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Reverse phase high performance liquid chromatography method Development and Validation for Simultaneous Determination of Hydroxychloroquine Sulphate and Nitazoxanide Bulk Drug and Their Formulation

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Abstract
The present study aimed to develop and validate the simultaneous determination of hydroxychroquine sulphate and nitazoxanide in bulk and their formulation. Under isocratic conditions, the samples were analysed by the HPLC equipment using a HiQ SiL C18 (250mm, × 4.6mm, D., 5µm). The mobile phase was composed of methanol, acetonitrile, and water (30:40:30) at an average flow rate of 0.8 ml/min. Orthophosphoric acid was used to bring the pH of the water up to 3. For both medications, the validation findings of this approach indicate a correlation value of 0.999. With orthophosphoric acid serving as a mobile phase and an isoabsorptive point wavelength of 340 nm, the HPLC technique was designed for methanol: acetonitrile: water (30:40:30) (pH of water adjusted up to 3). For hydroxychroquine sulphate, the linearity range was 4–20 µg/ml , while for nitazoxanide, it was 10–50 µg/ml . The devised technique was accurate, with an intra-day accuracy of 0.5% and an inter-day precision of 1.7%, with a percentage RSD value not exceeding 2. The percentage degradation of hydroxychloroquine sulphate and nitazoxanide by acidic, basic, oxidative, thermal, and photolytic processes. The findings were statistically evaluated in accordance with the principles outlined in ICH Q2 (R1).

Keywords: Hydroxychloroquine sulphate. Nitazoxanide, RP-HPLC, Force degradation. Ich guidelines. Method validation.

1. Introduction

Hydroxychloroquine sulphate (HCQS) is a solid, crystalline molecule with the CAS number 747-36-4 [1,2] and chemical formula $C_{18}H_{28}C_1N_3O_5.H_2SO_4$ (Fig. 1). For a long time, hydroxychloroquine (HQ) was used as a malaria chemoprophylaxis and, more recently, as a therapy for chronic autoimmune illnesses [3]. This active element in pharmaceuticals may prevent the SARS-CoV-2 infection, according to early in vitro [4]. For SARS-CoV-2, nitazoxanide and hydroxychloroquine sulphate (HCQ) are used [5]. With the chemical formula C12H9N3O5S and a molecular weight of 307.28 g/mol, nitazoxanide is a [2-[(5-nitro-1,3-thiazol 2yl) carbamoyl] acetate (Fig. 2). It serves as an antiprotozoal agent. The antiprotozoal medication nitazoxanide is a tiny chemical that is sold as 500 mg pills and solutions. Currently, it is authorised for the treatment of diarrhoea [6].

One such potential contender is the antiprotozoal medication nitazoxanide, which has FDA approval. According to specialists, nitazoxanide enhances the host's inherent reactions, preventing a potentially dangerous cytokine storm. The drug's potential to improve lung damage in addition to multiple organ damage and to be a valuable addition to COVID-19 patients with comorbidities are further noteworthy facts. In addition to having a positive anti-inflammatory reaction to the cytokine storm, nitazoxanide has direct antiviral activity [7]. Currently unclear is nitazoxanide's mode of action for SARS-CoV-2. On the other hand, it has been noted that influenza involves interfering with hemagglutinin's N-glycosylation. Similar cellular targets in the upper respiratory tract and heavy glycosylation of the SARS-CoV-2 spike protein may indicate a similar mode of action [8]. The quantification of hydroxychloroquine sulphate, either on its own

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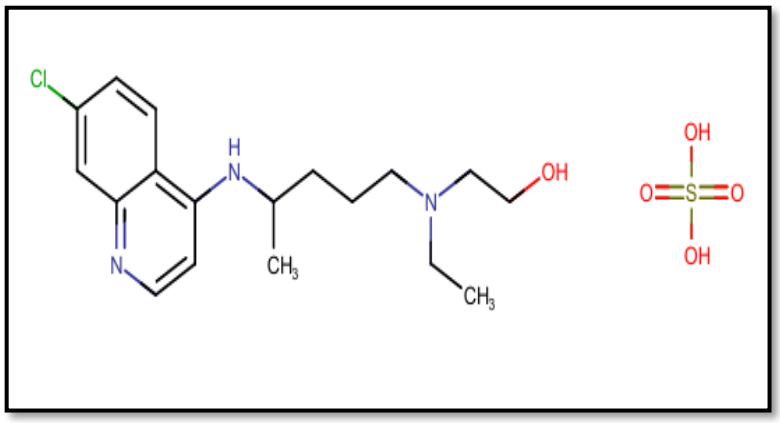
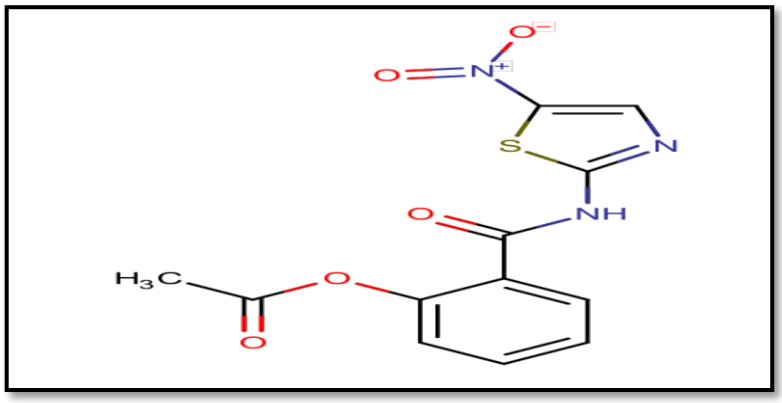
or in conjunction with other medications, has been documented using a number of HPLC techniques. Nitazoxanide can also be quantified using a number of approaches, either alone itself or in combination with other medications in different pharmacological formulations.
Determining the appropriate, straightforward, accurate, reliable, and repeatable HPLC technique for concurrently measuring hydroxychloroquine sulphate and nitazoxanide in a synthetic combination was the goal of the current study. Following ICH Guidelines, the procedure was verified Organization [9-11] \

Fig 1. Chemical structure of Hydroxychloroquine sulphate



IUPAC NAME:

Fig no. 2 Chemical structure of Nitazoxanide



7-Chloro-4-[4-(N-ethyl-N-b-hydroxyethylamino)-1-methylbutylamino]quinoline sulfate

IUPAC NAME: [2-[(5-Nitro-1,3-thiazol-2yl)carbamoyl]phenyl]ethanoate

2. Materials and methods

We acquired nitazoxanide and hydroxychloroquine sulphate standards from Yarrow Chemical Products in Mumbai, India. Hydroxychloroquine sulphate and nitazoxanide were separated chromatographically using HPLC-grade solvents and a 0.45 u membrane filter that was acquired from Millipore. Two substances were employed in the analysis: 200 mg of hydroxychloroquine sulphate and 100 ml of nitazoxanide.

2.1 Instrument

The HPLC equipment in use was the JASCO-4000 Extra, which included an autosampler and PDA detector in addition to ChromNav software for data processing. The Inertsil ODS-250 x 4.6 mm analytical column was used for optimal separation. The best conditions for drug separation were investigated using a variety of solvents with various characteristics.

2.2 UV analysis is used to choose the analytical wavelength.

Methanol is a blank or diluent.

2.2.1 Getting a standard solution ready for UV analysis

Two separate 10-ml volumetric flasks were filled with 10 mg of each of the following substances: nitazoxanide and hydroxychloroquine sulphate. I added 6 ml of diluent and sonicated to thoroughly dissolve. Diluent was added, and after

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properly mixing 1000 µg/ml, the amount was raised to 10 ml. The aforementioned stock was further diluted by 1 ml using a diluent of 10 µg/ml to make 100 ml in a volumetric flask.

2.2.2 Choice of a workable wavelength

The base was acquired by scanning 800 to 200 nm. The hydroxychloroquine sulphate and nitazoxanide standard solutions were scanned at 800 and 200 nm wavelengths in order to determine the working wavelength based on the spectra. After scanning, both UV spectra were superimposed to choose the working wavelength.

2.3 High Performance Liquid Chromatography method development

2.3.1 To prepare a typical stock solution:

Weighed 10 mg of hydroxychloroquine sulphate and 10 mg of nitazoxanide standard precisely and placed them in a 100ml amber volumetric flask. I sonicated the dissolved standard after adding roughly 100ml of diluent. Volume was adjusted to the mark using diluent and well mixed.

The standard stock concentration for hydroxychloroquine sulphate is 100 µg/ml.

Nitazoxanide Standard Stock Concentration: 100 µg/ml

Additionally, an aliquot of 1 mL of the aforesaid standard stock solution was pipetted into two separate 10 mL volumetric flasks. Its diluent is comparable to methanol in terms of strength, yielding a stock solution of 100 µg/ml . The stock solution of hydroxychloroquine sulphate and nitazoxanide yielded five standards with concentrations of 4, 8, 12, 16, 20, and 10 µg/ml for hydroxychloroquine sulphate and 10, 20, 30, 40, and 50 µg/ml for nitazoxanide.

2.3.2 Preparation of sample solution

We have produced a synthetic blend of hydroxychloroquine sulphate and nitazoxanide, which is currently undergoing clinical studies. To create the synthetic mixture, 200 mg of microcrystalline cellulose, 45 mg of lactose monohydrate, 100 mg of talc, and magnesium stearate were combined with 500 mg of nitazoxanide and 200 mg of hydroxychloroquine sulphate in a 1:2 ratio, which is equivalent to 20 tablets.

In a 100-ml volumetric flask, a synthetic combination corresponding to 10 mg of hydroxychloroquine sulphate and 25 mg of nitazoxanide was taken. dissolved in methanol and given a 15-minute sonication. Whatman filter paper was used to filter the mixture. 4 µg/ml of hydroxychloroquine sulphate and 10 µg/ml of nitazoxanide were obtained by diluting 0.5 ml of the filtrate solution with 10 ml of methanol. After that, 20 ug of the resultant solution were examined using the previously mentioned HPLC technique.

2.3.3 Optimization of mobile phase

In order to optimise the composition of the mobile phase and produce distinct, well-defined peaks, a number of experiments were conducted.

The selection of the mobile phase was based on many factors, such as theoretical plates, peak symmetry, purity index, and excellent separation peaks. Thus, several trials were conducted in order to choose a mobile phase. (Table 1)

Fig 3. Overly of spectrum of Hydroxychloroquine sulphate and Nitazoxanide

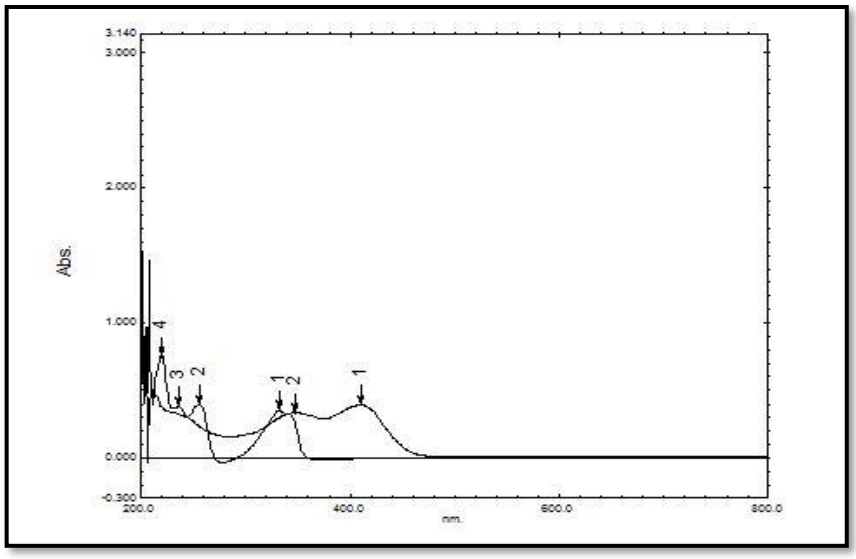

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Table 1- Composition Trails For The Mobile Phase

Sr No.	Composition of the mobile phase composition
1	ACN: Methanol (30:70)
2	Methanol: OPA ph. 3 adjusted of water (60: 40)
3	ACN: OPA (2.5) (40: 60)
4	Water: methanol: ACN (20:40:40)

2.4 Validating the High-performance liquid chromatography method
The developing analytical method was validated to confirm that it was acceptable for its intended purpose, as indicated in ICH guideline Q2(R1) [12]

2.4.1 System suitability
A system suitability test was performed to evaluate the chromatographic parameters (number of thermotical plates, separation factor, and capacity factor). Asymmetry in resolution and peak positions between two peaks. We looked at six injections of the reference solution, which included 10 µg/ml of hydroxychloroquine sulphate and 10 µg/ml of nitazoxanide.

2.4.2 Specificity
After injecting blank, standard, and sample solutions, any interference at the major peaks was seen.

2.4.3 Linearity
The process of determining linearity involved creating a standard solution containing the medicines at six distinct concentration levels: 4–20 µg/ml for hydroxychloroquine sulphate and 10–50 µg/ml for nitazoxanide. A linear equation that was produced by graphing the graph versus concentration vs. peaks was used to get the correlation coefficient value.

2.4.4 Accuracy
A recovery study was conducted to evaluate the method's accuracy. For every concentration level, three independent analyses of the sample solutions (80%, 100%, and 120%) were carried out.

2.4.5 Precision
The injection of six duplicates of the standard solution was used to achieve system accuracy, while the injection of six distinct sample solutions was used to achieve method precision. The RSD was computed.

2.4.6 Robustness
The method's resilience is its capacity to withstand little variation in a wide range of parameters. Three sample solutions were generated and assessed with three different parameters: flow rate ± 0.1 ml/min, oven temperature ± 5, and wavelength ± 3 nm. calculated the % RSD of the variable circumstance and looked at the effects.

2.4.7 The limits of detection and quantification are as follows:
The limit of quantitation, calculated using the formula below, is the smallest amount of analyte in the sample that can be quantified. The limit of detection refers to the smallest quantity of analysis in a sample that can be detected but not measured.
The limit of detection (LOD) is $3.3 \times \sigma$ per second.
LOQ is defined as $10 \times \sigma/s$.
Thus, σ denotes the standard deviation of a response. The calibration curve is shown by Sample S.

2.5 Force degradation
The new UV spectroscopy technique was tested on samples under stress settings including acid, oxidation, heat, and photolytic deterioration. The degradation percentage was computed overall.

2.5.1 Acid degradation
To 1 ml of standard stock solution, add 1 ml of 0.1 M HCL diluted in up to the volumetric flask mark with solvent. Solution kept for 24 hours. After 24 hours, the solution is filtered with a syringe filter, and a chromatogram is recorded.

2.5.2 Alkaline degradation
1 ml of standard stock solution was mixed, then 1 ml of 0.1 N NaOH was diluted up to the mark with solvent. Solution kept for 24 hours. After 24 hours, the solution is filtered with a syringe filter, and a chromatogram is recorded.

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2.5.3 Oxidation degradation

1 ml of standard stock solution was mixed with 1 ml of 3% H_2O_2 and diluted up to the mark with solvent. solution kept for 30 minutes. A 30-minute solution filter with a syringe filter and chromatogram are recorded.

2.5.4 Neutral degradation

1 ml of standard stock solution was mixed with 1 ml of distilled water and diluted up to mark with solvent and kept for 24 hours. After 24 hours, the solution is filtered with a syringe filter, and a chromatogram is recorded.

2.5.6 Thermal degradation

The drug sample should be kept on a petri plate and placed in a hot oven for 6 hours. After 6 hours, prepare a 10µg/mL solution. solution gets filtered and a chromatogram is recorded.

2.5.7 Photolytic degradation

The drug sample should be kept on a petri plate and placed in a UV chamber for 6 hours. after preparing 10µg/ml solution. solution, get a filter, and take a chromatogram

3. Result and discussions

3.1 Idealized chromatographic circumstances

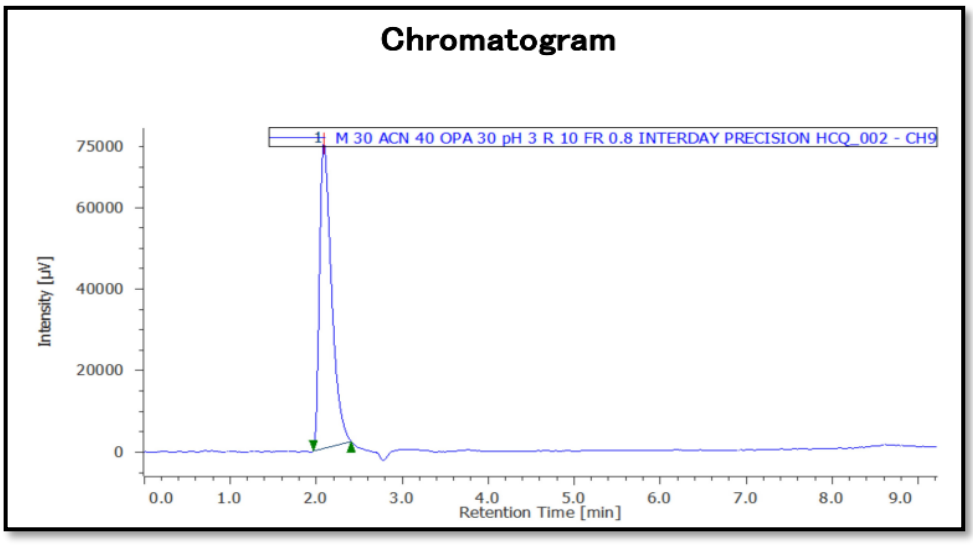
Since hydroxychloroquine sulphate and nitazoxanide had retention periods of 2.1 and 5.9 minutes, respectively, the mobile phase that was chosen was a combination of acetonitrile and water in the ratio of 30:40:30 v/v at a flow rate of 0.8 ml/min. Since the medication had a relatively excellent reaction with features of the UV spectrum as shown in (FIGURE. 3) and the chromatographic condition as tubulated in (Table II), standard drug solutions were scanned throughout a range of 800 to 200 nm. Detection was performed at 340 nm.

Table 2. Optimization Of Chromatographic Condition

Sr.No.	Specification	Description
1.	Equipment	Jasco Extrema LC system – 4000
2.	Diluent	Methanol
3.	Column	Inertsil ODS -2 (250 x 4.6mm id.,) µm
4.	Detector	Photo Diode Array (PDA)
5.	Elusion Mode	Isocratic
6.	Wavelength	(Isoabsorptive point) 340nm
7.	Flow rate	0.8 ml/min
8.	Run time	10 min
9.	Mobile phase	Water(Orthophosphoric acid) pH3:Acetonitrile:Methanol (30:40:30) v/v
10.	Injection volume	10 µl
11.	Retention time	Between 2 and 6 min
12.	Column temperature	25°C

3.2 Developing assay methods

Fig 4. Chromatogram of Hydroxychloroquine sulphate



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The analytical dosage form included 99.55% hydroxychloroquine sulphate and 100% nitazoxanide. The quantity of hydroxychloroquine sulphate and nitazoxanide present in dosage form was effectively measured using an RP-HPLC development assay. FIGURE 4,5, and 6 illustrate the chromatograms for the standard and sample solutions, respectively.

Fig 4. chromatogram of Nitazoxanide

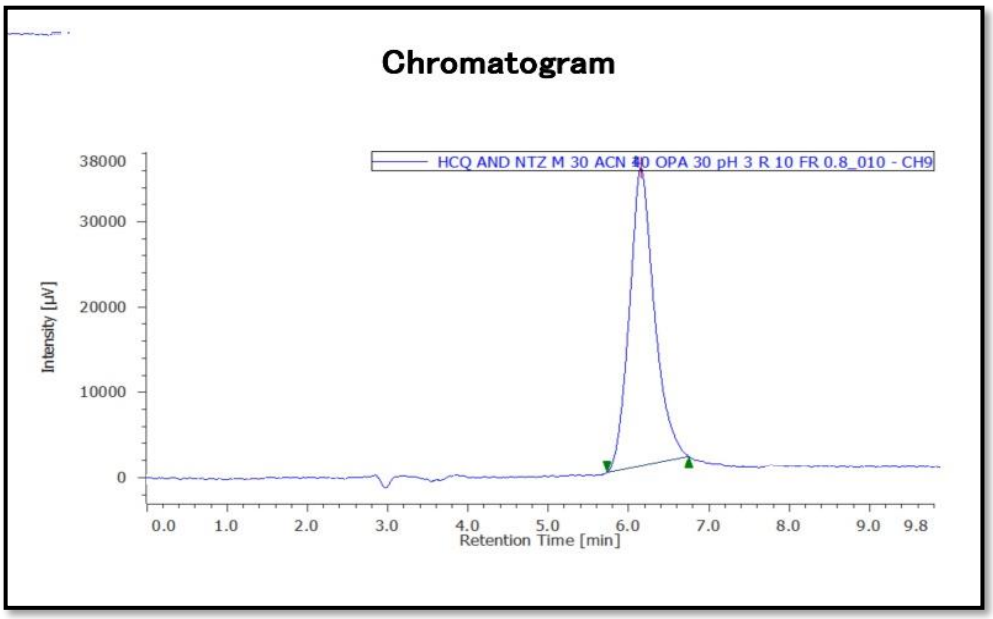
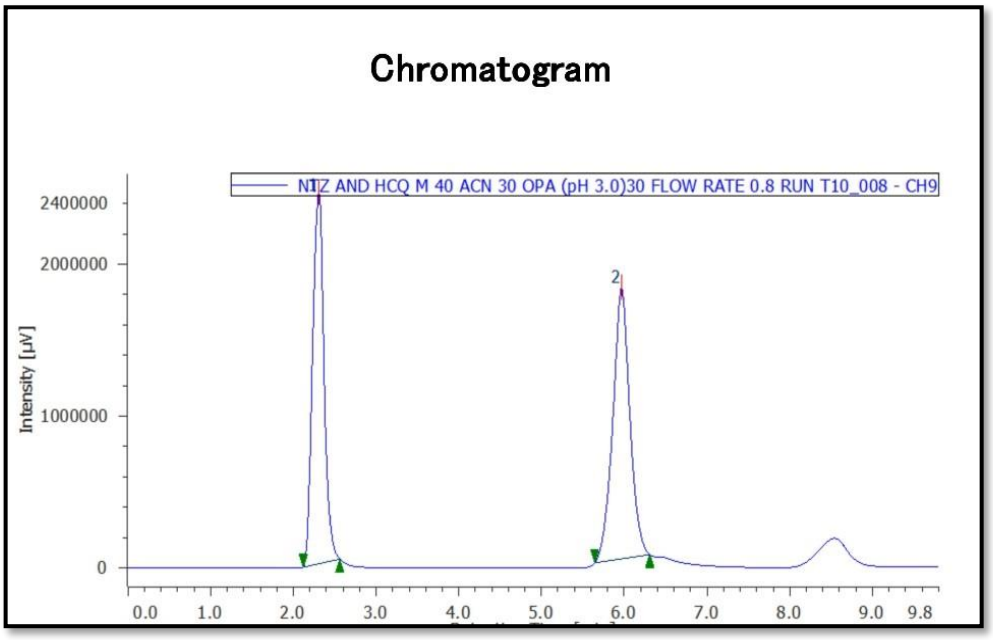


Fig 5. Chromatogram of Hydroxychloroquine Sulphate and Nitazoxanide



3.3 Validation of development

System appropriateness It was discovered that the tailing factor was less than 2.0. plate was larger than 1500 in theory. resolution for drug peaks of six standard replicates determined to be less than two was 3.5 min, which is greater than 2% RSD. Table 2 has a list of SST parameters.

3.3.1 specificity

It was found that neither contaminants nor the mobile-phase solvent diluent caused any interference. It proved to be particular as a result.

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3.3.2 Linearity

It was observed that the concentration ranges of nitazoxanide and hydroxychloroquine sulphate were linear, ranging from 10–50 µg/ml and 4–20 µg/ml, respectively. Table III and FIGURE -7 and 8 give the correlation coefficient value, which was determined to be 0.999.

Table 3 - Hydroxychloroquine Sulphate And Nitazoxanide

Sr. no	Hydroxychloroquine sulphate		Nitazoxanide	
	Concentration	Area	Concentration	Area
1	4	58638	10	249878
2	8	128925	20	522219
3	12	192125	30	757192
4	16	265994	40	1003238
5	20	332369	50	1261942

Fig 6. Linearity of Hydroxychloroquine sulphate

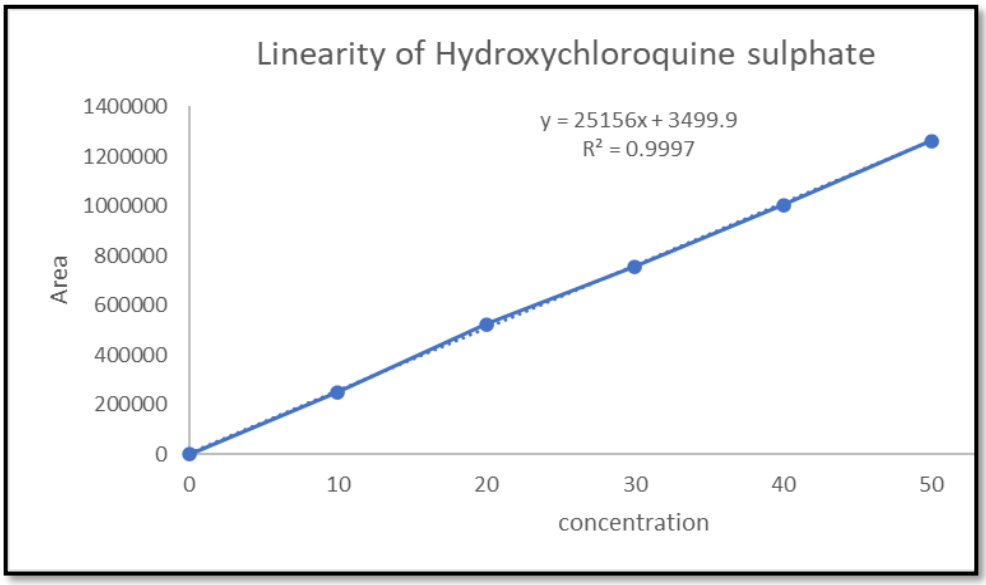
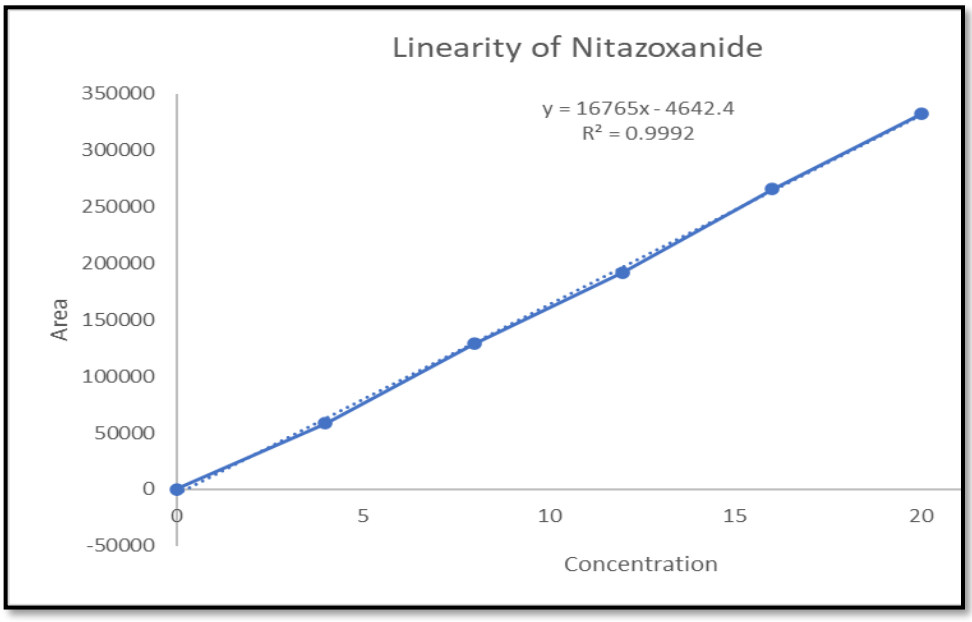


Fig 7. Linearity of Nitazoxanide



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3.3.3 Precision

The created technique was determined to be exact since the RSD for system and method precision was discovered to be less than 2%, namely 1.40% and 1.03% for hydroxychloroquine sulphate and 1.766% and 1.59% for nitazoxanide, respectively.(Table 4,5,)

Table 4. Precision Of Hydroxychloroquine Sulphate(N=6±Sd)

Sr no	Hydroxychloroquine sulphate	
	Intraday(Area)	Interday(Area)
1	193125	154456
2	190234	154578
3	193437	156874
4	188485	153845
5	194456	156469
6	188546	157682
	Std Deviation : 0.15 %RSD : 1.45	Std Deviation : 0.09 % RSD : 1.03

Table 5. Precision Of Nitazoxanide(N=6±Sd)

Sr no	Nitazoxanide	
	Intraday(Area)	Interday(Area)
1	760100	569193
2	726761	559861
3	759270	557061
4	762609	579804
5	751877	556548
6	752397	562564
	Std Deviation : 0.52 %RSD : 1.76	Std Deviation : 0.35 % RSD : 1.59

3.3.4 Accuracy

For hydroxychloroquine sulphate, the mean and recovery were determined to be 99.81% (80%), 100.41% (100%), and 99.57% (120%), whereas for nitazoxanide, the values were 99.09% (80%), 100.24% (100%), and 101.19% (120%). Table 9 provides the findings of the recovery research for determining accuracy. A range of 98% to 102% was determined to be the recovery percentage.(Table 6,7)

Table 6. Accuracy Data Of Hydroxychloroquine Sulphate(N=3)

Concentration(x)	Abs(Y)	Mean	X'	% Recovery
80%	128246			
80%	117571	125180.7	7.189876	99.85939
80%	129725			
100%	138677			
100%	139506	138662.3	7.994031	99.92539
100%	137804			
120%	159934			
120%	151765	151634	8.767766	99.63371
120%	143203			

Table 7. Accuracy Data Of Nitazoxanide (N=3)

Concentration	Area	Mean area	%RSD Concentration obtained	%RSD
80%	201275	202918	7.927258	99.09072
80%	202918			
80%	211475			
100%	255672	255672	10.02433	100.2433
100%	250539			
100%	266806			
120%	303736	308991	12.14387	101.1989
120%	310648			
120%	308991			

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3.3.5 Robustness

The robustness research conducted revealed that the devised technique is robust, with the RSD falling within the allowed range (less than 2%) at various wavelengths, flow rates, and column oven temperatures.(Table 8)

Table 8. Data For Robustness Study(N=3)

Parameter			%RSD	
			HCQ	NTZ
Flow rate	0.9 min/ml	AREA CURVE	0.56	0.27
		RT	1.03	1.04
		NTP	0.21	0.97
	0.7 min/ml	AREA CURVE	0.21	0.47
		RT	1.30	0.66
		NTP	0.99	0.85
Wavelength	343 nm	AREA CURVE	0.53	1.05
		RT	0.45	1.01
		NTP	0.92	1.08
	337 nm	AREA CURVE	0.18	0.97
		RT	0.23	1.01
		NTP	0.73	0.69
Temperature	20 ^{0}C	AREA CURVE	0.31	0.46
		RT	0.40	0.37
		NTP	0.76	0.39
	30 ^{0}C	AREA CURVE	1.18	0.93
		RT	0.65	0.66
		NTP	1.38	0.85

3.3.6Limit of detection and limit of quantification

The following medication concentrations were determined: nitazoxanide (0.857 µg/ml) and (2.59 µg/ml), and hydroxychloroquine sulphate (0.571 µg/ml) and (1.730 µg/ml)

.

3.4 Force degradation

To evaluated the stability condition of the development Hplc method stress in condition as acid base, oxidation, thermal and photolytic degradation. In all studies % degradation was calculated. (Table 9

Table 9. Force Degradation(N=3)

SR NO	Hydroxychloroquine Sulphate			Nitazoxanide		
	Degradation condition	time	% Degradation	Degradation condition	time	% Degradation
1	Oxidation	30min	Stable	Oxidation	30min	Stable
2	Acid	24hrs	Stable	Acid	24hrs	Stable
3	Base	24hrs	Stable	Base	24hrs	Stable
4	Neutral	24hrs	Stable	Neutral	24hrs	Stable
5	Thermal	6hrs	Stable	Thermal	6hrs	Stable
6	Photolytic	6hrs	Stable	Photolytic	6hrs	Stable

Conclusion

The ICH guideline for method validation was followed in order to identify which parameters for hydroxychloroquine sulphate and nitazoxanide were suitable for regular quantitative analysis. Since the bottom bounds of quantification and detection were found to be constant, the developed method is reliable, accurate, sensitive, and precise.

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Conflicts of interest

The authors hereby declare that they do not have any conflicts of interest. The authors are particularly responsible for the content and preparation of this article.

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