IJCRT.ORG ISSN : 2320-2882

INTERNATIONAL JOURNAL OF CREATIVE RESEARCH THOUGHTS (IJCRT)

An International Open Access, Peer-reviewed, Refereed Journal

"ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF IVERMECTIN AND NITAZOXANIDE IN SYN-THETIC MIXTURE"

Kshatriya Shivani Amit Kumar[1], Divya Jadav[2], Mitali Dalwadi[3], Dr. Umesh Upadhyay[4]
Student[1, 2], Professor[3], Principal[4]
Sigma Institute of Pharmacy, Bakrol, Vadodara, Gujarat, 390019, India

ABSTRACT

A simple, sensitive and accurate RP-HPLC method was developed for simultaneous estimation of Ivermectin and Nitazoxanide in syntheticmixture. The Reversed phase High Performance Liquid Chromatography Method was developed and validated for the determination of both the drugs. RP-HPLC method gives us good resolution and better separation for both the drugs. The separation was carried out by using Cybersil C18 Column. (250 mm× 4.6mm × 5 µm) with mobile phase consisting of Methanol: Acetonitrile: phosphate buffer (35:35:30) % v/v/v (pH :3). The Mobile Phase was delivered at the Flow Rate of 1mL/min. The Eluent was monitored at wavelength 235nm and sharp and symmetrical peak for Ivermectin and Nitazoxanidewas found at 2.98 min and 7.38 min respectively. The method wasvalidated for Linearity, Accuracy, Precision, Robustness, Specificity and System suitability parameters. The method was found to be linear over the concentration range of (6-36 µg/mL) for Ivermectin and (100-600 µg/mL) for Nitazoxanide with coefficient R^2 0.9988 and 0.9927 respectively. Therefore, proposed method can be successfully used for routine analysis of Ivermectin and Nitazoxanide in Bulk as well as Synthetic Mixture. respectively. Therefore, proposed method can be successfully used for routine analysis of Ivermectin and Nitazoxanide in Bulk as well as Synthetic Mixture.

Key Words: Ivermectin, Nitazoxanide, RP-HPLC method, Stationary phase, Mobile Phase,

Column, Wavelength.

INTRODUCTION:

INTRODUCTION TO DISEASE: [1-3]

In December 2019, a new infectious respiratory disease emerged inWuhan, Hubei province, China. An initial cluster of infections was linked to Huanan seafood market, potentially due to animal contact.Subsequently, human-to-human transmission occurred and the disease, now termed coronavirus disease 19 (COVID-19) rapidly spread within China and all over the world. A novel coronavirus, SARS-coronavirus 2 (SARS-CoV-2), which is closely related to SARS-CoV, was detected in patients, and is believed to be the etiologic agent of the new lung disease.The causative agent of the current COVID-19 pandemic, SARS-CoV-2, is a single stranded positive sense RNA virus that is closely related to severe acute respiratory syndrome coronavirus (SARS-CoV).

The World Health Organization (WHO) has declared the coronavirus disease 2019 (COVID- 19) a pandemic.

DRUGS USE IN COVID-19:

The reports suggested that Ivermectin's nuclear transport inhibitory activity may be effective against SARS-CoV-2. Interestingly, it has been postulated that the FDA-approved drug Ivermectin inhibits the replication of SARS-CoV-2 in vitro whereas a single treatment was able to provoke approximately 5000-fold reduction in viral load within 48h.

Nitazoxanide is originally developed as an antiprotozoal agent and has a broad-spectrum antiviral activity undergoing development for thetreatment of influenza and other viral respiratory infections. In addition to its antiviral activity, Nitazoxanide 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.

Nitazoxanide could improve outcomes in patients infected with MERS- CoV by suppressing overproduction of pro-inflammatory cytokines, including IL-6. Nitazoxanide has been tested in clinical setting for the treatment of acute uncomplicated influenza.

The rationale of the use of Ivermectin and Nitazoxanide combination for treatment of COVID-19 infected patients is based on the antiviral and anti-inflammatory activity of the selected drugs. Since the two drugs exhibit different modes of action, it would be of value in containing the viral infection through targeting different sites in the pathophysiology of the disease.

INTRODUCTION OF DRUGS: [3-5]

INTRODUCTION OF IVERMECTIN:

Ivermectin has anti-parasitic effect along with anti-viral activity against a broad range of viruses in vitro. Ivermectin was identified as an inhibitor of interaction between the human immunodeficiency virus-1 (HIV-1) integrase protein (IN) and the importin (IMP) α/β1 heterodimer responsible for IN nuclear import. Ivermectin has since been confirmed to inhibit HIV-1 replication. Importantly, Ivermectin has been demonstrated to limit infection by RNA viruses such as West Nile Virus and influenza. This broad-spectrum activity is

believed to be due to the reliance by many different RNA viruses on IMPα/β1 during infection. Ivermectin has similarly been shown to be effective against the DNA virus pseudorabies virus (PRV) both in vitro and in vivo. Finally, Ivermectin was the focus of a phase III clinical trial in Thailand against DENV infection (dengue infection), in which a single daily oral dosewas observed to be safe and resulted in a significant reduction in serum levels of viral NS1 protein, but no change in viremia or clinical benefit was observed.

The causative agent of the current COVID-19 pandemic, SARS- CoV-2, is a single stranded positive sense RNA virus that is closely related to severe acute respiratory syndrome coronavirus (SARS-CoV). Studies on SARS-CoV proteins have revealed a potential role for IMPα/β1 during infection in signal-dependent nucle-ocytoplasmic shutting of the SARS-CoV nucleocapsid protein that may impact host cell division.

Taken together, these reports suggested that Ivermectin's nuclear transport inhibitory activity may be effective against SARS-CoV-2. Interestingly, it has been postulated that the FDA-approved drug Ivermectin inhibits the replication of SARS-CoV-2 in vitro whereas a single treatment was able to provoke approximately 5000-fold reduction in viral load within 48h.

INTRODUCTION OF NITAZOXANIDE:

Nitazoxanide is a small-molecule antiprotozoal drug marketed as tablets (500mg) and suspension is currently approved for treating diarrhea caused by the protozoa cryptosporidium or Giardia inimmunocompetent adult and children.

Nitazoxanide (NTZ) a US-FDA approved antiprotozoal drug, as one such promising candidate.

Nitazoxanide which is reported to exert broad-spectrum antiviral activity against various viral infections, revealed good in vitro activity against SARS-CoV-2 in cell culture assays, suggesting potential for repurposing in COVID-19.

NTZ displays the potential to boost host innate immune responsesand thereby tackle threatening cytokine storms. The possibilities of improving lungs as well as multiple organ damage and providing value addition to COVID-19 patients with comorbidities are other important facets of the drug.

The main role of NTZ in fighting COVID19 pathogenesis at multiple levels highlighting the great promise the drug exhibits. The in- silico data and in vitro efficacy in cell lines confirms the promise of Nitazoxanide. Several approved clinical trials world over further substantiate leveraging Nitazoxanide for COVID-19 therapy.

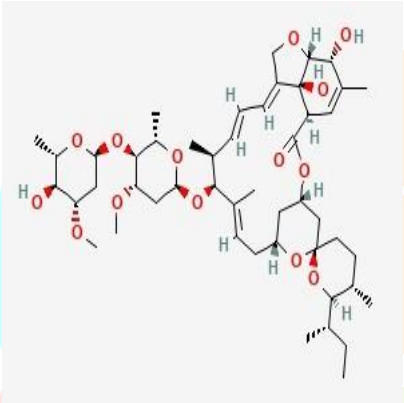
Nitazoxanide exhibits direct antiviral action and hosts a beneficial anti-inflammatory response to the cytokine storm. The mechanism of action of Nitazoxanide for SARS-CoV-2 is currently unknown. However, for influenza it has been reported to involve interference with N- glycosylation of hemagglutinin. Since the SARS-CoV-2 spike protein is also heavily glycosylated with similar cellular targets in the upper respiratory tract, a similar mechanism of action may be expected.

Reason for selecting NTZ against 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. In addition, it 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.

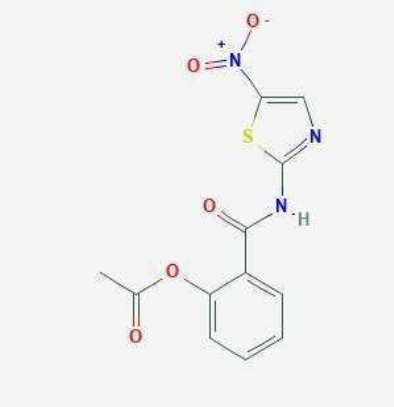
Ivermectin is official in IP, BP and USP [11-13] which describes HPLC method for its estimation. Various methods like UV Spectroscopy, HPLC Method, RP-HPLC method, UPLC Method, HPTLC Method [14-29] for determination of Ivermectin are reported in literatures.

Nitazoxanide is not official in any of the pharmacopoeias but literature survey revealed that V Spectroscopy, HPLC Method, RP-HPLC method, HPTLC Method, TLC methods [30-42] have been developed for the estimation of Nitazoxanide.

Structure of



Ivermectin



Nitazoxanide

IUPAC NAME OF IVERMECTIN: [6-10] - 6'-[(2S)-butan-2-yl]-21,24-dihydroxy-12-[(2R,4S,5S,6S)-5-[(2S,4S,5S,6S)-5-hydroxy-4-methoxy-6-methyloxan-2- yl] oxy-4-methoxy-6-methyloxan-2-yl]oxy-5',11,13,22-tetramethylspiro [3,7,19-trioxatetracyclo [15.6.1.14,8.020,24] pentacosa-10,14,16,22-tetraene-6,2'-oxane]-2-one.

IUPAC NAME OF Nitazoxanide: - 2-[(5-nitro-1,3-thiazol-2-yl) carbamoyl] phenyl] acetate

MATERIALS AND METHODS: - API: Sample of Ivermectin (IVE) procured from Zota life sciencePvt Ltd, Surat, Gujarat. Nitazoxanide (NIT) as procured from Globela life science Pvt Ltd, Surat, Gujarat.

Experimental Condition:

Table:1 List of Instruments and Apparatus:

Sr. No.	Instrument	Model No	Manufacturer
1	**HPLC Column:** Cyber-Sil, C18 (250mm x 4.6mm, 5 µg) **Injector:** Rheodyne injector**Software:** Cyberlab-100 **Detector:** UV Detector	LC-100	Cyber Lab
2	UV Visible Spectrophotometer	UV 1700	Shimadzu
3	FT-IR	Cary 630	Agilent Technologies
4	pH meter	MAC/SR No. 1706	Digital pH meter
5	Analytical Weighing Balance	AUW 220D	Shimadzu
6	Ultra Sonicator	-	Trans-o-sonic

CHEMICALS AND REAGENTS:

Table: 2 List of reagents:

Sr. No,	Reagent	Grade	Manufacturer
1	Methanol	HPLC	Ranchem Ltd.
2	Acetonitrile	HPLC	Ranchem Ltd.
3	Water	HPLC	Ranchem Ltd.
4	Potassium Dihydrogen Phosphate	AR Grade	Ranchem Ltd.
5	Sodium Hydroxide	AR Grade	Ranchem Ltd.
6	Triethyl amine	AR Grade	Ranchem Ltd.

IDENTIFICATION OF API:

Melting point of Ivermectin (IVE) and Nitazoxanide (NIT) was carried out by Capillary Tube Method in paraffin bath. The melting point studywas performed in Thieles tube that was filled with liquid paraffin. 50 mg of powdered drug was filled in capillary that was attached with the tip of thermometer with the help of thread. Then thermometer was placed in Thiele's tube and was heated. Temperature at which the drug powder melted was noted down. It was performed in triplicate.

SOLUBILITY STUDY:

Solubility of Ivermectin (IVE) and Nitazoxanide (NIT) was performedusing various solvents like water, methanol, acetonitrile etc.

Table: 3 Solubility Criteria as Per Pharmacopoeia:

Descriptive term	Parts of solvent required for part of solute
Very soluble	Less than 1
Freely soluble	From 1 to 10
Soluble	From 10 to 30
Sparingly soluble	From 30 to 100
Slightly soluble	From 100 to 1000
Very slightly soluble	From 1000 to 10000
Practically Insoluble	10000 or more

IR SPECTRA:

Drug was placed in sample compartment of FT-IR instrument, where it was scanned in the range of 4000 - 650 cm^{-1}. Principle IR peaks were observed for drug are shown in table and from this, it was concluded that drugs were found to be authentic.

UV ABSORPTION STUDY:

Accurately weighed 10 mg of Ivermectin (IVE) and Nitazoxanide (NTZ) were transferred separately in 10 ml volumetric flasks, dissolved in small volume of methanol and then volume was adjusted to the mark with methanol to obtain concentration of 1000 µg/ml. These solutions were further diluted to obtain concentration of 10 µg/ml. These standard solutions of Ivermectin (IVE) and Nitazoxanide (NTZ) in methanol were scanned in UV range, 200-400 nm in 1 cm cell using methanol as blank and maximum absorbance was measured for selection of λ max of Ivermectin (IVE) and Nitazoxanide (NTZ)

METHOD DEVELOPMENT AND VALIDATION:

METHOD DVELOPMENT

Establishment of Optimal Conditions for the Development of HPLC Methods Various conditions should be optimized throughout the development of analytical methods in order to develop sensitive, accurate, and repeatable methods.

Selection of Diluent:

Based on solubility, Ivermectin (IVE) and Nitazoxanide (NTZ) were slightly soluble in methanol. Hence, methanol was selected as diluent.

Selection of Mobile Phase:

The mobile phase selection method took into account water, buffer, buffer pH, organic solvent, and buffer-to-solvent ratio. The nature, physicochemical qualities, molecular weight, and solubility of the sample all

influence the HPLC procedure selection. The use of a buffer is required for pH regulation. The acidic component's pH is kept low, while the base component's pH is kept high. The mobile phase for the HPLC system was optimized using separation, peak purity, tailing factor, theoretical plate, and other parameters. To achieve a sharp peak of Ivermectin (IVE) and Nitazoxanide (NTZ), numerous mobile phasesin varied compositions and pH levels were attempted.

Selection of Wavelength:

An ideal wavelength is the one that gives Maximum response for the drugs that was to be detected. For selection of wavelength U.Vspectrophotometer is used or using HPLC assisted with UV detector, UV overlay spectra Ivermectin (IVE) and Nitazoxanide (NTZ) were obtained. For High Performance Liquid Chromatography 235 nm was selected wavelength where both drug show good absorbance.

Preparation of Mobile Phase:

Preparation of Buffer: Dissolve 3.40 g of potassium dihydrogen phosphate R in 900 ml of water R. Adjust to pH 3.0 with phosphoric acid and dilute to 1000.0 ml with water R. Mix well and sonicate. Filter through 0.45 µm membrane filter paper.

Prepare a mixture of Methanol: Acetonitrile: phosphate buffer (35:35:30) in the volume ratio 35:35:30 v/v/v. Adjust pH 3 with ortho phosphoric acid. Filter and mix well and sonicate to degas the mixture.

Selection of column:

Ivermectin (IVE) and Nitazoxanide (NTZ) are polar in nature. So, C18analytical column were selected for HPLC method. The column was used Cybersil C18 column (250 mm × 4.6 mm, 5 µm) was used for the development of the method.

Preparation of Stock solution:

Accurately weighed and transferred about 12 mg of Ivermectin (IVE) and 100 mg of Nitazoxanide (NTZ) in to 100 ml of volumetric flask, 50 ml of methanol was added and sonicated to dissolve. Volume was made up to the mark with diluent. Concentration of Ivermectin (IVE)is 120 µg/ml and Nitazoxanide (NTZ) 1000 µg/ml. Further diluted 10 ml of Ivermectin (IVE) solution to 100 ml volumetric flask and volume was make up to the mark with diluent. Further diluted 5 ml of Nitazoxanide (NTZ) solution to 100 ml volumetric flask and volume was make up to the mark with diluent. Concentration of Ivermectin (IVE) is 12 µg/ml and Nitazoxanide (NTZ) 100 µg/ml. The optimum wavelength was selected for the estimation was 235 nm where gives maximumabsorbance, which was obtained by scanning solution in the range of 200-400 nm in UV spectrophotometer.

Preparation of Ivermectin (IVE) solution:

Pipette out 0.5, 1.0, 1.5, 2.0, 2.5, and 3 ml of Ivermectin (IVE) from 120 µg/ml in 10 ml volumetric flask and diluted up to 10 ml with the diluent to get Concentration of Ivermectin (IVE) i.e. 6, 12, 18, 24, 30and 36 µg/ml.

Preparation of Nitazoxanide (NIT) solution:

Pipette out 1, 2, 3, 4, 5 and 6 ml of Nitazoxanide (NIT) from 1000 µg/ml in 10 ml volumetric flask and diluted up to 10 ml with diluent to get Concentration Nitazoxanide (NIT) is 100, 200, 300, 400, 500 and 600 µg/ml.

System suitability test:

System suitability test was performed every time a method was used either before or during analysis. After completion of method, the results of each system suitability test were compared with defined acceptance criteria and if they passed, the method is deemed satisfactory on that occasion. Capacity factor (k'), theoretical plate (N), tailing factor (T) and RSD were evaluated for six replicate injections of the IVE and NTZ at a concentration of 12 µg/ml and 100 µg/ml. Acceptance criteria: tailing factor < 2, theoretical plate > 2000.

METHOD VALIDATION

LINEARITY AND RANGE:

The linearity is expressed in terms of correlation co-efficient of linear regression analysis. The linearity response was determined by analyzing 6 independent level of calibration curve in the range of 06-36µg/ml for IVE and 100-600 µg/ml for NTZ, in five replicates.

The calibration curve of peak area vs. concentration was plotted andcorrelation coefficient was determined and regression line equation obtained when scanned in HPLC at 235 nm.

LIMIT OF DETECTION AND LIMIT OF QUANTITATION:

The limit of detection (LOD) and the limit of quantification (LOQ) ofthe drug were derived by calculating the signal-to-noise ratio (S/N, i.e.,

3.3 for LOD and 10 for LOQ) using the following equations designatedby International Conference on Harmonization (ICH) guidelines.

LOD = 3.3 x (σ/S)LOD = 10 x (σ/S)

Where, σ - intercepts of the 5 calibration curves; S = Mean slope of the5 calibration curves.

PRECISION:

The precision of method was evaluated in terms of repeatability andintermediate precision.

REPEATABILITY:

The repeatability was checked by scanning and measurement of the responses of solution of IVE (18 µg/ml) and NTZ (300 µg/ml) without changing the parameters of the proposed methods. The procedure was repeated six times and % RSD was calculated.

INTERMEDIATE PRECISION:

The intraday and inter day precision was determined by analyzing corresponding response in triplicate on the same day and on 3 different days over a period of 1 week for 3 different concentrations of standard solution of IVE (06, 18, 36 µg/ml) and NTZ (100, 300, 600 µg/ml).

ACCURACY:

It is carried out to determine the suitability and reliability of the proposedmethods.

The Accuracy was determined by calculating the % recovery of IVE and NTZ from the formulation prepared by the standard addition method in which known amount of standard sample of IVE (6, 12, 18 µg/ml) and NIT (200, 250, 300 µg/ml) at 80%, 100%, 120% levels were added tothe pre analysed samples of IVE (06 µg/ml) and NTZ (250 µg/ml).

The procedure was repeated 3 more times and the recovered amounts of IVE and NTZ was calculated at each level and % recovery was reported.

SPECIFICITY:

The specificity of the method was ascertained by comparing retention time of IVE and NTZ in chromatogram obtained from synthetic mixture and standard drug.

The standard concentration of IVE and NTZ taken was 18 and 300 µg/mlrespectively.

ROBUSTNESS:

The small deliberate variations in liquid chromatography conditions were used to evaluate the robustness of the assay method.

Robustness of the method was determined in triplicate at concentration levels of three changes were measured for both IVE and NTZ taken was 18 and 300 µg/ml respectively.

Effect of change in flow rate (0.9 ml/min, 1.0 ml/min, 1.1 ml/min), effectof change in detection wavelength (230 nm, 235nm, and 240 nm), and effect of change in volume of phosphate buffer (27 ml, 30 ml, 33ml) in mobile phase was studies. The standard concentration of IVE and NTZ taken was 18 and 300 µg/ml respectively injected. The mean and % RSD values of the peak area were calculated.

APPLICATION OF ANALYTICAL METHOD FOR ANALYSIS OF SYNTHETIC MIXTURE DOSAGE FORM:

Table:4 Preparation of Synthetic Mixture

Sr. No.	Excipients/API	Concentration for(01 Tab)	Concentration for (05 Tab)
1.	Ivermectin	12 mg	0.06 gm
2.	Nitazoxanide	500 mg	2.5 gm
3.	Starch	25.6 mg	0.128 gm
4.	MCC (Avicel PH-101)	15.36 mg	0077 gm
5.	Mg. Stearate	5.12 mg	0.026 gm
6.	Lactose Powder q.s. to600 mg	41.92 mg	0209 gm

From the synthetic mixture, powder equivalent to 12 mg of IVE and 500 mg of NTZ was transferred to 10 ml volumetric flask, dissolved and diluted up to the mark with methanol. This solution was sonicated for 15 min. The solution was filtered through Whatman filter paper no. 42.

Pipette out 1 ml of solution and transferred to the 10 ml volumetric flask and diluted up to the mark with methanol. Furthermore, pipette out 1 ml of solution and transferred to 10 ml volumetric flask and diluted up tothe mark with methanol to get strength of 12 µg/ml of IVE and 500 µg/mlof NTZ. The 20 µl of sample were injected as per optimizedchromatographic conditions and detection was carried out at 235nm. The areas were determined and quantification was carried out by keeping this value in regression equation.

RESULTS AND DISCUSSION:

MELTING POINT STUDY:

The observed melting point of each mentioned drugs were similar to the standard melting point reported for respective drugs as evident from Table.

Table: 5 Melting Point Study:

Drugs	Reported Melting Point (°C)	Observed Melting Point (°C)
Ivermectin (IVE)	155 °C [11]	154-156 °C
Nitazoxanide (NTZ)	198-200 °C [13]	196-199 °C

N = 3, Mean of 3 replicates.

SOLUBILITY STUDY:

The solubility of substance fundamentally depends on the physical and chemical properties of the solute and solvent as well as temperature, pressure and the Ph of the solution. The solubility profile is used for solvent selection in method development. The solubility of each drug in different solvent in shown in Table 6.

Table: 6 Solubility Study:

DRUGS	IVERMECTIN (IVE)	NITAZOXANIDE (NTZ)
Water	Insoluble	Slightly soluble
Ethanol, Methanol	Slightly soluble	Slightly soluble
Acetonitrile	Slightly soluble	Slightly soluble

UV ABSORPTION STUDY:

UV spectra of drugs in methanol depicted that the wavelength maximaof Ivermectin (IVE) and Nitazoxanide (NTZ) were at 235 nm and 344nm respectively as shown in Figure 1.

For High Performance Liquid Chromatography both drug Ivermectin(IVE) and Nitazoxanide (NTZ) show good absorbance at 235 nm, so it was selected as detection wavelength.

For High Performance Liquid Chromatography both drug Ivermectin(IVE) and Nitazoxanide (NTZ) show good absorbance at 235 nm, so it was selected as detection wavelength.

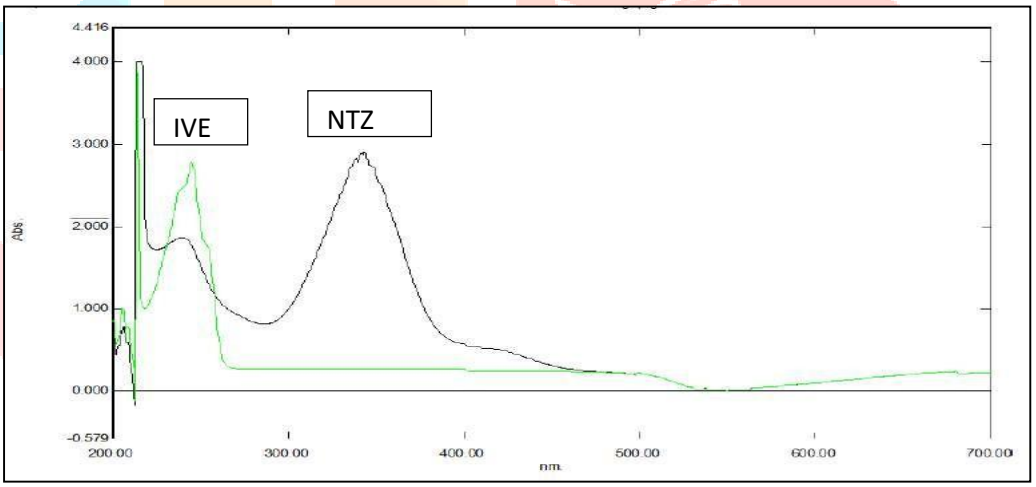


Figure 1: Overlain UV Spectrum in methanol

IR SPECTRA:

An IR spectrum of Standard sample shown in figure 2 and reference sample figure 3 observed frequency of Ivermectin was within the standard frequency range.

So, concluded that given sample content was Ivermectin (IVE) and Nitazoxanide (NTZ) results are shown in table 7 and 8.

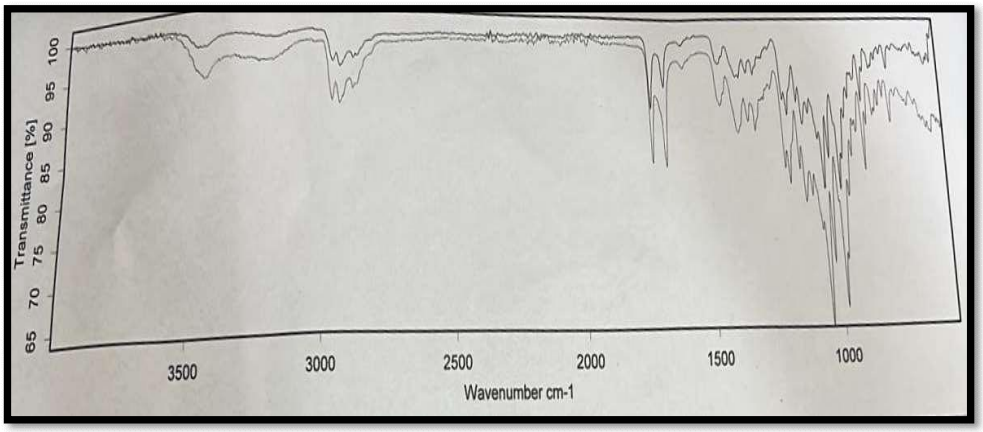


Figure: 2 FT-IR spectra of Standard IVC [15]

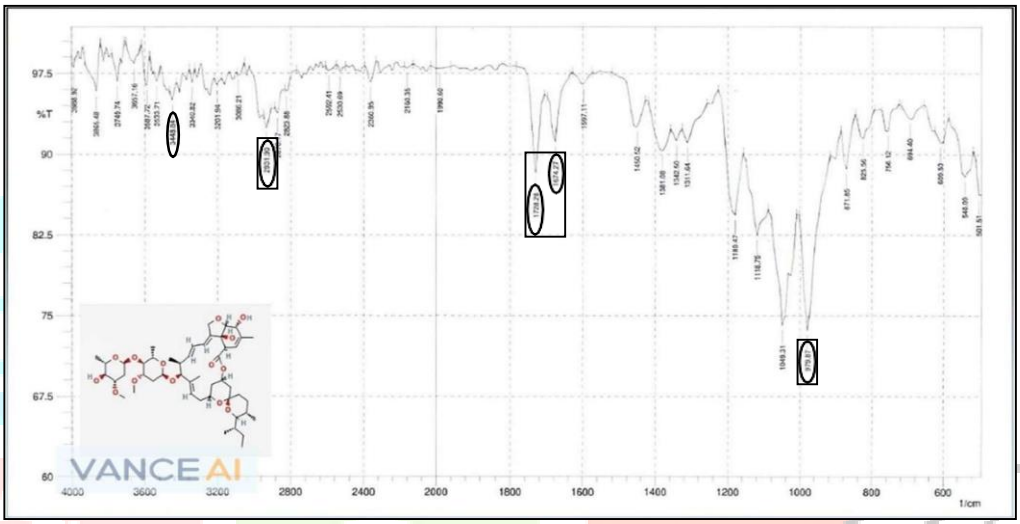


Figure: 3 Observed IR Spectrum of Ivermectin (IVE)Table 6.3 IR value for Ivermectin (IVE)

Table:7 IR Value for Ivermectin (IVC):

Sr. No.	Functional Group	Reported Wavenumber (cm $^{-1}$)	Observed Wavenumber (cm $^{-1}$)
1.	O-H stretching	3550-3200	3443.84
2.	O-H stretching Carboxylic acid	3300-2500	2931.90
3.	C=O stretching	1750-1725	1728.28
4.	C=O stretching conjugated ketone	1685-1666	1674.27
5.	Alkene	980-960	979.87

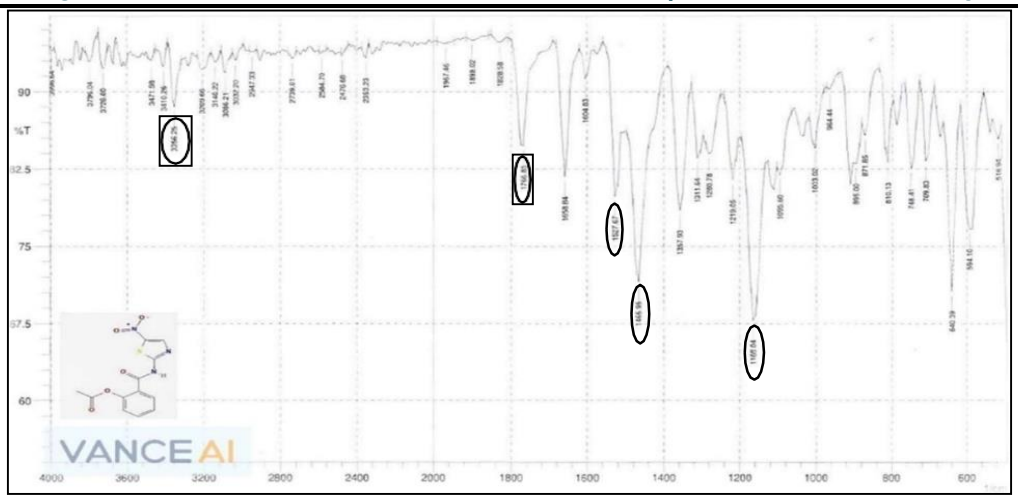


Figure: 4 Observed IR Spectrum of Nitazoxanide (NTZ)

Table 8 IR Value for Nitazoxanide (NTZ):

Sr. No.	Functional Group	Reported Wavenumber (cm^{-1})	Observed Wavenumber (cm^{-1})
1.	N-H stretching	3500-3300	3356.25
2.	Carboxylic acid C=O	1770-1750	1766.85
3.	N=O stretch	1600-1500	1527.67
4.	O-H bending	1480-1395	1465.95
5.	C-N stretching	1250-1020	1165.04

CONCLUSION:

- From the obtained results of identification study, it can be interpretedthat the Ivermectin (IVE) and Nitazoxanide (NTZ) are pure and authentic.

METHOD DEVELOPMENT AND VALIDATION:

Table:9 Method Development Trial:

TRIAL NO.	CONDITION	OBSERVATION
1	**Column:** C_{18} (250 mm x 4.6 mm),5 µm **Mobile Phase:** Acetonitrile: Water (90:10 V/V) **Flow Rate:** 1 ml/min **Wavelength:** 235 nm **Injection Volume:** 20 µl	Peak Splitting
2	**Column:** C 18 (250 mm x 4.6 mm),5 µm **Mobile Phase:** Acetonitrile: Water (60:40) **Flow Rate:** 1 ml/min	Peak splitting was observed.

	Wavelength:235nm Injection **Volume:** 20 µl	
3	**Column:** C 18 (250 mm x 4.6 mm),5 µm **Mobile Phase:** Acetonitrile: Water: Methanol (50:5:45 v/v/v) **Flow Rate:** 1 ml/min **Wavelength:** 235 nm **Injection Volume:** 20 µl	Peak shape was not proper and peak tailing observed
4	**Column:** C 18 (250 mm x 4.6 mm),5 µm **Mobile Phase:** Acetonitrile: Water: Methanol (50:5:45 v/v/v) (PH:5) **Flow Rate:** 1 ml/min **Wavelength:** 235 nm **Injection Volume:** 20 µl	Peak shape was not proper.
5	**Column:** C 18 (250 mm x 4.6 mm),5 µm **Mobile Phase:** Methanol: Water: Acetonitrile (50:5:45 v/v/v) (PH:6) **Flow Rate:** 1 ml/min **Wavelength:** 235 nm **Injection Volume:** 20 µl	Peak tailing observed and resolution is less
6	**Column:** C 18 (250 mm x 4.6 mm),5 µm **Mobile Phase:** Acetonitrile: Methanol: Phosphate buffer (40:30:30 % V/V/V) 7.5 Ph of buffer **Flow Rate:** 1 ml/min **Wavelength:** 235 nm **Injection Volume:** 20 µl	Shorter retention time of Drug and peak shape was proper, resolution is less and theoretical plate is less than 2000
7	**Column:** C 18 (250 mm x 4.6 mm),5 µm **Mobile Phase:** Methanol: Acetonitrile: phosphate buffer (35:35:30) (Ph :3) **Flow Rate:** 1 ml/min **Wavelength:** 235 nm **Injection Volume:** 20 µl	Shorter retention time of Drug and peak shape was proper, resolution good

- **CHROMATOGRAMS OF TRIALS:**

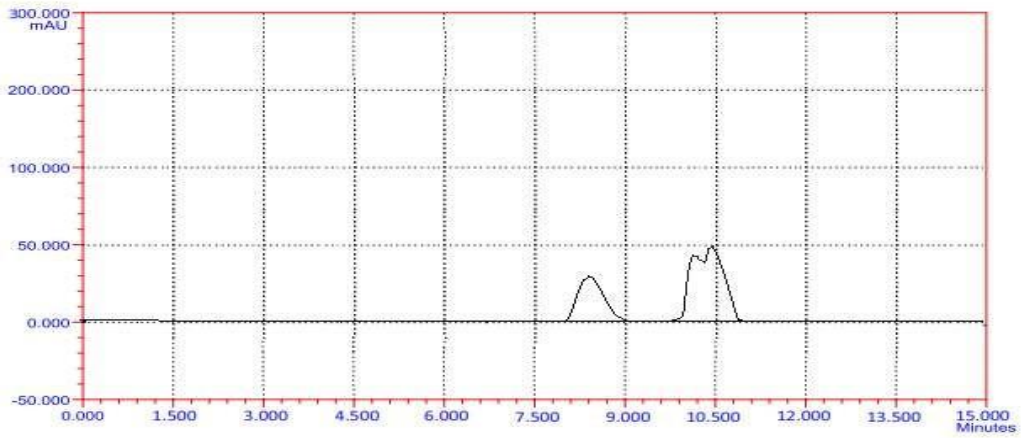


Figure 5 Trial 1: HPLC Chromatogram, Mobile Phase:Acetonitrile: Water (90:10 V/V)

Observation: Peak splitting.

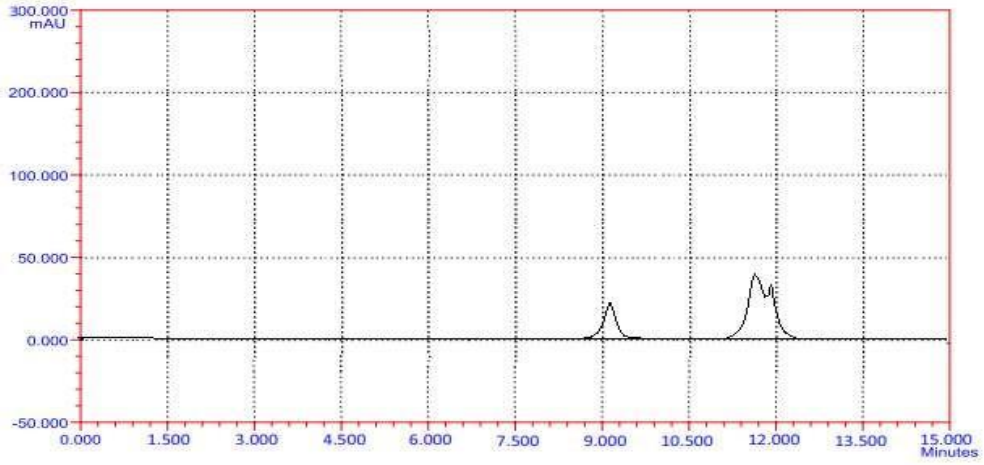


Figure 6 Trial 2: HPLC Chromatogram, Mobile Phase:Acetonitrile: Water (60:40)

Observation: Peak splitting

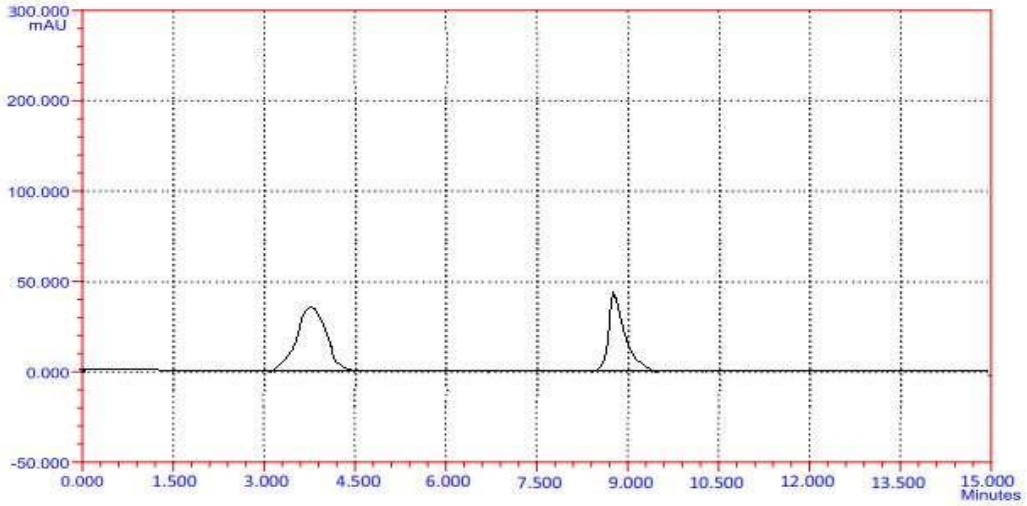


Figure 7 Trial 3: HPLC Chromatogram, Mobile Phase:Acetonitrile: Water: Methanol (50:5:45 v/v/v)

Observation: Peak shape was not proper and peak tailing observed

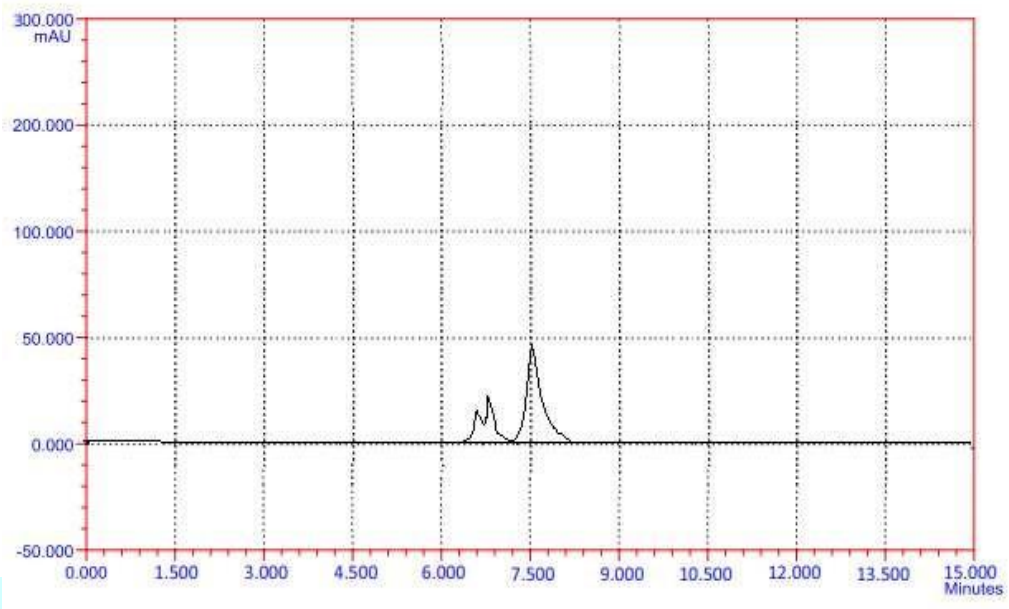


Figure 8 Trial 4: HPLC Chromatogram, Mobile Phase:Acetonitrile: Water: Methanol (50:5:45 v/v/v) (Ph:5)

Observation: Peak shape was not proper.

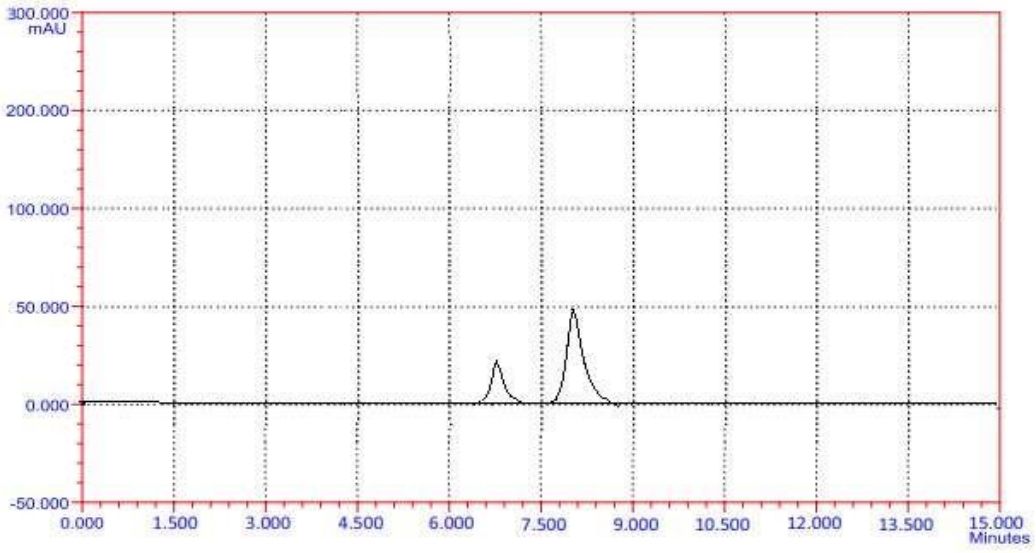


Figure 9 Trial 5: HPLC Chromatogram, Mobile Phase:Methanol: Water: Acetonitrile (50:5:45 v/v/v) (pH:6) Observation: Peak tailing observed and resolution is less

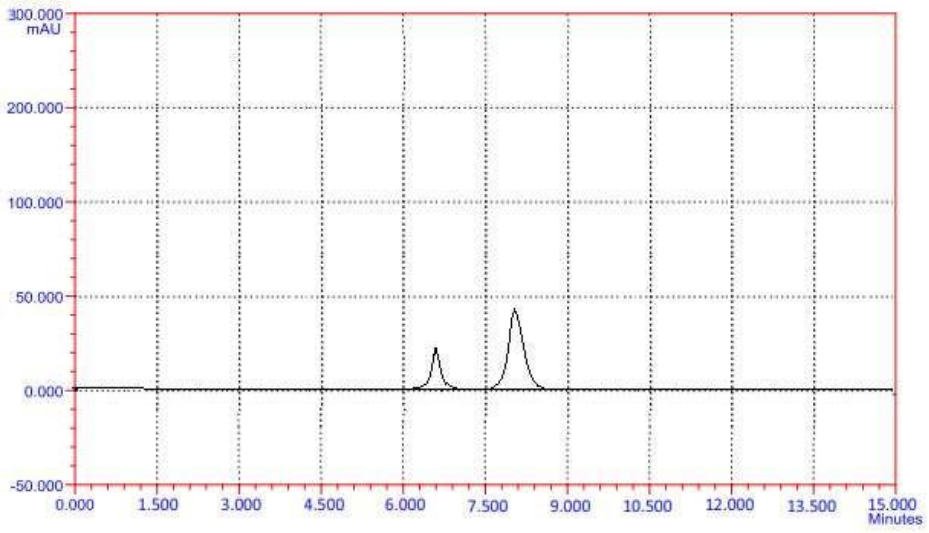


Figure 10 Trial 6: HPLC Chromatogram, Mobile Phase: Acetonitrile: Methanol: Phosphate buffer (40:30:30 % V/V/V) 7.5 pH of buffer

Observation: Shorter retention time of Drug and peak shape wasproper; resolution is less and theoretical plate is less than 2000

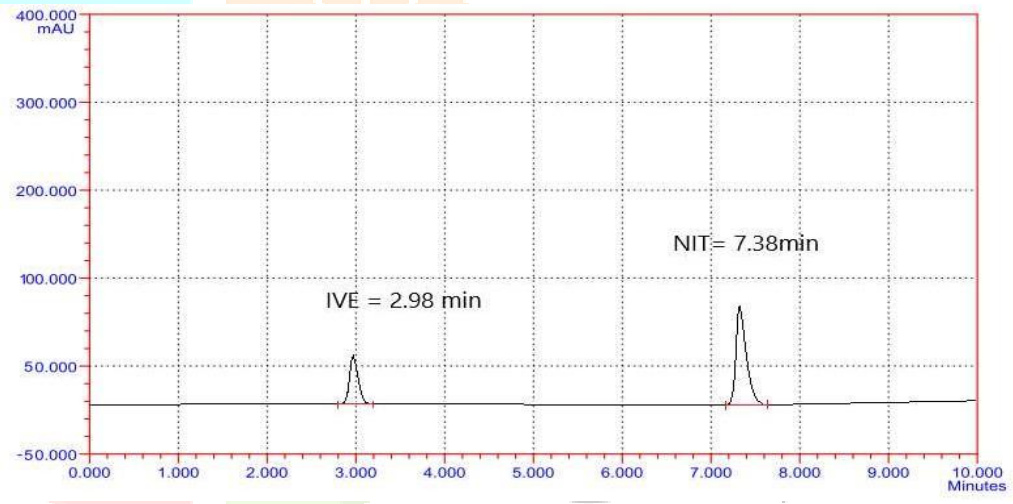


Figure 11 Trial 7: HPLC Chromatogram, Mobile Phase: Methanol: Acetonitrile: phosphate buffer (35:35:30) (Ph :3)

Observation: Shorter retention time of Drug and peak shape wasproper, resolution good.

Table:10 Optimization of RP-HPLC chromatographic condition:

SR. NO.	CHROMATOGRAPHIC PARAMETER	OPTIMIZED CONDITION	
1	Flow Rate	1 ml/min	
2	Detection Wavelength	235nm	
3	Mobile Phase composition	Methanol: Acetonitrile: phosphate buffer (35:35:30) (Ph :3)	
4	Column	C18 (250 mm×4.6 mm×5 µm)	
5	Injection Volume	20 µl	
6	Ph of buffer	7.5 ± 0.02	
7	Retention time (min)	IVE	NTZ
		2.98 min	7.38 min

OPTIMIZATION OF MOBILE PHASE COMPOSITION:

- From the above trial, mobile phase optimized was Methanol: Acetonitrile: phosphate buffer (35:35:30) (Ph :3) showed well resolved peak of IVE and NTZ eluted at 2.98 and 7.38 min respectively.
- Peak resolution 9.65 and tailing factor for IVE and NTZ 1.64 and 1.25 respectively (Figure 11) detection at 235nm.
- Individual drug peak was confirmed by separately injecting drug solution like IVE and NTZ on optimized mobile phase condition and detection was carried out at 235nm (figure 12 and 13)

Figure 12 Chromatogram of Ivermectin (IVE) on optimizedmobile phase.

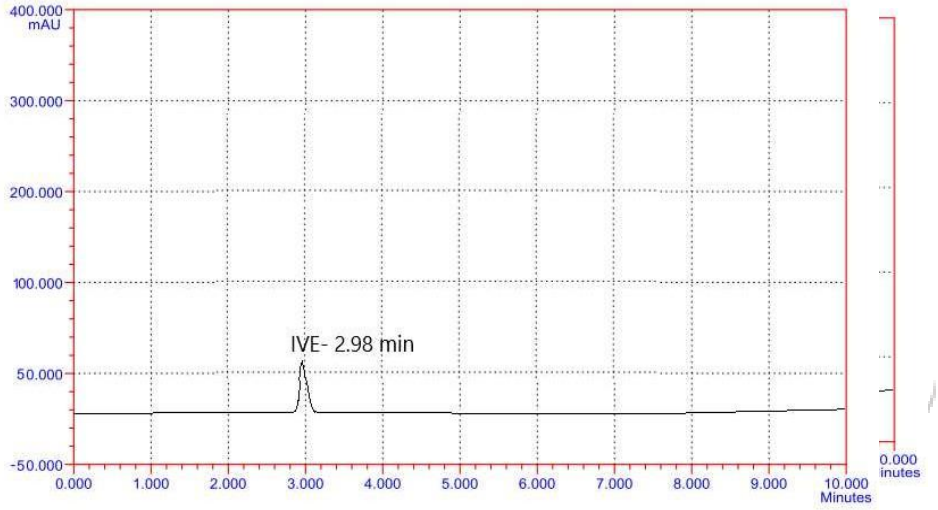


Figure 13 Chromatogram of Nitazoxanide (NTZ) on optimizedmobile phase.

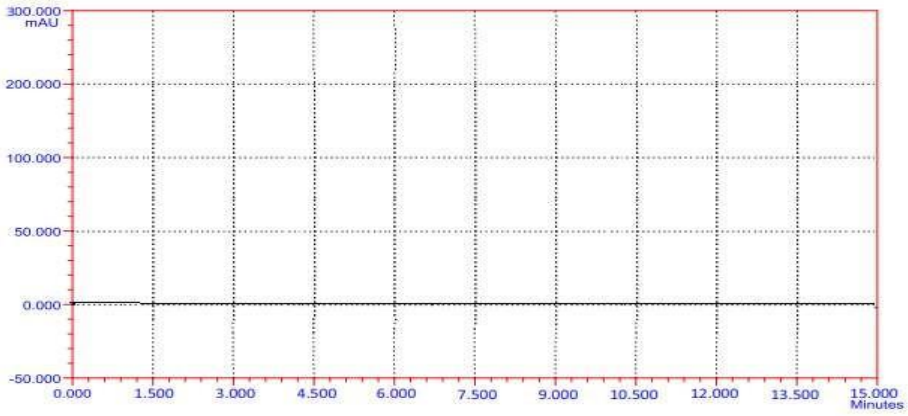


Figure 14 Blank chromatogram on optimized mobile phase.

SYSTEM SUITABILITY TEST:

The acceptance criteria were Relative standard deviation (RSD) less than 2.00% for the peak area and Retention time, Column plates greater than 2000, Tailing factor less than 2.00.

The results of system suitability analysis are as shown in Table 6.7 were well within acceptance criteria, revealing that method and system were adequate for the analysis to be performed

Table: 11 System Suitability Parameter:

PARAMETER	IVE	NTZ
Retention Time(min)	2.98 min	7.38 min
Resolution	0.00	9.6
Theoretical plate	4020	7188
Symmetric Factor	1.64	1.25

CONCLUSION:

RP-HPLC method was developed for the simultaneous estimation of Ivermectin and nitazoxanide in synthetic mixture.

All System Suitability Parameters were considered during each trial of Ivermectin and Nitazoxanide.

RESULTS AND DISCUSSION:

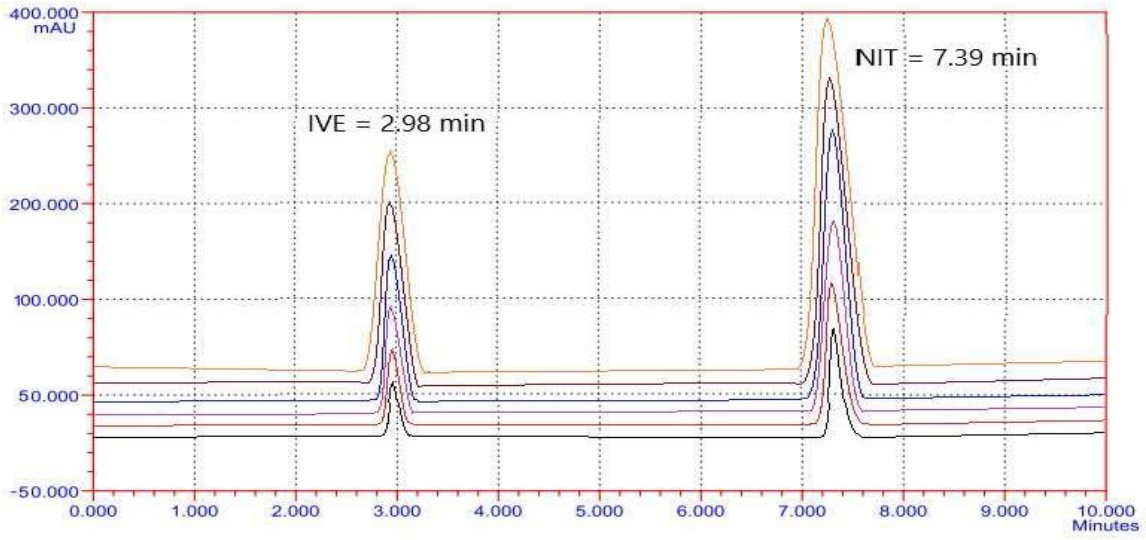
LINEARITY:

The linear concentration range of IVE and NTZ was in the range of 06- 36 µg/ml and 100- 600 µg/ml respectively (Table 12 and 13, Figure 15).

As shown in Table 12 and 13, regression coefficients of determination (r2) ≥ 0.9900 and y intercepts that were not significantly different from zero at the 95% confidence level (Figure 16- Figure 17).

These data indicate acceptable linearity. The data showed satisfactory linear relationship between the peak areas and the solution concentrations across the evaluated range were verified asshown in Table 10.

Figure 15 Chromatogram of calibration curve for mixture of IVE(6 -36 µg/ml) and NTZ (100– 600



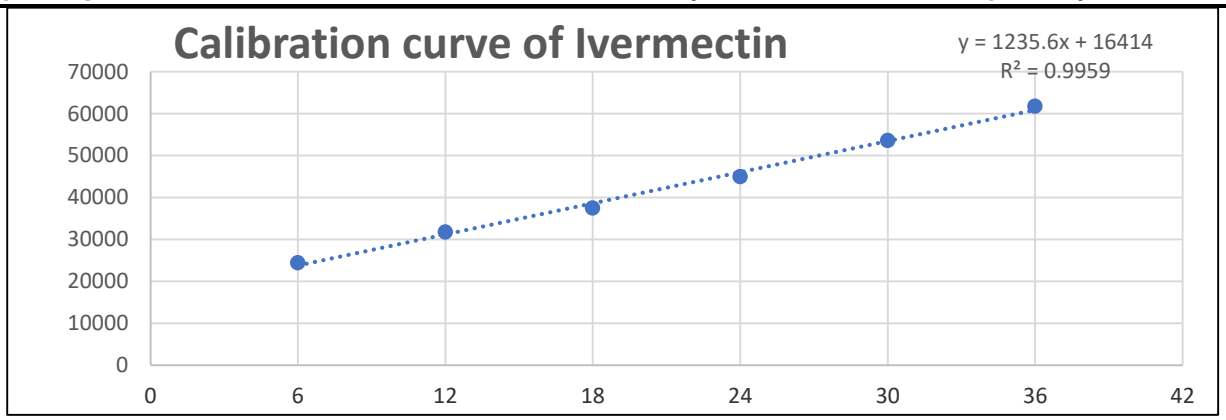


Figure 16 Calibration Curve of IVE at 235 nm

Table: 12 Linearity of HPLC method for IVE:

Concentration (µg/ml)	Average Peak Area (n)	Standard Deviation	% RSD
6	24475	448.46	1.83
12	31792	187.08	0.59
18	37503	561.25	1.50
24	45004.33	777.58	1.73
30	53621	748.33	1.40
36	61773	935.41	1.51

*n = 3 replicate

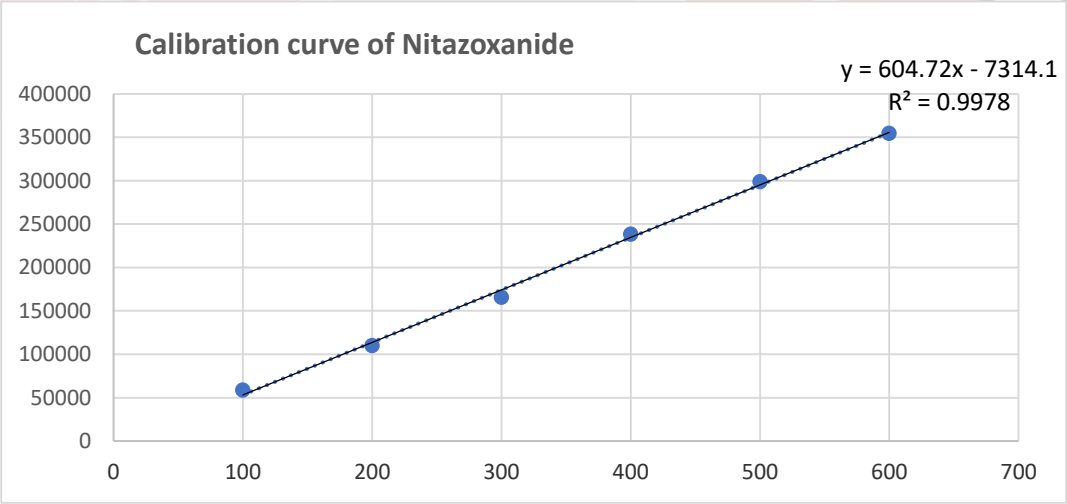


Figure 17 Calibration Curve of NTZ at 235 nm

Table:13 Linearity of HPLC method for NTZ

Concentration (µg/ml)	Average Peak Area (n)	Standard Deviation	% RSD
100	58840.5	864.63	1.50
200	110029.66	1131.42	1.02
300	165776.5	3287.05	1.98
400	238230	1833.41	0.77
500	298687.66	5717.63	1.91
600	349456.83	5416.39	1.55

***n = 3 replicates**

Table: 14 Linear regression parameters for estimation of IVE and NTZ

Parameters	IVE	NTZ
Calibration range (µg/ml) (a)	6-36	100-600
Regression equation	y = 1235.6x + 16414	y = 604.72x + 7314.1
Standard deviation of slope	251.49	28.26
Standard deviation of intercept	151.41	237.62
Correlation coefficient (r2)	0.9959	0.9978

LOD and LOQ:

LOD and LOQ of IVE and NTZ were determined by equation according to ICH guideline.

LOD for IVE and NTZ was found to be 1.98 and 27.75 µg/ml respectively.

LOQ for IVE and NTZ was found to be 6.00 and 84.06 µg/ml respectively as shown in Table 15 indicating sensitivity of the method.

Table: 15 LOD and LOQ for HPLC method

Parameters	IVE (µg/ml)	NTZ (µg/ml)
LOD	1.98	27.75
LOQ	6.00	84.06

PRECISION:

The precision was determined by studying the Repeatability, intraday and interday precision. The % RSD was calculated for drugs and the results are as shown in Table 16..

The intraday precision was determined at three concentrations of IVE (06, 18 and 36 µg/ml) and NTZ (100, 300 and 600 µg/ml) were analyzed three times on same days. Table 6.7 shows that % RSD values were not more than 2% that indicates acceptable precision of the method.

The interday precision was determined at three concentrations of IVE (06, 18 and 36 µg/ml) and NTZ (100, 300 and 600 µg/ml) were analyzed three times on different days. Table 16 shows that % RSD values were not more than 2% that indicates acceptable precision of the method.

Table: 16 Precision data:

Drug	Conc. (µg/ml)	Average(n)	SD	%RSD
Repeatability				
IVE	18	18.31	0.3	1.67
NTZ	300	301.52	0.78	0.28
Inter-day precision				
IVE	06	6.05	0.07	1.15
	18	18.6	0.31	1.63
	36	36.33	0.27	0.77
Intraday precision				
IVE	06	6.00	0.11	1.82
	18	18.71	0.41	0.88
	36	35.9	0.45	1.29
Inter-day precision				
NTZ	100	101.18	1.18	1.18
	300	305.18	2.82	0.95
	600	611.23	7.80	1.30
Intraday precision				
NTZ	100	100.85	1.15	1.14
	200	300.58	3.25	1.01
	600	603.4	4.35	0.72

ACCURACY:

To check the accuracy of the developed method, analytical recovery study experiments were carried out by the standard addition method.

The accuracy of the method was evaluated at 80, 100 and 120% for the IVE and NTZ. As indicated in Table 6.9, the average percent recoveries ranged from the 97.81-102.76 % for IVE and 98.78 -102.13% for NTZ. The accuracy of the method was considered acceptable based on its intended use.

Table: 17 Accuracy study of HPLC method:

% Of Spike	Conc. OfSample (µg/ml)	Conc. Of Std Spiked (µg/ml)	Total Conc. Ofdrug taken (µg/ml)	Total Conc. Of drug foundn (µg/ml)	Mean % Recovery	SD	%RSD
IVE							
80	6	4.8	10.8	10.49	99.96	0.66	0.66
100	6	12	12	12.17	101.43	0.92	0.91
120	6	18	13.2	13.3	100.76	0.83	0.83
NTZ							
80	250	200	450	458.32	101.85	0.95	0.93
100	250	250	500	510.65	102.13	0.40	0.39
120	250	300	550	543.29	98.78	0.62	0.63

n=3 replicate, S.D = standard deviation, %RSD= relative standard deviation

SPECIFICITY:

The specificity of the method was ascertained by comparing retention time of IVE and NTZ in chromatogram obtained from synthetic mixture and standard drug.

The retention time of the standard drug and the formulation was found to be same for both the drugs, so the method was found to be specific. Moreover, there was no interference from excipients at the peaks observed from the proposed formulation (Figure 6.18).

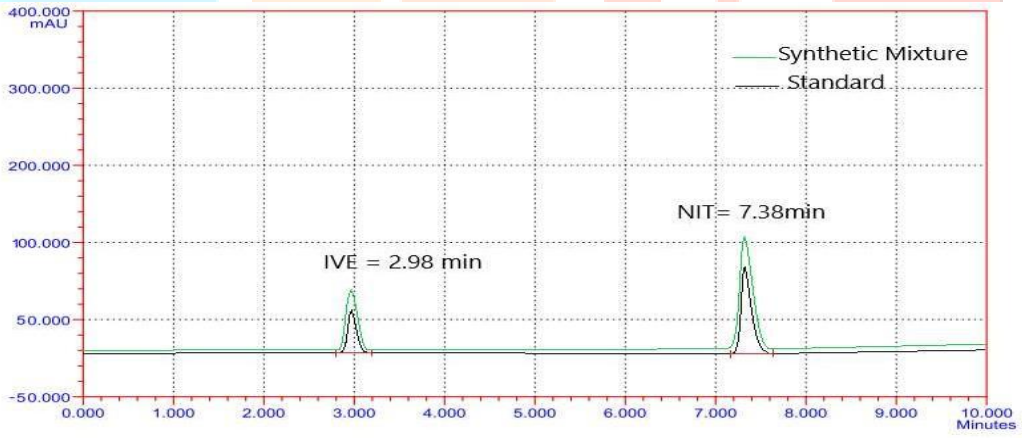


Figure.18 Chromatogram of mixture of IVE and NTZ in synthetic mixture and standard

ROBUSTNESS:

Table:18 Robustness study for HPLC method:

EFFECT OF CHANGE IN VOLUME OF PHOSPHATE BUFFER									
	27 ml			30 ml			33 ml		
Drug	Peak Area	%RSD	Rt (min)	Peak Area	%RSD	Rt (min)	Peak Area	%RSD	Rt (min)
IVE	37045	0.25	2.75	37605	0.444	2.99	37469	0.864	3.15
NTZ	161584	1.23	7.96	165584	0.906	7.39	167584	0.122	8.56
EFFECT OF CHANGE IN FLOW RATE									
	0.9 ml/min			1.0 ml/min			1.1ml/min		
Drug	Peak Area	%RSD	Rt (min)	Peak Area	%RSD	Rt (min)	Peak Area	%RSD	Rt (min)
IVE	37696	0.439	3.12	37605	0.444	2.99	38617	0.949	2.65
NTZ	160584	1.177	8.45	165584	0.906	7.39	163584	0.179	6.75
EFFECT OF CHANGE IN DETECTION WAVELENGTH									
	230nm			235nm			240nm		
Drug	Peak Area	%RSD	Rt (min)	Peak Area	%RSD	Rt (min)	Peak Area	%RSD	Rt (min)
IVE	34786	0.247	2.98	37605	0.444	2.95	34247	0.218	2.96
NTZ	162584	0.49	7.36	165584	0.906	7.41	168584	0.242	7.44

CONCLUSION:

RP-HPLC methods were developed for the simultaneous estimation of for Ivermectin and nitazoxanide in synthetic mixture.

Proposed method is developed for the identification and quantification of Ivermectin and nitazoxanide in bulk and marketed dosage form. The developed method is simple, accurate, less time consuming, economical and sensitive when compared to other reported analytical methods.

According to ICH guideline the method was found to be accurate, sensitive and precise. Statistical analysis proved that the method was repeatable and selective for the analysis of Ivermectin and nitazoxanide without any interference of excipients.

This method was successfully used in determination Ivermectin and nitazoxanide in marketed dosage form.

In HPLC (High Performance Liquid Chromatography) by using Cyber C-18 column (250 mm × 4.6 mm, 5 µm) equilibrated with mobile phase Methanol: Acetonitrile: phosphate buffer (35:35:30) (Ph :3), flow rate was maintained to 1ml/min and analysis was carried out by using UV detector. The common detection wave-length was found to be 235 nm.

The method was validated for linearity, precision, accuracy and robustness, limit of detection and limit of quantification.

The linear range of Ivermectin and nitazoxanide was found to be (6- 36µg/ml) and (100-600 µg/ml) and regression coefficient was found to be 0.9988 for IVE and 0.9927 for NTZ respectively.

The % recovery for Ivermectin and nitazoxanide were found to be and 97.81-102.76 % and 98.78 -102.13% respectively.

REFERENCES:

1. Anonymous, “Corona virus Diseases”, November 2020 https://physiopedia.com/
2. WHO advises that ivermectin only be used to treat COVID-19 within clinical tri-alshttps://www.who.int/news-room/feature-stories/detail/who-advises-that ivermectin-only-be-used-to-treat-covid-19-within-clinical-trials Access on October 2021.
3. The COVID-19 Treatment Guidelines Panel (the Panel) recommends against the useof nitazoxanide for the treatment of COVID-19. https://www.covid19treatmentguide-lines.nih.gov/therapies/antiviral- therapy/nitazoxanide/ Access in October 2021.
4. Bryant A, Lawrie TA, Dowswell T, et al. “Ivermectin for Prevention and Treatment of COVID-19 Infection: A Systematic Review, Meta-analysis, and Trial Sequential Analysis to Inform Clinical Guidelines.” *Am J Ther*. **2021**, 28(4), e434-e460. DOI: 10.1097/MJT.0000000000001402
5. Manisha Khatri, Payal Mago, “Nitazoxanide/Camostat combination for COVID-19:An unexplored potential therapy”. *Chem. Biol. Lett,* **2020**, 7(3), 192-196. http://thescien-cein.org/cbl
6. Drug Bank Ivermectin (Access in October 2021). https://go.drugbank.com/drugs/DB00602
7. Pub Chem Ivermectin (Access in October 2021). https://pubchem.ncbi.nlm.nih.gov/compound/6321424
8. Wikipedia Nitazoxanide (Access in October 2021) https://en.wikipedia.org/wiki/
9. Pub chem Nitazoxanide (Access in October 2021). https://pubchem.ncbi.nlm.nih.gov/compound/Nita-zoxanide
10. Drug Bank Nitazoxanide (Access in October 2021). https://go.drugbank.com/drugs/DB00507

11. Indian Pharmacopoeia; The Indian Pharmacopoeia Commission, Ghaziabad-201 002, Govt. of India Ministry of Health and Family welfare, IP 2018, Volume I 8th Edn
12. British Pharmacopoeia, The British Pharmacopoeia Commission, London, Medicines and Healthcare products Regulatory Agency, British Pharmacopoeia 2018, TSO-2017.
13. The United State Pharmacopoeia (USP 40 NF 35) Edn, US Pharmacopeial Convention, Global Expertise Trusted Standards Improved Health, 2017.
14. Limbani R K, Modi J, Pashat Y, "Simultaneous Equation Method for The Estimation of Ivermectin and Clorsulon in Their Combined Pharmaceutical Dosage Form By UV-Visible Spectrophotometry." *International Bulletin of Drug Research*. **2014**, 4(6), 131-139.
15. Gandla K, Lalitha R, et. Al. "Analytical Method Validation of Ivermectin and Praziquantel in Bulk and Pharmaceutical Dosage Form by UPLC." *International Journal of Research Publication and Reviews*. **2021**, 2(4), 573-583.
16. Kavitha J, Lakshmi KS, Madhan S, "Multivariate Calibration Technique for The Spectrophotometric Quantification of Ivermectin in Pharmaceutical Formulation." *Asian J Pharm Clin Res.* **2019**, 12(2), 444-451.
17. Daoli Z, Wimalasinghe R M, Wang L, Abu M R, "Development and Validation of a Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC) Method for Identification, Assay and Estimation of Related Substances of Ivermectin in Bulk Drug Batches of Ivermectin Drug Substance." *Journal of Chromatographic Science.* **2021**, bmab112. https://doi.org/10.1093/chromsci/bmab112
18. Padivitage N, Adhikari S, &Abu M R, "Assay of Ivermectin Including Determination and Identification of Its Related Substances in Ivermectin Injectable Product by a Stability-Indicating RP-HPLC Method." *Chromatographia.* **2021**, 84, 989–997.
19. Oltean E G, Nica A, "Development and Validation of A RP- HPLC Method for The Quantitation Studies Of Ivermectin In Solutions Dosage Forms." *Medicamentul Veterinar / Veterinary Drug.* **2011**, 5(2), 68-70
20. Bangchang K Na, Banmairuroi V and Choemung A, "High-Performance Liquid Chromatographic Method for The Determination of Ivermectin in Plasma." *Southeast Asian J Trop Med Public Health.* **2006**, 37 (5), 848- 857.
21. Wimalasinghe RM, Daoli Z, Wang L, Rustum A, "Development and Validation of a Stability-Indicating Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC) Method for Identification, Assay, and Estimation of Related Substances of Ivermectin Drug Substance." *AOAC Int*. **2021**, 104(6), 1505-1513. Doi: 10.1093/jaoacint/qsab088.
22. Maher S, et.al. "Development and Validation of a New HPLC-UV Method for the Simultaneous Determination of Triclabendazole and Ivermectin B1a in a Pharmaceutical Formulation." *Journal of AOAC INTERNATIONAL*. **2010**, 93(6), 1868–1873 https://doi.org/10.1093/jaoac/93.6.1868
23. Vegad K L, Paranjape D B., Shah D, et. Al. "Development and Validation of RP-HPLC Method for Simultaneous Estimation of Ivermectin and Clorsulon in Ivercam Injection." *Indo American Journal of Pharmaceutical Research.* **2017**, 7(08), 523-531. www.iajpr.com
24. Nischal K, Somshekar B, Abhilekha P M, et. Al. "A Simple RP-HPLC Method for Estimation of Triclabendazole and Ivermectin in a Pharmaceutical Suspension Dosage Form." *CPR.* **2011**, 1(4), 306-310. DOI:10.33786/JCPR. https://www.researchgate.net/publication/301701854

25. Bhavya B, Nagaraju P, Mounika V, Priyadarshini G I, "Stability Indicating RP-HPLC Method Development and Validation for Simultaneous Estimation of Albendazole and Ivermectin in Pharmaceutical Dosage form*." Asian J. Pharm. Ana*. **2017**, 7(1), 6-14. Doi: 10.5958/2231-5675.2017.00002.3
26. Rao B S, Reddy M V, Rao B S, "HPLC Determination of Fenbendazole and Ivermectin Simultaneously in Bulk and Pharmaceutical Dosage Forms." *IAJPS*. **2017**, 4(05), 1194-1204.
27. Harod S, Dubey P K, "Development and Validation of Analytical Method For Fluconazole and Ivermectin In Tablet Formulation by Using RP-HPLC." *IRJP*. **2018**, 3(8), 257-261.
28. Ali M, Alam S, Ahmad S, et.al. "Determination of Ivermectin stability by High-Performance Thin-Layer Chromatography." *Int J. Drug Dev. & Res.* **2011**, 3(2), 240-247.
29. Wani G P, Jadhav S B, "RP-HPLC and HPTLC Stability Indicating Assay Methods for Ivermectin in Bulk and Tablet Dosage Form." *Indian Drug.* **2018**, 55(03), 32-42. https://doi.org/10.53879/id.55.03.11143
30. Agrawal A, Bichala P K, Singh S, "Simple and sensitive analytical method development and validation of Nitazoxanide and Ofloxacin by RP-HPLC." *Asian Journal of Pharmaceutical Analysis*. **2021**, 11(2), 32-42. http://www.anvpublication.org/ , http://www.asianpharmaonline.org/
31. Gandhi S V, Khairnar P L, Chaudhari A P, "RP-HPLC Method for Simultaneous Estimation of Nitazoxanide and Ofloxacin from Bulk and Tablet Dosage Form." *Indian Research Journal of Pharmacy and Science*. **2018**, 5(1),1392- 1398. DOI: 10.21276/irjps.2018.5.1.17 https://www.irjps.in
32. Sharma S, Sharma M, Sahu N K, "Simultaneous determination of Nitazoxanide and Ofloxacin in pharmaceutical preparations using UV-Spectrophotometric and High-Performance Thin Layer Chromatography Methods." *Arabian Journal of chemistry*. **2017**, 10, S62-S66. http://dx.doi.org/10.1016/j.arabjc.2012.07.009
33. Sultana S, Ahammad T, et.al. "RP-HPLC Method Development and Validation for Nitazoxanide In Powder For Suspension Dosage Form." *IJPSR*. **2013**, 4(1), 301-305 http://www.ijpsr.com/
34. Gadapa N and Tripathi UM, "RP-HPLC Analytical Method Development and Validation for Simultaneous Estimation of two Drugs Nitazoxanide, Ofloxacin and its Pharmaceutical Dosage Forms." *International Journal of Chemtech Research.* **2012**, 4(2), 775-783.
35. Nouruddin W A, et. Al. "Validated stability indicating methods for determination of Nitazoxanide in presence of its degradation products." *Journal of Pharmaceutical analysis.* **2012**, 2(2), 105-116. http://www.elsevier.com/locate/jpa , http://www.sciencedirect.com/
36. Ahir et al. "Development of a Validated Stability- Indicating HPTLC Method for Nitazoxanide in Pharmaceutical Formulation." *J Chromat Separation Technique*. **2012**, 3(5), 1-5. DOI:10.4172/2157-7064.1000138
37. Deshpande MM, Soni CS, Kasture VS, Rohom SS, Chaudhari SR, "UV Spectrophotometric Estimation of Nitazoxanide in Tablet Dosage Form." *Research J. Pharm. And Tech.* **2011**, 4(11), 1714-1716. http://www.rjptonline.org/
38. Sharma, et al. "RP-HPLC Method for Simultaneous Estimation of Nitazoxanide and Ofloxacin in Tablets." *Indian Journal of Pharmaceutical Sciences.* **2011**, 73(1), 84-88. http://www.ijpsonline.com/

39. Premanand DC, Senthilkumar KL, Senthilkumar B, Saravanakumar M, Thirumurthy R, "A Validated RP-HPLC Method for Simultaneous Estimation of Nitazoxanide and Ofloxacin in Pharmaceutical Formulation." *Pelagia Research Library*. **2010**, 1(2), 1-5. http://www.pelagiaresearchlibrary.com/
40. Ranea VP, and Shindea DB, "Simultaneous High-Performance Liquid Chromatographic Determination of Nitazoxanide and Ofloxacin in Tablet Formulation." *Eurasian J. Anal. Chem*. **2009,** 4(3), 304-310.
41. Malesuik MD, Cardoso SG, Steppe M, "Development of a Validated Stability-Indicating LC Method for Nitazoxanide in Pharmaceutical Formulations." *Chroma.* **2008**, 67. https://doi.org/10.1365/s10337-007-0454-9
42. Kapse GK, Prabhakar G and Appala RS, "Spectrophotometric Methods for the Estimation of Nitazoxanide in Pharmaceutical Formulations." *Indian Journal of Pharmaceutical Sciences.* **2006**, 68(3), 403-406. http://www.ijpsonline.com/