were analyzed. For the U.S. pharmaceutical industry, 100 ± 2% is typical for an assay of an active ingredient in a drug product over the range of 80 to 120% of the target concentration [42].

Preparation of standard: Weighed and transferred accurately about 50 mg of doxycycline hyclate working standard into a 100 ml volumetric flask.

Preparation of sample for 80 % recovery: Weighed accurately about 40 mg of doxycycline hyclate standard and 87.04 mg placebo and transferred into a 100 ml volumetric flask.

Preparation of sample for 100% recovery: Weighed accurately about 50 mg of doxycycline hyclate standard and 108.81 mg placebo and transferred into a 100 ml volumetric flask.

Preparation of sample for 120% recovery: Weighed accurately about 60 mg of doxycycline hyclate standard and 130.57 mg placebo and transferred into a 100 ml volumetric flask. For both standard and sample preparation, same procedures were performed as other parameters analysis.

2.2.5.5. Intermediate precision

Intermediate precision of an analytical procedure expresses intra-laboratory variations of the repeatability test performed by a different analyst, on a different day.

Preparation of standard: Weighed and transferred accurately about 50 mg of doxycycline hyclate standard into a 100 ml volumetric flask.

Preparation of sample: Weighed accurately about 50 mg of doxycycline hyclate standard and 108.81 mg placebo and transferred into a 100 ml volumetric flask. For both standard and sample preparation, same procedures were performed as it was done for specificity, linearity, repeatability and accuracy analysis.

3. RESULTS

3.1. Method of optimization

According to the United States Pharmacopoeia (USP), Doxycycline hyclate has potency not less than 80% and not more than 92% [33]

whereas it should be approximately 84.7% declared in British Pharmacopoeia (BP) [29]. For UV assay of sample 1, Weight of SS standard = 0.05084 g, Weight of C standard = 0.05085 g, Sample weight = 0.05093 g, Average area of SS standard = 0.668, Average area of C standard = 0.666, Sample absorbance = 0.664. By putting these values into the calculating formula, assay result for sample 1 was 85.80 % on dried basis and repeated the formula for rest of the samples (Table 1, Fig. 3).

3.2. Assay validation

3.2.1. Specificity

The specificity (Table 2) of the method can be determined with the addition of impurities and degradation products, obtained experimentally or by inducing their formation [40]. Here, the specificity of the chromatographic method was determined by the screening of a placebo solution and the assay solution. The placebo solution was prepared in the same manner as the investigated solution but without doxycycline hyclate. It would be investigated by injecting of the extracted placebo to demonstrate the absence of interference with the elution of analyte.

3.2.2. Linearity

The linearity (Table 3, Fig. 4) of an assay method is its ability to elicit test results, which are directly proportional to the concentrations of drug actives in samples in a given range.

Table 1. Summary report of pH, water content, impurities and UV assay.

Sample	Water by KF 1.40%-2.80%	pH at 1% aqueous 2.0-3.0	Light absorbing Impurities ≥ 0.07	Assay at 349nm 80.0%-92.0%
1	2.41%	2.15	0.018	85.80%
2	2.16%	2.32	0.020	85.24%
3	2.08%	2.39	0.019	85.53%
4	2.81%	1.97	0.022	84.31%
5	2.85%	1.98	0.017	84.03%
6	2.11%	2.31	0.022	85.76%
7	2.83%	1.99	0.019	84.52%
8	2.07%	2.02	0.016	84.71%
9	2.19%	2.29	0.017	84.95%
10	2.05%	2.25	0.020	84.05%

Table 2. Placebo interference/Specificity study.

Serial No.	Weight of Placebo (mg)	Absorbance	Weight of Standard (mg)	Absorbance of Standard	% Placebo Interference
1.	108.72	0.009	60.55	0.695	0.60
2.	108.75	0.005			0.34
3.	108.90	0.002			0.13
		Average			0.36

Table 3. Linearity study.

Serial no.	Concentration %	Sample weight (mg)	Concentration (ppm)	Absorbance
1	80%	47.94	16.06	0.566
2	90%	54.04	18.10	0.635
3	100%	60.55	20.28	0.697
4	110%	65.99	22.10	0.784
5	120%	71.53	23.95	0.848

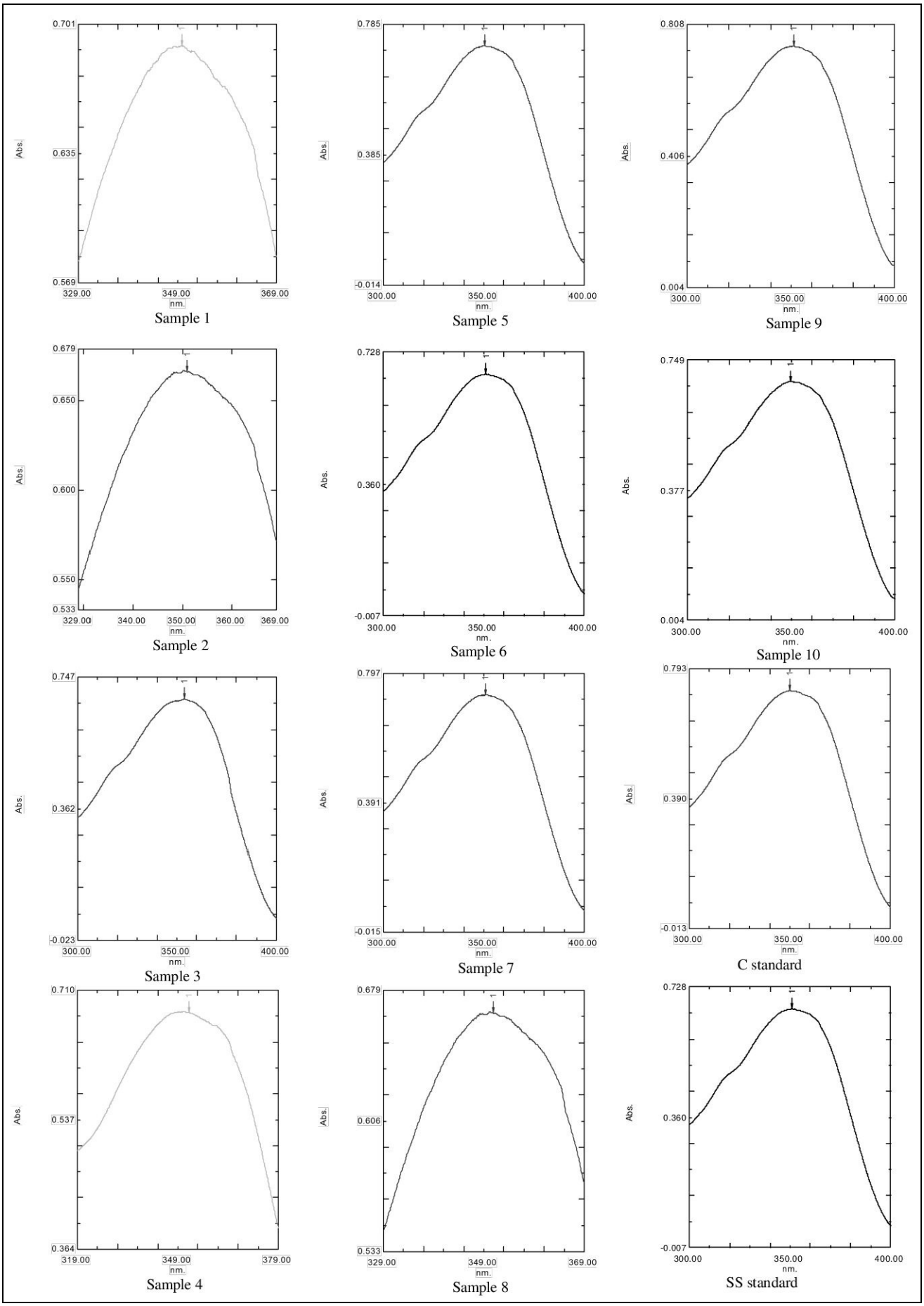

Figure 3. Ultraviolet spectrum of ten samples and standards.

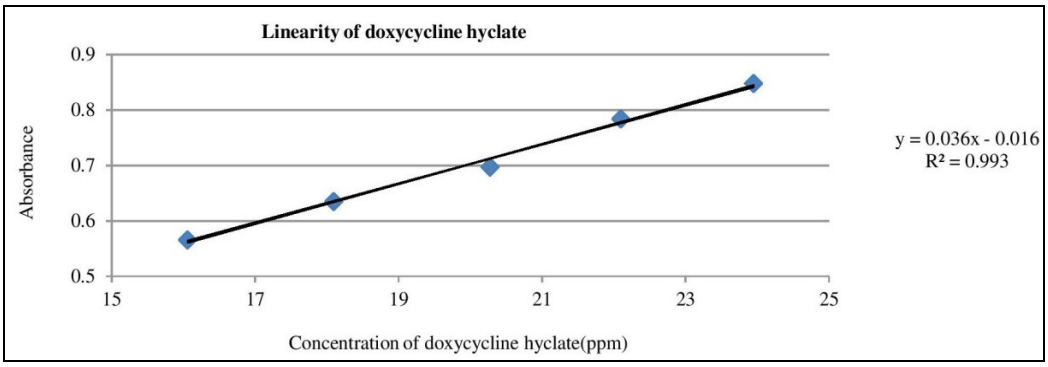

Figure 4. Linearity of doxycycline hyclate.

Table 4. Accuracy study.

Sample No.	Level	Sample Weight (mg)	A (mg)	B (mg)	B/A×100 (% Recovered)	Mean Value %	%RSD
1.	80%	166.24	80.66	80.82	100.20	101.22	0.91
2.		166.24	80.66	82.27	102.00		
3.		166.24	80.66	81.84	101.46		
1.	100%	165.93	98.92	100.36	101.46	101.11	0.59
2.		165.93	98.92	99.34	100.42		
3.		165.93	98.92	100.36	101.46		
1.	120%	168.30	119.82	119.47	99.71	99.43	0.26
2.		168.30	119.82	119.04	99.35		
3.		168.30	119.82	118.89	99.22		

A = Theoretical concentration, B = Concentration recovered, RSD = Relative standard deviation.

Proof of linearity justifies the use of single-point calibrations. The linearity was checked on samples of standard doxycycline hydrochloride at five different concentrations (16.06-23.95 ppm). A regression curve was constructed: y = 0.036x - 0.016, with R^2 = 0.993; where *x* represents concentration in ppm, *y* represents the peak area, and *R* is the correlation coefficient.

3.2.3. Accuracy

The accuracy (Table 4) of the method was checked by determining recovery values. Series of solution were made containing 80, 100 and 120 % of doxycycline hyclate. Recoveries for different concentrations ranged from 99.58 to 101.93% for the determination of doxycycline in bulk drug and in tablets [34].

3.2.4. Repeatability

The % RSD (Table 5) due to doxycycline hyclate concentration for the six samples was found to be less than 2.0%. Six separated sample preparations were analysed according to the method of analysis. The % of RSD due to doxycycline hyclate concentration for the assay meets the requirements. The obtained results are given in Table 5, together with the calculated values of their standard deviation, *SD*, and relative standard deviation, *RSD*.

3.2.5. Intermediate precision

According to the International Conference on Harmonisation (ICH) and United States Pharmacopeia (USP) guidelines [30, 31, 33] intermediate expresses "within laboratories" variation (e.g., different days, different analysts and different equipment). For intermediate precision (Table 6), we checked % RSD of six replicates and overall % RSD of twelve replicates of repeatability and intermediate precision separely. The assay results obtained by two operators on different days should have a statistical RSD ≤ 2% [42, 43].

Table 5. Repeatability study.

Injection No.	Level	Sample Weight (mg)	Absorbance	Assay (%)	Mean Assay (%)	Standard Deviation	%RSD
1.	100%	166.36	0.705	98.70	99.80	0.72	0.65
2.		166.83	0.715	100.10			
3.		166.76	0.712	99.68			
4.		167.06	0.718	100.52			
5.		166.65	0.711	99.54			
6.		165.92	0.716	100.24			

Table 6. Intermediate precision study.

Serial no.	Weight of sample (mg)	Absorbance	Assay %	Weight of sample (mg)	Absorbance	Assay %
1.	166.36	0.705	98.70	167.80	0.705	100.55
2.	166.83	0.715	100.10	168.40	0.711	101.40
3.	166.76	0.712	99.68	168.21	0.707	100.83
4.	167.06	0.718	100.52	167.50	0.700	99.83
5.	166.65	0.711	99.54	168.60	0.712	101.55
6.	165.92	0.716	100.24	167.30	0.704	100.41
Mean assay (n=6)			99.80	Mean assay (n=6)		100.76
Standard deviation (n=6)			0.72	Standard deviation (n=6)		0.43
Relative standard deviation (n=6)			0.65	Relative standard deviation (n=6)		0.63
Mean assay (Analyst 1 & Analyst 2) n=12						100.28
Standard deviation n=12						0.72
Relative standard deviation n=12						0.79

Table 7. Summary report of method validation.

Serial no.	Analytical performance parameter	Acceptance limit		Result
1	Specificity/Placebo interference	NMT 2%		0.36
2	Accuracy	98%-102%	80%	101.22
			100%	101.11
			120%	99.43
3	Repeatability	% RSD NMT 2%		0.65
4	Linearity(Correlation Coefficient)	R^2 Value NLT 0.99		0.99
5	Intermediate Precision	% RSD NMT 2%		0.79

NMT = Not more than, NLT = Not less than.

Table 8. Comparison of the proposed method with other methods.

Reference	Concentration (ppm)	RSD (%)	Recovery (%)	Conditions
This paper	15.0-25.0	0.65	100.59	UV detection
[24]	2.0-100.0	5.05	99.3	Sequential injection chromatography
[25]	-	5.0	95	Derivative spectrophotometry
[26]	10.0-80.0	1.40	2.3[a]	FIA-spectrophotometry
[27]	10.0-30.0	-	100.3	Spectrophotometry
[28]	20.0-100.0	-	99.6	Spectrophotometry

[a] = Relative error.

4. DISCUSSION

In this study, there was no residual effect that could be harmful to humans. In 2001, Zarghi et al. developed a method for determination of doxycycline in human plasma using HPLC assay based on a mbondapak C18 column and a lambda (347 nm) UV detector [9]. Reagents like actonitrile, potassium dihydrogen phosphate, 24% perchloric acid aqueous solution were utilized there. In 2011, Ramesh *et al* developed and validated three cost-effective spectrophotometric methods for the determination of doxycycline in bulk drug and in tablets where HCl, NaOH, H_2SO_4 medium and iron(III) chemicals were used [34]. In 2012, Kogawa et al. developed and validated an accurate, sensitive, precise and rapid gradient reversed-phase high-performance liquid chromatographic method for the determination of doxycycline hyclate in bulk drug and tablets [44]. CN Luna column at 360 nm was utilized as stationary phase and water, 0.1% TFA-acetonitrile and 0.1% TFA were used as the mobile phase at a flow rate of 1.0 mL/min. In 2016, Kogawa et al. developed and validated an eco-friendly method of infrared spectroscopy for quantification of doxycycline in raw material [45].

The proposed spectrophotometric methods do not require any expensive equipment and specialized technicians when compared alongside HPLC and bioassay. Besides, other characteristics of these methods are the short time and less space required for performance and handling respectively than other methods. Although there is not enough evidence of pH determination of antibiotics and drug products, here it was performed because doxycycline hyclate causes ulcer in stomach due to acid reflux and heartburn. For this reason, pH level should be within the range of acceptance. Here, sample 4, 5 and 7 had slightly higher acidic pH levels that would be a concern of health safety issues. Beside, stability of doxycylcine hyclate in aqueous solutions depends on the pH value. On the other hand, Karl Fischer titration method in analytical chemistry to determine trace amounts of water in a sample [32]. In the environmental science, it is used to test the percent (%) of water content in soil and cosmetic industry to test the percent (%) of moisture content in cosmetics like soap, detergents, cream etc. After the analysis of ten different samples of doxycycline hyclate, sample no. 4, 5 and 7 showed results those had some differences considering the specification limit. Due to the high moisture content value, these samples potency might be lost before the given expiry date.

A linear regression curve was constructed and the correlation coefficient (R^2) and assessment value calculated. Here, a plot of concentration against peak area shows a straight line where R^2 value is 0.993. Precision determined the repeatability of assay results under the same operating conditions over a short period of time. The % RSD due to doxycycline hyclate concentration for the six samples was 0.65%. For accuracy analysis, if the release specification for a product is 100 ± 2%, the accuracy criterion should be ~1%. Furthermore, if it is 100 ± 5%, the accuracy specification should be ± 2% to 2.5% [46]. In other words, acceptance criteria for each individual sample recovery should lie within the range of 98-102% [47]. From the accuracy results, percent recovery values for doxycycline hyclate satisfy the acceptance criteria (98-102%) across the range of 80-120%. A comparison of the proposed method with other methods is given in Table 8.

The results obtained by the proposed method have an RSD of 0.65 %, better than that reported by Šatínský et al. [24], Salinas et al. [25] and Lopez-Paz et al. [26]. The first two methods [24, 25] have relatively high RSD values, higher than RSD_{max}. This indicates that the proposed method is more precise and accurate than some of the aforetime published methods. Mahrous and Abdel-Khalek [27] described a long pre-treatment of the drug through mixing with acetic acid and sodium cobalt nitrite, then boiling the mixture, followed by cooling. In addition, the same author [28] described a determination employing ammonium vanadate but the later has less recovery percentage than the earlier one. In comparison of both methods [27, 28], this proposed method have the higher recovery rate (100.59%). Here, the validated methods yielded good results and suggest their application in the quality control laboratories where the modern and expensive instruments are not available.

5. CONCLUSION

In this work, an analytical UV method was developed for the quantitative determination of doxycycline hyclate in samples. Its advantages over other existing method include its simplicity, speed and low cost. The proposed method not only provides a linear relation between absorbance and concentration in 349 nm wavelength, but also ensures a simple, sensitive, accurate, and repeatable determination of doxycycline hyclate in pharmaceutical samples. Doxycyline was shown to be stable during all the procedure. Thus, the result parameters demonstrated that the spectrophotometric method could be applied for the analysis of the pharmaceutical formulations assuring the quality and efficacy of the doxycycline hyclate under investigation.

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AUTHORS' CONTRIBUTION

KMMH: Data analysis, data interpretation and drafting the article; MH: Conception of the work; SMAK: Data collection; MAH: Critical revision of the article. The final manuscript has been read and approved by all authors.

TRANSPARENCY DECLARATION

The authors declare that there is no conflict of interest.

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