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UV-SPECTROPHOTOMETRIC METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF HYDROXYCHLOROQUINE SULFATE AND NITAZOXANIDE IN SYNTHETIC MIXTURE

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Keywords:

UV spectrophotometric, Hydroxychloroquine sulfate (HCQ), Nitazoxanide (NTZ), Simultaneous equation method, Firs- order derivative method.

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ABSTRACT: A simple, accurate, precise, reproducible, economical UV spectrophotometric method has been developed and validated for simultaneous estimation of Hydroxychloroquine sulfate (HCQ) and Nitazoxanide (NTZ) in a synthetic mixture. Method A by simultaneous equation method, Method B by the first-order derivative method. Method A: simultaneous equation method was based on the measurement of absorbance of nitazoxanide at 345 nm and Hydroxychloroquine Sulfate at 220 nm. Method B: first-order derivative method was based on the measurement of absorbance of HCQ measure at 226 nm (ZCP of NTZ) and absorbance of NTZ measure at 375.50 nm (ZCP of HCQ). The calibration curve was found to be linear in the concentration range 2-10 µg/ml and 5-25µg/ml for HCQ and NTZ, respectively (n=6), with their correlation coefficient of 0.999. The developed method was validated according to the ICH guideline.

INTRODUCTION: 1, 5 Hydroxychloroquine sulfate chemically 2-[4-[(7-chloroquinoline-4-yl) amin] phenylethylamine] ethanol; sulfuric acid **Fig. 1**. Chemical formula $C_{18}H_{28}C_lN_3O_5S$ and Molecular weight 434 g/mol. It is used AS anti-malarial drug [1]. Hydroxychloroquine sulfate (HCQ) was used in combination with Nitazoxanide for SARS-corona virus 2 (SARS-CoV-2) [2]. The ACE2 undergoes glycosylation for it to convert to an active form. When SARS-CoV-2 S protein bind to it, the ACE2 receptor undergoes glycosylation and gets activated here, HCQ prevents the glycosylation of ACE2 receptors. So, HCQ preventing entry of SARS-CoV-2 into the host organisms [3]. Nitazoxanide chemically [2- [(5-nitro-1, 3-thiazol 2yl) carbamoyl] acetate, Chemical formula $C_{12}H_9N_3O_5S$, and Molecular weight 307.28 g/mol. It is used as antiprotozoal [3]. The reason behind selecting NTZ for SARS-CoV-2 could be derived from its impact on the immune system in potentiating the production of type 1 interferon and bronchodilation of the airways through inhibition of TMEM16A ion channels. NTZ inhibits the production of pro-inflammatory cytokines TNFα, IL-2, IL-4, IL-5, IL-6, IL-8, and IL-10 in peripheral blood mononuclear cells [5].

A review of the literature revealed that only a few chromatographic and spectrophotometric methods were reported for estimation of HCQ and NTZ

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individually or it's combination with other drugs, no method was found for simultaneous estimation of HCQ and NTZ. So, it was thought of interest to develop simple, accurate, precise, reproducible, and economic UV-Spectrophotometric methods for simultaneous estimation of HCQ and NTZ. Methods were validated as per ICH guideline [Q2 (R1)] [6].

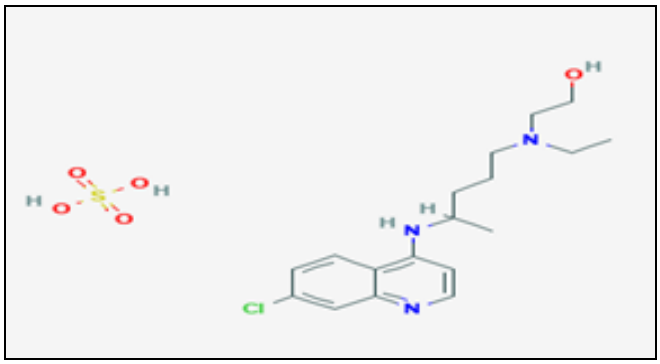

FIG. 1: STRUCTURE OF HYDROXYCHLOROQUINE SULPHATE

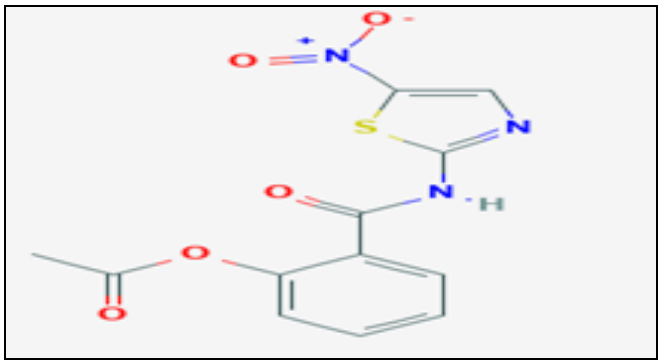

FIG. 2: STRUCTURE OF NITAZOXANIDE

MATERIALS AND METHODS:

Reagents and Material: Nitazoxanide and Hydroxychloroquine sulfate were received as gift samples from Globela Pharma Pvt. Ltd. Methanol AR (Advent Chem bio Pvt. Ltd) use as a solvent for the development of the method.

Instrumentation: Adoublebeam UV/ Visible spectrophotometer (Shimadzu UV-1700) with spectral width of 2 nm, 1 cm path length quartz cells was used to measure the absorbance of all the solutions. The spectra obtained by UV-Probe 2.50 software

Solvent Selection for UV-Method: Solubility of NTZ and HCQ in methanol. So, methanol was selected as a solvent for analysis of NTZ and HCQ.

Preparation of Standard Solutions: Accurately weighed quantity of Nitazoxanide and Hydroxychloroquine sulfate 25 mg was transferred to separate 25 ml volumetric flask, add some methanol and sonicate for 10 min and diluted up to the mark with methanol to give a stock solution having the strength of 1000 µg/ml. An aliquot of 2.5 ml from the above standard stock solution was pipette out into 25 ml of volumetric flask and diluted up to the mark with methanol to give a stock solution having the strength of 100 µg/ml.

Preparation of Test Solution: Take synthetic mixture equivalent to 20 mg HCQ and 50 mg NTZ in 100 ml volumetric flask and add methanol up to the mark to give solution strength (200, 500 µg/ml) sonicate for 10 min.

Take 1 ml from the above solution and transfer in 10 ml volumetric flask and make the volume up to mark with methanol give solution strength (20, 50 µg/ml). Take 5 ml again from the above solution and transferred in 10 ml volumetric flask and diluted up to the mark so, the final concentration of HCQ was 10 µg/ml, and NTZ was 25 µg/ml.

TABLE 1: FORMULATION OF SYNTHETIC MIXTURE

S. no.	Ingredient	Quantity (mg)	Role
1	Nitazoxanide	500	Treat inflammation
2	Hydroxychloroquin sulfate	200	ACE2 inhibitor
3	Microcrystaline cellulose	20	Disintegrant
4	Hydroxypropyl methylcellulose	15	Binder
5	Lactose monohydrate	45	Diluent
6	Magnesium stearate	10	Lubricant
7	Talc	10	Glidant

Procedure for Determination of Wavelength for Measurement: 1 ml of stock solution of NTZ (100 µg/ml) and 1 ml of stock solution of HCQ (100 µg/ml) were pipette out into two separate 10 ml volumetric flasks. Volume was adjusted to the mark with methanol to get 10 µg/ml of NTZ and 10

µg/ml of HCQ. Each solution was scanned between 200-800 nm against methanol as a blank reagent. The spectrum of each solution was obtained. The wavelength maximums were found to be 345 nm and 220 nm for NTZ and HCQ, respectively.

Stability of Solution: The stability of the solution in methanol was done for 48 h at room temperature. The absorbance of the solution of NTZ and HCQ was taken at 345 nm and 220 nm in the interval of 0, 24, and 48 h **Table 2.**

TABLE 2: STABILITY OF SOLUTION

S. no.	Time(hr)	HCQ	NTZ
1	**0**	0.193	0.186
2	**24**	0.190	0.184
3	**48**	0.186	0.179

METHOD A:

Simultaneous Equation Method: The simultaneous equation method based on the absorbance of both drug NTZ and HCQ at their λ_{max}. There are two wavelengths selected for the development of simultaneous equation method λ_{max} of the Nitazoxanide and Hydroxychloroquine Sulfate at 345nm and 220 nm respectively in methanol **Fig. 3.**

Validation Parameters:

Linearity and Range: The Different concentrations of HCQ (2-10 µg/ml) and NTZ (5-25 µg/ml) were prepared from respective stock solutions (100 µg/ml). The absorbance was observed at 220 nm and 345 nm. At the wavelengths 220 nm and 345 nm, good linearity was observed, and hence these wavelengths were fixed for their simultaneous estimation. The absorptivities were calculated for Hydroxychloroquine sulfate and nitazoxanide at the 220nm and 345nm wavelengths **Table 3.** The Correlation coefficient (r2) for the calibration curve of HCQ and NTZ was found to be 0.999 and 0.999, respectively. The regression line equation for HCQ and NTZ are as following,

y = 0.077 × + 0.006 for HCQ, y = 0.043 ×+ 0.00416 for NTZ

TABLE 3: ABSORBANCE FORHCQ ANDNTZAT220NMAND345NM RESPECTIVELY (N=6)

HCQ			NTZ		
Conc. µg/ml	**Mean Abs. At 220nm**	**Mean Abs. At 345nm**	**Conc. µg/ml**	**Mean Abs. At 220nm**	**Mean Abs. At 345nm**
2	0.154±0.0015	0.042±0.0005	5	0.1684±0.0015	0.1722±0.0015
4	0.308±0.0020	0.095±0.0005	10	0.2950±0.0010	0.3784±0.0015
6	0.454±0.0020	0.143±0.0011	15	0.4250±0.00208	0.5988±0.0010
8	0.608±0.0047	0.215±0.0015	20	0.5960±0.0010	0.8128±0.0030
10	0.777±0.0042	0.276±0.0025	25	0.7230±0.0100	1.0340±0.0050

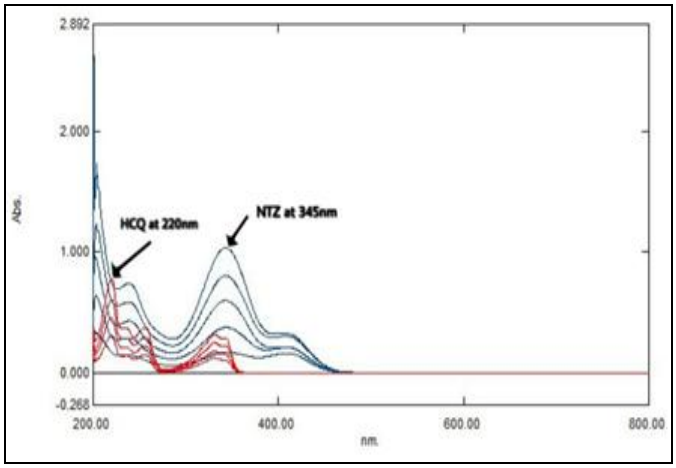

FIG. 3: OVERLAY OF DIFFERENT CONCENTRATION OFHCQ AT 220NM AND NTZ AT 345 NM

TABLE 4: ABSORPTIVITIES AT 220 AND 345NM

At 220nm		At 354nm	
ax_1	0.077	**ax_2**	0.029
ay_1	0.027	**ay_2**	0.043

Precision:

Repeatability: The concentration of solutions 2, 6, 10µg/ml and 5, 15, 25 µg/ml for HCQ and NTZ respectively and the same solutions were analyzed seven times at 220 nm and 345 nm. The %RSD was found to be 0.25-0.99% for HCQ and 0.16-. 089% for NTZ. These % RSD value was found to beless than ± 2.0 indicated that the method is precise **Table 5.**

Intraday Precision: The concentration of Solutions 2, 6, 10 µg/ml and 5, 15, 25 µg/ml for HCQ and NTZ respectively series were analyzed three times on the same day using the developed spectroscopic method, and % RSD was calculated. The % RSD was found to be 1.1-1.2% for HCQ and 1.0- 1.1% for NTZ. These % RSD value was found to be less than ± 2.0 indicated that the method is precise **Table 6.**

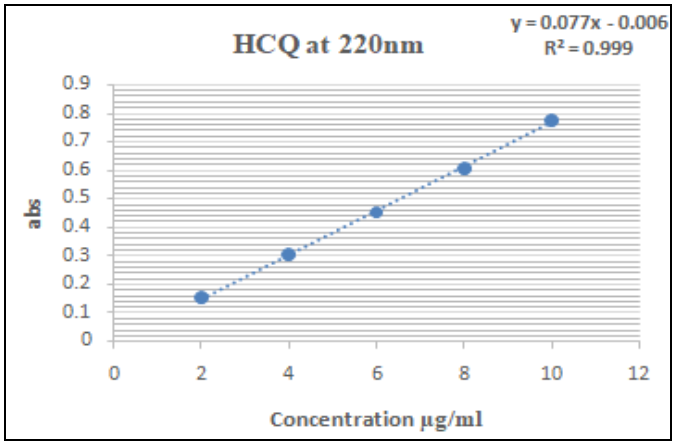

FIG. 4: CALIBRATION CURVE OF HCQ AT 220NM

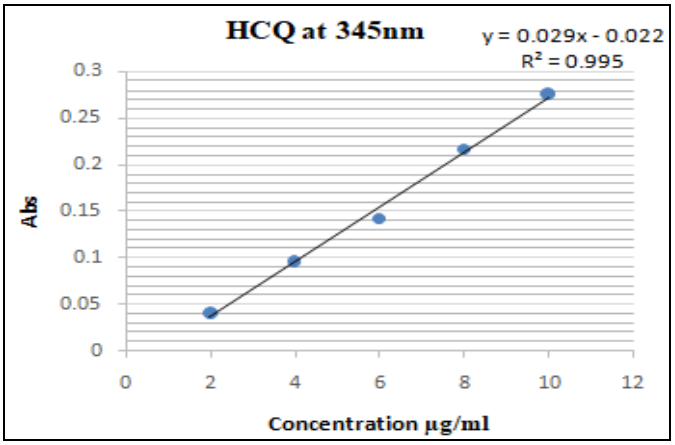

FIG. 5: CALIBRATION CURVE OF HCQ AT 345NM

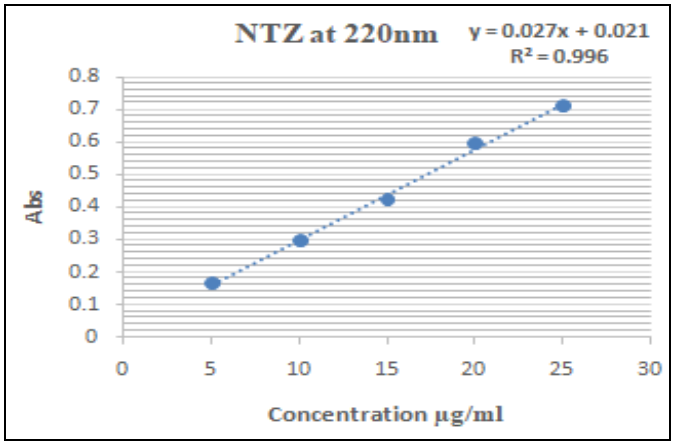

FIG. 6: CALIBRATION CURVE OF NITAZOXANIDE AT 220NM

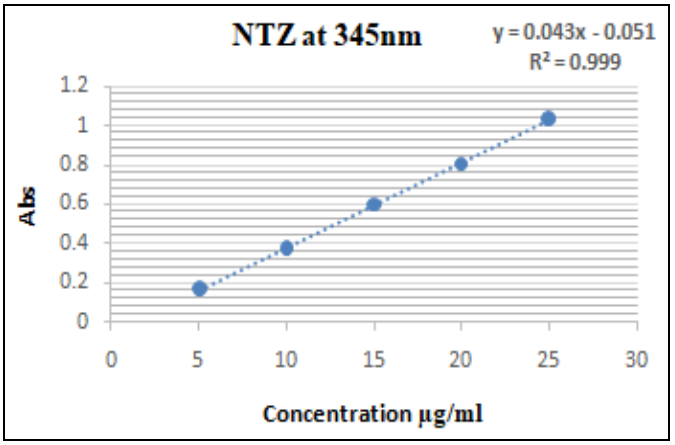

FIG. 7: CALIBRATION CURVE OF NITAZOXANIDE AT 345NM

TABLE 5: REPEATABILITY DATA FOR ESTIMATION OFHCQ AND NTZ (N=7)

Conc. (µg/ml)		Mean Abs. ±SD HCQ	% RSD	Mean Abs. ± SD NTZ	% RSD
HCQ	NTZ				
2	5	0.153 ± 0.0015	0.9962	0.170 ± 0.0015	0.8967
6	15	0.453 ± 0.0010	0.3278	0.597 ± 0.0010	0.1675
10	25	0.776 ± 0.002	0.2577	1.027 ± 0.0050	0.4897

TABLE 6: INTRADAY PRECISION DATA FOR ESTIMATION OF HCQ AND NTZ (N=3)

Conc. (µg/ml)		Mean Abs. ± SD HCQ	% RSD	Mean Abs. ± SD NTZ	% RSD
HCQ	NTZ				
2	5	0.155 ± 0.002	1.2903	0.172 ± 0.002	1.1627
6	15	0.452 ± 0.0052	1.1706	0.590 ± 0062	1.0584
10	25	0.765± 0.0096	1.2555	1.023 ± 0.011	1.124

Inter Day Precision: The concentration of solutions 2, 6, 10µg/ml and 5, 10, 25 µg/ml for HCQ and NTZ respectively series were analyzed three times on a different day using the developed spectroscopic method, and % RSD was calculated. The % RSD was found to be 1.6 –1.7% for HCQ and 1.7- 1.8% for NTZ. These % RSD value was found to be less than ± 2.0 indicated that the method is precise **Table 7.**

TABLE 7: INTERDAY PRECISION DATA FOR ESTIMATION OFHCQ ANDNTZ (N=3)

Conc. (µg/ml)		Mean Abs. ± SD HCQ	% RSD	Mean Abs. ± SD NTZ	% RSD
HCQ	NTZ				
2	5	0.156 ± 0.0026	1.6959	0.173 ± 0.00306	1.7625
6	15	0.454 ± 0.0080	1.7621	0.6057 ± 0.0109	1.8111
10	25	0.754 ± 0.0135	1.7998	1.0310 ± 0.0183	1.7805

Accuracy: The developed UV spectroscopic method was checked for the accuracy. It was determined by calculating the recovery of HCQ and NTZ. The spiking was done at three levels 50 %, 100 %, and 150 % **Table 8, 9.**

Procedure: From the test solution, take 2 ml and transfer in 10 ml volumetric flask and make the volume up to the mark with methanol. So, the final Concentration of HCQ is 4µg/ml, and NTZ is 10 µg/ml.

Solution - A 20 µg/ml + 50 µg/ml (2ml), Solution-B(HCQ): 100 µg/ml, Solution - C(NTZ): 100µg/ml

This method's percentage recovery for HCQ and NTZ was found in the range of 100.51 to 100.88% and 99.97 to 101.33%, respectively.

TABLE 8: RECOVERY DATA OFHCQ (N=3)

Level	Conc. of HCQ from Synthetic mixture (µg/ml)	Amount of Std.HCQ added (µg/ml)	Total amount of HCQ (µg/ml)	Total amount of HCQ Recovered (µg/ml) Mean± SD	% Recovery
0%	4	0	4	-	-
50%	4	2	6	2.01 ± 0.052	100.51%
100%	4	4	8	4.03 ± 0.011	100.83%
150%	4	6	10	6.05 ± 0.095	100.88%

TABLE 9: RECOVERY DATA OFNTZ (N=3)

Level	Conc. of NTZ from Synthetic mixture (µg/ml)	Amount of Std. NTZ added (µg/ml)	Total amount of NTZ (µg/ml)	Total amount of NTZ Recovered (µg/ml) Mean± SD	% Recovery
0%	10	0	10	-	-
50%	10	5	15	4.99±0.068	99.800%
100%	10	10	20	10.13±0.11	101.33%
150%	10	15	25	14.99±0.085	99.97%

Limit of Detection and Quantitation: The Limit of detection (LOD) and Limit of Quantification (LOQ) of the developed method was calculated from the five-calibration curve **Table 10.** The LOD and LOQ were calculated by using this formula.

$$LOD = 3.3 \times \sigma/Slope, \quad LOQ = 10 \times \sigma/Slope$$

Where, σ = standard deviation of intercept of 5 calibration curves. Slope =the mean slope of the 5 calibration curves

TABLE 10: LOD ANDLOQ DATA OF HCQ ANDNTZ (N=5)

	HCQ (µg/ml)	NTZ(µg/ml)
LOD	0.0501	0.0334
LOQ	0.152	0.1014

Application of the Proposed Method for Analysis of HCQ and NTZ in Synthetic Mixture: The zero-order spectrum of test solution was recorded and measured the absorbance at 220 nm and 345 nm to estimate HCQ and NTZ.

The concentrations of HCQ and NTZ in the synthetic mixture were determined using the simultaneous equation. The % assay value sare given in **Table 11.**

TABLE 11: ANALYSIS DATA OF SYNTHETIC FORMULATION (N=3)

S. no.	Drug	Concentration(µg/ml)	% Assay ± SD	% R.S.D
1	HCQ	4	101.25 ± 0.05	0.56
2	NTZ	10	101.33 ± 0.01	0.24

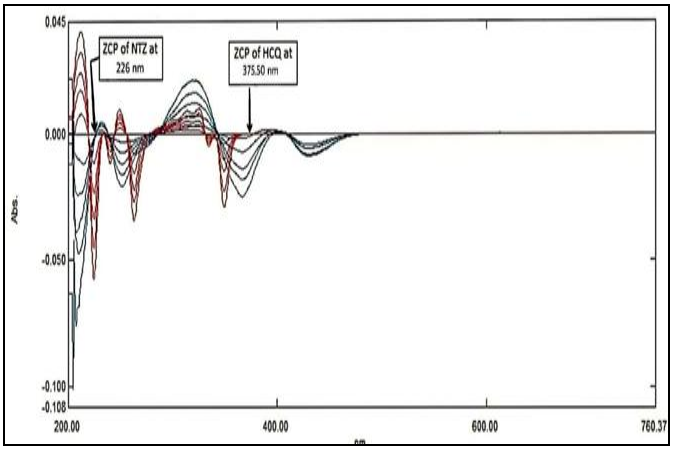

FIG. 8: OVERLAY FIRST-ORDER DERIVATIVE SPECTRA OF DIFFERENT CONCENTRATIONS OF HCQ (2-10MG/ML) AND NTZ (5-25MG/ML)

METHOD B:

First Order Derivative Spectroscopy Method: Selection of wavelength for estimation of HCQ and

NTZ simultaneously**.** To determine wavelength for estimation, standard spectra of HCQ and NTZ were scanned between 200-800 nm against methanol. The zero-order absorption spectra were derivatized in first-order using software UV probe 2.50. The zero-crossing point was obtained at 375.50 nm and 226 nm for the estimation of HCQ and NTZ.

Linearity and Range: The Different concentrations of HCQ (2-10 µg/ml) and NTZ (5-25 µg/ml) were prepared from respective stock solutions (100 µg/ml). The absorbance was observed at 226 nm and 375.50 nm. It was observed that at the wavelengths 226 nm is ZCP of NTZ and at 375.50 nm ZCP of HCQ. So, the absorbance of HCQ measure at 226 nm (ZCP of NTZ) and the absorbance of NTZ measure at 375.50 nm (ZCP of HCQ), linearity was observed wavelengths were fixed for first Oder derivative spectroscopy method **Table 12.**

The Correlation coefficient (r2) for the calibration curve of HCQ and NTZ was found to be 0.9988and 0.996, respectively **Fig 9** and **10.** The regression line equation for HCQ and NTZ are as following,

y = 0.0052 × + 0.004 for HCQ, y = 0.0009 ×+ 0.0041 for NTZ

TABLE 12: ABSORBANCE FORHCQAT 226 (ZCP OF NTZ) AND NTZ AT 375.50 (ZCP OF HCQ) (N=6)

HCQ at 226 (ZCP of NTZ) (n=6)				NTZ at 375.50 (ZCP of HCQ) (n=6)		
S. no.	Conc. µg/ml	Mean Abs. At 226 nm	%R.S.D	Conc. µg/ml	Mean Abs. At 375.50 nm	% R.S.D
1	2	0.0101 ± 0.0005	0.3876	5	0.006 ± 0.0006	1.0304
2	4	0.0207 ± 0.0005	0.2431	10	0.0046 ± 0.0005	0.7048
3	6	0.0301 ± 0.0004	0.329	15	0.0099 ± 0.0005	0.4268
4	8	0.0407 ± 0.0002	0.4627	20	0.0149 ± 0.0005	0.2240
5	10	0.0516 ± 0.0003	0.5166	25	0.0182 ± 0.0002	0.8738

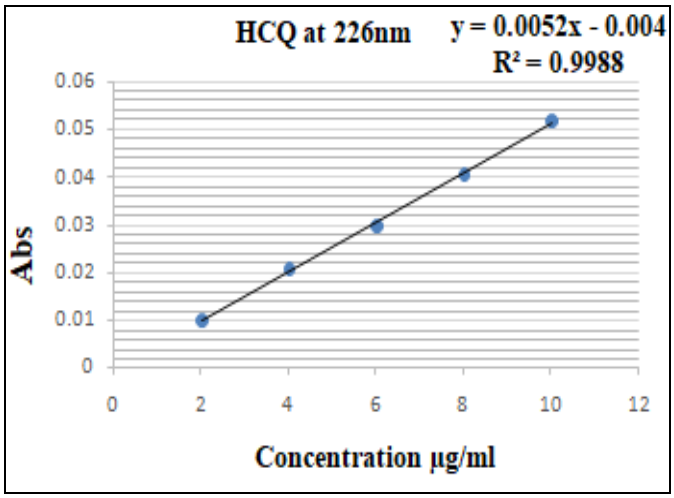

FIG. 9: CALIBRATION CURVE OF HCQ AT 226 NM (ZCP OF NTZ)

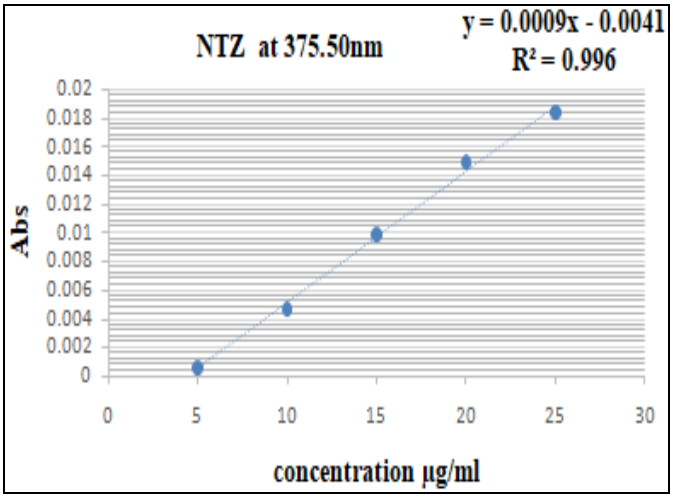

FIG. 10: CALIBRATION CURVE OF NTZ AT) 375.50 NM (ZCP OF HCQ

TABLE 14: REPEATABILITY DATAFOR ESTIMATION OFHCQ ANDNTZ (N=7)

Conc. (µg/ml)		Mean Abs. ± SD	% RSD	Mean Abs. ± SD	% RSD
HCQ	NTZ	HCQ at 226 nm (ZCP of NTZ)		NTZ at 375.50 nm (ZCP of HCQ)	
2	5	0.0101 ± 0.000039	0.3875	0.00058 ± 0.00006	1.0904
6	15	0.0301 ± 0.00005	0.2430	0.0046 ± 0.00004	0.7048
8	20	0.0515 ±0.00026	0.5166	0.01821 ± 0.00015	0.8738

TABLE 13: REGRESSION LINE EQUATION, REGRESSION COEFFICIENT FOR HCQ AND NTZ

S. no.	Drug	Regression line equation	Regression coefficient (R^2)
1	HCQ	y = 0.0052 x - 0.004	0.9988
2	NTZ	y = 0.0009 x - 0.0041	0.996

Precision

Repeatability: The concentration of Solutions 2, 6, and 10 µg/ml and 5, 15, 25 µg/ml for NTZ and HCQ, respectively, and the same solution was analyzed seven times at 226nm and 375.50 nm. The

% RSD was found to be 0.2-0.51% for HCQ and 0.7-1.09% for NTZ **Table 14.**

Intraday Precision: The concentration of Solutions 2, 6, 10 µg/ml and 5, 15, 25 µg/ml for HCQ and NTZ respectively series were analyzed three times on the same day using the developed spectroscopic method, and % RSD was calculated. The % RSD was found to be 0.3-0.7% for HCQ and 0.9- 1.6% for NTZ **Table 15.**

TABLE 15: INTRADAY PRECISION DATA FOR ESTIMATION OF HCQ ANDNTZ (N=3)

Conc. (µg/ml)		Mean Abs. ± SD	% RSD	Mean Abs. ± SD	% RSD
HCQ	NTZ	HCQ at 226 nm (ZCP of NTZ)		NTZ at 375.50 nm (ZCP of HCQ)	
2	5	0.0101 ± 0.000041	0.4105	0.006 ± 0.00001	1.6949
6	15	0.0302 ± 0.000010	0.3049	0.0098 ± 0.00009	0.98
10	25	0.0518 ± 0.00041	0.7954	0.0182 ± 0.000018	0.991

Interday Precision: The concentration of solutions 2, 6, 10 µg/ml and 5, 15, 25 µg/ml for HCQ and NTZ respectively series were analyzed three times daily using a developed spectroscopic method, and %RSD was calculated. The %RSD was found to be 1-1.3% for HCQ and 1.3-1.7% for NTZ **Table 16.**

TABLE 16: INTERDAY PRECISION DATA FOR ESTIMATION OFHCQ ANDNTZ (N=3)

Conc. (µg/ml)		Mean Abs. ± SD	% RSD	Mean Abs. ± SD NTZ at	% RSD
HCQ	NTZ	HCQ at 22 6nm (ZCP of NTZ)		375.50 nm (ZCP of HCQ)	
2	5	0.0108 ± 0.0001	1.0121	0.00058 ± 0.00001	1.7241
6	15	0.0309 ± 0.0001	1.3814	0.00994 ± 0.0002	1.6899
10	25	0.0519 ± 0.0005	1.0015	0.01825 ± 0.0002	1.3332

Accuracy: The developed UV spectroscopic method was checked for accuracy. It was determined by calculating the recovery of HCQ and NTZ. The spiking was done at three levels 50 %, 100 %, and 150 %. Percentage recovery for HCQ and NTZ by this method was found in the range of 99.54-101.67 and 99.85-101.7%, respectively **Table 17** and **18.**

TABLE 17: RECOVERY DATA OF HCQ (N=3)

Level	Conc. Of HCQ from Synthetic mixture (µg/ml)	Amount of Std. HCQ added (µg/ml)	Total amount of HCQ (µg/ml)	Total amount of HCQ Recovered(µg/ml) Mean ± SD	% Recovery
0%	4	0	4	-	-
50%	4	2	6	2.03± 0.012	101.67%
100%	4	4	8	4.03± 0.084	100.91%
150%	4	6	10	5.97±0.012	99.54%

TABLE 18: RECOVERY DATA OF NTZ (N=3)

Level	Conc. Of NTZ from Synthetic mixture (µg/ml)	Amount of Std. NTZ added (µg/ml)	Total amount of NTZ (µg/ml)	Total amount of NTZ Recovered (µg/ml) Mean ± SD	% Recovery
0%	10	0	10	-	-
50%	10	5	15	5.085±0.45	101.7%
100%	10	10	20	9.98±0.084	99.85%
150%	10	15	25	15.01±0.39	100.1%

Limit of Detection and Quantitation:

Application of the Proposed Method for Analysis of HCQ and NTZ in Synthetic Mixture: The first-order spectrum of test solution was recorded and measured the absorbance at 226 nm and 375.50 nm for estimation of HCQ and NTZ. The concentrations of HCQ and NTZ in synthetic mixture were determined using the First order derivative method. The% assay values are given in **Table 20.**

TABLE 19: LOD ANDLOQ DATA OF HCQ ANDNTZ (N=5)

	HCQ (µg/ml)	NTZ(µg/ml)
LOD	0.0906	0.01893
LOQ	0.2746	0.05737

TABLE 20: ANALYSIS DATA OF FORMULATION (N=3)

S. no.	Drug	Concentration (µg/ml)	% Assay* ± SD	% R.S.D
1	HCQ	4	101.22 ± 0.0067	0.16
2	NTZ	10	100.22 ± 0.078	0.77

Statistical comparison of developed first-order derivative method and simultaneous equation method by F test F calculated was less than F critical for both HCQ and NTZ, thus indicating no significant difference observed in assay result among the two methods. Hence it was concluded that both methods do not differ significantly **Table 21.**

TABLE 21: F TEST FOR DEVELOPED METHODS

Method	% HCQ	% NTZ
	100.5	100.13
	101.08	100.7
Simultaneous	99.17	99.4
Equation Method	99.96	99.76
	100.16	100.24
	100.83	100.22
	100.19	99.94
Derivative method	99.85	99.83
	99.39	100.88
	99.4	99.94
F calculated	1.364248	1.216042
F critical	6.388233	6.388233

METHOD A:

Simultaneous Equation Method: Based on results, obtained from the analysis of HCQ and NTZ in their synthetic mixture and bulk using the Simultaneous Equation Method, the method has linearity in the range of 2-10 µg/ml for HCQ and 5-25 µg/ml for NTZ. The regression coefficient (R2) was found to be 0.999 and 0.999for HCQ and NTZ at 220 nm for HCQ and 345nm for NTZ respectively **Table 3, 4.** Further % R.S.D. was found to be less than 2% for precision, repeatability intraday and interday study **Table 5, 7.** The %recovery for HCQ and NTZ was found to be 100.51-101.25 and 99.97-101.33 %, respectively. **Table 8, 9.** The limit of detection for HCQ and NTZ was found to be 0.0501 µg/ml and 0.0334 µg/ml, and the limit of quantification for HCQ and NTZ was found to be 0.152µg/ml and 0.1014µg/ml, respectively (Table 10). The % assay was found to be 101.25% and 101.33 % for HCQand NTZ, respectively **Table 11.**

METHOD B:

First Order Derivative Method: On the basis of results obtained from the analysis of HCQ and NTZ in their synthetic mixture and bulk using the first-order derivative method, the method has linearity in the range of 2-10 µg/ml for HCQ and 5-25 µg/ml for NTZ. The regression coefficient (R2) was found to be 0.9988 and 0.996 for HCQ and NTZ at 226 nm for HCQ and 375.50 nm for NTZ, respectively. (Table 12, 13). Further % R.S.D. was found to be less than 2% for precision, repeatability intraday, and interday study **Table 14, 16.** The %recovery for HCQ and NTZ was found to be 99.54-101.67 and 99.85-101.7 %, respectively **Table 17, 18.** The limit of detection for HCQ and NTZ was found to be 0.0906 µg/ml and 0.01893 µg/ml, and the limit of quantification for HCQ and NTZ was found to be 0.2746 µg/ml and 0.05737µg/ml, respectively (Table 19). The % assay was found to be 101.22% and 100.22% for HCQ and NTZ, respectively **Table 20.**

TABLE 22: SUMMARY OF VALIDATION PARAMETERS

PARAMETERS	Simultaneous equation method		First-order derivative method	
	HCQ	NTZ	HCQ	NTZ
Concentration range(µg/ml)	2-10	5-25	2-10	5-25
Wavelength(nm)	220	345	375.50	226
Regression equation	y = 0.077x - 0.006	y = 0.043x - 0.051	y = 0.0052x - 0.004	y = 0.0009x - 0.0041
Correlation Coefficient(r^2)	0.999	0.999	0.9988	0.996
Accuracy(%Recovery) (n=3)	100.51-100.88	99.97 -101.33	99.54-101.67	99.85-101.7
Repeatability (%RSD) (n=7)	0.25-0.99	0.16- 0.89	0.2-0.51	0.7-1.09
Intra-dayPrecision (%RSD) (n=3)	1.1-1.2	1.0-1.1	0.3-0.7	0.9- 1.6
Inter-dayprecision (%RSD)(n=3)	1.6-1.7	1.7-1.8	1-1.3	1.3-1.7
Assay(n=3)	101.25	101.33	101.22	100.22
LOQ(µg/ml)	0.152	0.1014	0.2746	0.05737
LOD(µg/ml)	0.0501	0.0334	0.0906	0.01893

CONCLUSION: HCQ and NTZ have been simultaneously estimated by simultaneous equation method and first-order derivative method for the synthetic mixture. The developed methods were validated according to ICH guidelines. All validation parameters like linearity, precision, accuracy compliance with ICH guidelines. The overall result obtained for both drugs suggested that both proposed methods are specific for the estimation of HCQ and NTZ. So, the development method is accurate, sensitive, and precise.

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CONFLICTS OF INTEREST: Nil

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