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Development and Validation of a Stability-Indicating Rapid RP-HPLC Method for the Quantification of Nifurtimox in Bulk Drug and Pharmaceutical Formulations

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Keywords: Nifurtimox, RP-HPLC, Method Validation, Stability-Indicating Method, Forced Degradation, ICH Guidelines.

Abstract:

Background:

Analytical method development and validation are essential components of pharmaceutical research, quality control, and quality assurance to ensure the safety, quality, and stability of drug products. The present study aimed to develop and validate a simple, accurate, precise, and stability-indicating reverse-phase high-performance liquid chromatography (RP-HPLC) method for the estimation of Nifurtimox in bulk drug and marketed tablet formulations. Nifurtimox is an antiprotozoal drug widely used in the treatment of Chagas disease by eliminating the causative parasite.

Methods:

Chromatographic separation was performed using a Spherisorb ODS2 C18 column (250 × 4.5 mm, 5 µm) with an isocratic mobile phase comprising methanol:water:0.1% orthophosphoric acid (75:22:3, v/v). The flow rate and other optimized chromatographic conditions provided efficient separation, and detection was carried out at 244 nm. The developed method was validated according to ICH guidelines by evaluating specificity, system suitability, linearity, precision, accuracy, robustness, limit of detection (LOD), limit of quantification (LOQ), and forced degradation studies.

Results:

The developed RP-HPLC method demonstrated excellent linearity over the concentration range of 15–90 µg/mL. Validation results confirmed that the method was specific, accurate, precise, robust, and suitable for routine analysis. The method also effectively separated Nifurtimox from its degradation products generated under various stress conditions, confirming its stability-indicating capability.

Conclusion:

The developed RP-HPLC method demonstrated excellent analytical performance with rapid analysis (retention time 3.54 min), high sensitivity, accuracy, precision, robustness, and specificity in accordance with ICH guidelines. The method effectively separated Nifurtimox from its degradation products under all stress conditions, confirming its stability-indicating capability. These analytical advantages make the method reliable for routine quantitative estimation of Nifurtimox in bulk drugs and pharmaceutical formulations. Therefore, the

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developed method can be successfully applied in pharmaceutical quality control laboratories, stability studies, formulation development, and shelf-life evaluation of Nifurtimox products.

Introduction:

Analytical chemistry may be derived as the science and art of determining the composition of material in terms of the elements of compounds contained. By means of analytical techniques both qualitative analysis (the presence or absence of one or more elements) and quantitative analysis (how much amount is present) can be done [1]. Analytical chemistry involves the application of range of techniques and methodologies to obtain and access quantitative and structural information on the nature of matter [2,3]. Analytical information and level of accuracy required. Costs, timing, availability of laboratory instruments and facilities [4,5]. The most important aspect of analysis is quantitative chemical analysis. In the present age, the physical, chemical and biological analysis, involve computerized techniques to facilitate better result [6-9].

Analytical techniques are used to identify, separate, characterize, and quantify chemical substances in pharmaceuticals, biological samples, and materials. They include spectroscopic, chromatographic, thermal, X-ray, microscopic, electrochemical, and mass spectrometric techniques for structural, elemental, and compositional analysis. These methods are widely applied in drug analysis, quality control, material characterization, environmental monitoring, polymer studies, and biomedical research [10-12]. The validation should follow a plan that includes the scope of the method, the method performance characteristics and acceptance limits. Parameters usually examined in the validation process are limits of detection and quantitation, accuracy, precision, selectivity/specificity, linearity, range and ruggedness [13-16]. System suitability test parameters to be established for a particular procedure depend on the type of procedure being validated [17-19]. The term 'Chromatography' covers those processes aimed at the separation of the various species of a mixture on the basis of their distribution characteristics between a stationary and a mobile phase [20-24]. The chromatographic separation carried out with such silica is referred to as reversed-phase chromatography [25,26]. The retention decreases in the following order: aliphatics > induced dipoles (i.e. CCl4) > permanent dipoles (e.g. CHCl3) > weak lewis bases (ethers, aldehydes, ketones) > strong lewis bases (amines) > weak lewis acids (alcohols, phenols) > strong lewis acids (carboxylic acids). Also the retention increases as the number of carbon atoms increases [27]. Non- polar solutes are squeezed out of the mobile phase and are relatively insoluble in it but with the hydrocarbon moieties of the stationary phase, they get adsorbed [28]. The elution time (retention time) in reversed phase chromatography increases with increasing amount of water in the mobile phase [29]. In HPLC, the mobile phase is pumped through the column under high pressure, and its polarity and solvent properties help achieve efficient separation of sample components [30]. HPLC systems are also provided with an online degassing system, which continuously removes the dissolved gases from the mobile phase [31,32].

Drug Profile

Nifurtimox, sold under the brand name Lampit, which is in a class of medications called antiprotozoals. It works by killing

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the organism that can cause Chagas disease [33].

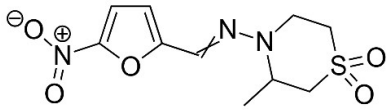


Figure 1: Molecular structure of Nifurtimox

Nifurtimox is an antiprotozoal drug used to treat Chagas disease and is also used with melarsoprol for African sleeping sickness. It works by producing free radicals that damage the parasite's DNA, leading to its death [8,35,36]. Common side effects include loss of appetite, weight loss, nausea, vomiting, headache, and dizziness, while less common effects include rash, fever, seizures, tremors, numbness, and allergic reactions, especially with long-term use [34,37–39].

Gnoth et al. (2015) [40] developed a simple, sensitive, and reproducible HPLC-MS/MS method for the determination of nifurtimox in human plasma with a quantification range of 10–5000 µg/L, making it suitable for bioanalytical studies. Perez et al. (2019) [41] developed a rapid and sensitive UHPLC-MS/MS method for the identification of nifurtimox metabolites in urine samples from pediatric patients with Chagas disease, which was useful for pharmacokinetic studies, therapeutic drug monitoring, and evaluation of adverse drug reactions. Samanta et al. (2019) [42] developed an HPLC method to estimate nifurtimox concentrations in plasma and breast milk of lactating women with Chagas disease and reported low drug exposure in breastfed infants with no observed adverse effects, indicating that nifurtimox treatment during breastfeeding is generally safe. These studies demonstrate that HPLC, UHPLC-MS/MS, and HPLC-MS/MS are reliable, accurate, and sensitive analytical techniques for the estimation of nifurtimox in biological samples [40–42].

Aim and Objectives

This study aims to develop and validate a simple, accurate, precise, rapid, economical, and stability-indicating HPLC method for the estimation of Nifurtimox in bulk drug and pharmaceutical formulations. The developed method will be validated according to ICH guidelines by evaluating parameters such as accuracy, precision, linearity, LOD, LOQ, specificity, robustness, and system suitability. In addition, forced degradation studies will be carried out to evaluate the stability of Nifurtimox and its degradation products.

Materials and Methods

The study was carried out using a PEAK HPLC system equipped with a UV detector, manual injector, C18 column, UV-Visible spectrophotometer, pH meter, sonicator, weighing balance, and membrane filtration assembly. Nifurtimox working standard (98.57% purity), Lampit® 120 mg tablets, HPLC-grade methanol, acetonitrile, water, and analytical-grade reagents were used throughout the study. Standard and sample solutions were prepared using suitable dilutions, and different chromatographic conditions were evaluated by varying the mobile phase composition, pH, wavelength, and stationary phase to obtain the optimized HPLC method.

Method Validation

HPLC Method Development:

Selection of wavelength:

To select an appropriate monitoring wavelength, the standard solutions of 10 µg /mL were prepared and scanned by the UV-Vis spectrophotometer. The obtained wavelength maxima were selected as suitable wavelength for the detection.

Selection of stationary phase:

Since the Nifurtimox is a Polar drug, a non-polar C18 column was selected for the

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separation of the drug. Different columns of different companies, manufactures and configurations were tested.

Method Development trails:

Trail 1:

S.No	Parameter	Condition
1	MP	Water : acetonitrile in 20:80 (v/v)
2	Wavelength	269 nm
3	Stationary Phase	Spherisorb ODS2 C18 column (250mm × 4.5 mm; 5µm)
4	Flow Rate	1.0 mL/min

Trail 2:

S.No	Parameter	Condition
1	MP	Water : acetonitrile in 80:20 (v/v)
2	Wavelength	244 nm
3	Stationary Phase	Kromasil C18 column (250 mm × 4.5 mm; 5µm)
4	Flow Rate	1.0 mL/min

Trail 3:

S.No	Parameter	Condition
1	MP	Water: acetonitrile in 60:40 (v/v)
2	Wavelength	244 nm
3	Stationary Phase	Spherisorb ODS2 C18 column (250mm × 4.5 mm; 5µm)
4	pH of MP	5.2
5	Flow Rate	1.0 mL/min

Trail 4:

S.No	**Parameter**	**Condition**
1	MP	methanol: water 70:30 (v/v)
2	Wavelength	244 nm
3	Stationary Phase	Spherisorb ODS2 C18 column (250mm × 4.5 mm; 5µm)
4	pH of MP	6.0
5	Flow Rate	1.0 mL/min

Trail 5:

S.No	**Parameter**	**Condition**
1	MP	methanol: water: % ortho phosphoric acid in 75:24:1 (v/v)
2	Wavelength	244 nm
3	Stationary Phase	Spherisorb ODS2 C18 column (250mm × 4.5 mm; 5µm)
4	pH of MP	6.1
5	Flow Rate	1.0 mL/min

Trail 6:

S.No	Parameter	Results
1	MP	methanol: water: 0.1 % ortho phosphoric acid in 75:22:3 (v/v)
2	Wavelength	244 nm
3	Stationary Phase	Spherisorb ODS2 C18 column (250mm × 4.5 mm; 5µm)
4	pH of MP	6.1
5	Flow Rate	1.0 mL/min
6	Pump Mode	Isocratic

4.6 Analytical Method Validation:

The method was validated with respect to linearity, accuracy, precision, repeatability, selectivity, and specificity, according to the ICH guidelines. Validation studies were carried out by replicate injections of the sample and standard solutions into the chromatograph.

Specificity:

Specificity of the method was checked by injecting the solution into the chromatograph. Specificity of the method was assessed by comparing the

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chromatogram of Nifurtimox (standard), blank and sample solutions to those obtained for tablet solutions. Retention time of the Nifurtimox in standard solution, and in the sample solution was compared to determine the specificity of the method.

System suitability:

The system suitability was determined by making six replicate injections of the standard solution and analyzing Nifurtimox for its peak area, peak USP tailing factor, and number of theoretical plates. The proposed accepted criteria are not more than 2% for RSD%, not less than 2 for resolution, not more than 2 for USP tailing factor, and not less than 2000 for the number of theoretical plates.

Sensitivity of the method:

The limit of detection (LOD) and limit of quantitation (LOQ) were defined as the lowest concentration of analyte in a sample that can be detected and quantified. The standard solutions of Nifurtimox for LOD and LOQ were prepared by diluting them with suitable solvent. The LOD and LOQ were determined by the signal-to-noise (S/N) ratio for each compound through analyzing a series of diluted solutions until the S/N ratio yield 3 for LOD and 10 for LOQ, respectively.

Linearity and Range:

The calibration curve in the developed method was constructed from LOQ concentration. Nifurtimox standard stock solution of 1 mg/mL was used for preparation of subsequent aliquots. Various aliquots were prepared by serial dilution as given in table 5.2. Sample solution was loaded and 20 µL was injected into column. All measurements were repeated for each concentration. The calibration curve of the area under curve versus concentration were recorded. Form the calibration curve, correlation and regression values were calculated for Nifurtimox.

Precision:

The precision studies were carried out by estimating response of Nifurtimox six times at a standard concentration of 45 µg/mL and results are reported in terms of %RSD. The intra-day and inter-day precision studies were carried out by estimating the corresponding responses six times on same day for intraday and interday for three different days and it was expressed as the percentage relative standard deviation (%RSD) which was calculated as per the following expression

%RSD = (standard deviation / mean) x 100.

Accuracy/ Recovery:

Accuracy of method was observed by recovery result from two placebos preparations accurately spiked with different concentration of Nifurtimox. Recovery assessment was obtained by using standard addition technique which was by adding known quantities of pure standards at three different levels in 50%, 100% and 150% to the pre analyzed sample formulation. From the amount of drug found, amount of drug recovered and percentage recovery were calculated by using the formula.

%RSD = (standard deviation / mean) x 100.

Ruggedness:

Two laboratory analysts carried out the precision of Nifurtimox at a standard concentration of 45 µg/ml was prepared by different analysts in the laboratory conditions, the prepared solution were analyzed in the optimized conditions. Peak area that obtained was used for the determination of ruggedness of the method.

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Ruggedness was expressed in terms of %RSD which must be less than 2.

Robustness:

Robustness of the proposed method included six deliberate variations to some chromatographic parameters. The modifications include different mobile phase ratios and different detector wavelengths and different percentage in the mobile phase (in the range of ± 5 of the nominal value and the normal %). The % change in each of the changed condition was calculated.

4.7 Methodology for Forced degradation study:

Acid Hydrolysis:

50 mg of drugs were mixed with 50 mL of 0.1N HCl solution. The solution was neutralized and diluted up to standard concentration (45 µg/mL) and was analyzed in the developed method condition

Base Hydrolysis:

50 mg of drugs were mixed with 50 mL of 0.1N NaOH solution. The solution was neutralized and diluted up to standard concentration i.e 60 µg/mL and was analyzed in the developed method condition

Oxidative Degradation:

50 mg of drugs were with 50ml of 3% Peroxide solution. The solution was neutralized and diluted up to standard concentration (45 µg/mL) and was analyzed in the developed method condition

Photolytic Degradation:

50 mg of drug sample was kept in UV light [254 nm]. After the selected time of light expose, the drug solution was prepared and was analyzed

Thermal Degradation:

50 mg of drug sample was kept in oven at 60 ^{0}C. After the selected time of light expose, the drug solution was prepared and was analyzed.

Results and Discussion

HPLC Method Development:

The spectrum of diluted solutions of the Nifurtimox in methanol was recorded separately on UV spectrophotometer. The ultraviolet absorption spectra of the Nifurtimox demonstrated that the maximum absorption at a wavelength near 244 nm, and it was therefore chosen during the entire study. The wavelength scanning overlay spectra was given in figure 2

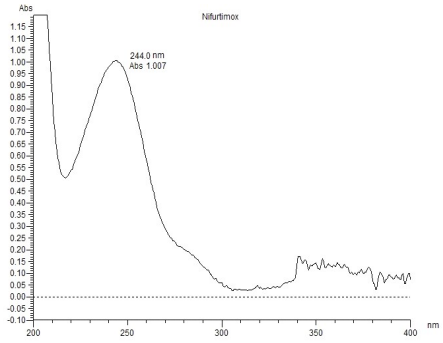


Figure 2: wavelength scanning spectra for Nifurtimox

Method Development Trials

During HPLC method development, six chromatographic trials were performed by varying the mobile phase composition and other chromatographic conditions. Trials 1–5 produced unsatisfactory results, including broad and asymmetric peaks, fluctuating baseline, low peak response, and excessive peak tailing. In Trial 6, a sharp, symmetric peak with acceptable system suitability was obtained, and therefore these chromatographic conditions were selected as the optimized method for the estimation of Nifurtimox.

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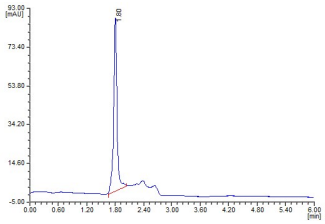


Figure 3: Chromatogram observed for Nifurtimoxin trail 1

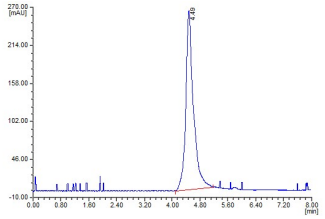


Figure 4: Chromatogram observed for Nifurtimox in trail 2

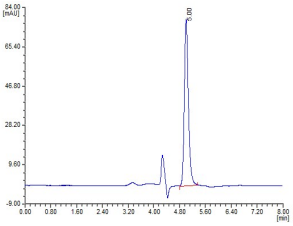


Figure 5: Chromatogram observed for Nifurtimox in trail 3

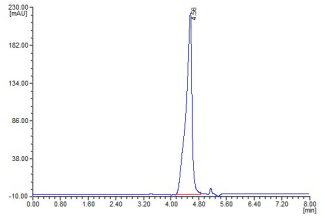


Figure 6: Chromatogram observed for Nifurtimox in trail 4

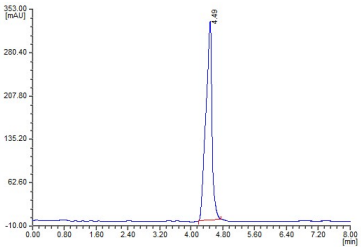


Figure 7: Chromatogram observed for Nifurtimox in trail 5

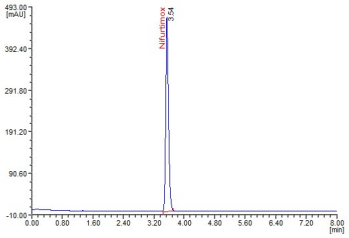


Figure 8: Optimized Chromatogram observed for Nifurtimox in trail 6

Method Validation

The developed HPLC method was validated according to ICH guidelines by evaluating specificity, system suitability, linearity, precision, accuracy, ruggedness, robustness, sensitivity, and forced degradation studies. The method validation was performed according to the latest ICH Q2(R1) was followed.

Specificity

Chromatograms of blank, standard, and sample solutions demonstrated no interference from tablet excipients. A well-resolved peak of Nifurtimox was obtained at 3.54 min, confirming excellent specificity.

System suitability:

In the optimized conditions, peak tailing factors of was found to be 1.06 for Nifurtimox whereas the number of theoretical plates was found to be 6652. This method met the accepted requirements. Table1 shows the results of the system suitability results of the proposed method.

S. No	Parameter	Results
1	Api Concentration	45 µg/mL
2	RT	3.54 min
3	Resolution	Not applicable
4	Area	455565.1
5	Theoretical Plates	6652
6	Tailing Factor	1.06

Table 1: System suitability results of Nifurtimox

Linearity

Excellent linearity was observed over the concentration range of 15–90 µg/mL with regression equation

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$$y = 10104x + 6149.6$$
$$R^2 = 0.9993$$

The calibration curve demonstrated a direct proportional relationship between concentration and peak area.

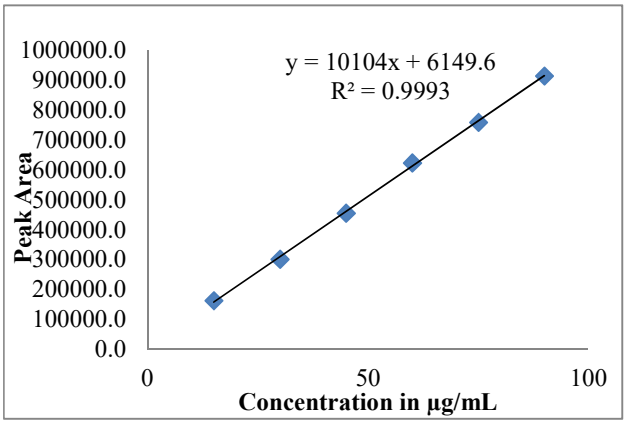


Figure 9: Linearity graph

Precision

The developed method showed excellent repeatability.

S. No	Intraday Precision	Interday Precision
1	451251.9	449446.9
2	456925.7	456011.8
3	452152.6	449439.7
4	452325.9	450968.9
5	457484.6	453367.2
6	455265.8	451168.4
% RSD	**0.59**	**0.56**

Table 2: Precision results

Both values were well below the acceptable limit of 2%.

Accuracy

Recovery studies performed at 50%, 100%, and 150% levels produced recoveries ranging from 98.13% to 101.32%, indicating excellent accuracy.

Ruggedness

The method remained reproducible when performed by different analysts with an overall %RSD of 0.69, demonstrating good ruggedness.

S. No	Peak area observed
1	447649.1
2	455099.8
3	446743.0
4	449616.0
5	449286.9
6	447107.9
RSD	**0.69**

Table 3: Ruggedness results

Robustness

Minor deliberate changes in mobile phase composition, wavelength, and pH produced negligible variation (% change **0.28–1.76%**), confirming that the method is robust (Table 4).

Sensitivity

The sensitivity of the method was confirmed by determining the detection limit (LOD) and quantification limit (LOQ). LOD and LOQ were found to be 0.015 µg/mL and 0.05 µg/mL for Nifurtimox. Results confirmed that the method was sensitive and can be useful for the detection and analysis of drugs at very lowest concentrations.

Parameter	Value
LOD	0.015 µg/mL
LOQ	0.05 µg/mL

Table 5: Sensitivity Results of the Developed HPLC Method.

S.No	Condition	Change	Area	% Change
1	Standard	No change	455565.1	0.00
2	MP 1	methanol: water: 0.1 % ortho phosphoric acid in 70:27:3 (v/v)	447559.2	1.76
3	MP 2	methanol: water: 0.1 % ortho phosphoric acid in 80:17:3 (v/v)	452135.7	0.75
4	WL 1	259 nm	447641.9	1.74
5	WL 2	239 nm	450788.5	1.05

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S.No	Condition	Change	Area	% Change
6	pH 1	6.2	454274.0	0.28
7	pH 2	6.3	452521.9	0.67

Table 4: Robustness result

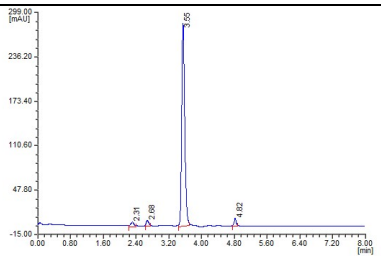


Figure 10: Chromatogram observed in Acid hydrolysis degradation

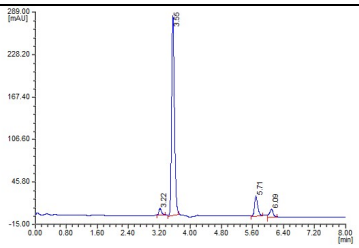


Figure 11: Chromatogram observed in base hydrolysis degradation

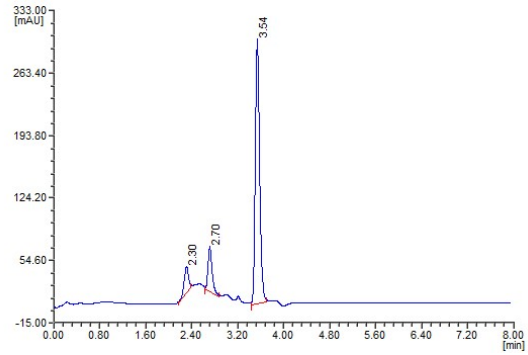


Figure 12: Chromatogram observed in thermal degradation

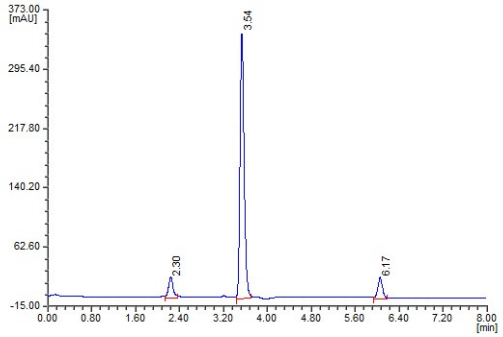


Figure 13: Chromatogram observed in photolytic degradation

Forced Degradation Study and Formulation Analysis

Forced degradation studies were carried out under acidic, alkaline, oxidative, thermal, and photolytic conditions to evaluate the stability-indicating capability of the developed method. In all stress conditions, Nifurtimox and its degradation products were well separated without interference, confirming the specificity of the assay (Figure 10, 11, 12, 13).

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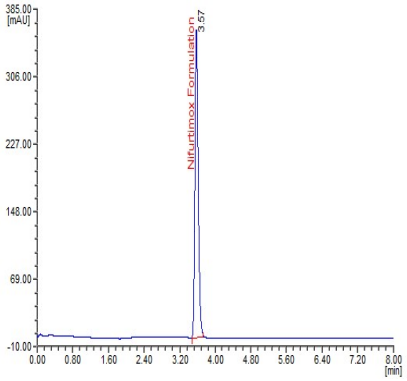


Figure 14: Formulation chromatogram

Conclusion

The developed RP-HPLC method provides several analytical advantages for the estimation of Nifurtimox in bulk drug and pharmaceutical formulations. The method offers a relatively short retention time (3.54 min) with an overall run time of 8 min, enabling rapid analysis and improved sample throughput. The optimized chromatographic conditions produced a sharp and symmetrical peak with excellent system suitability characteristics (tailing factor 1.06 and theoretical plates 6652), indicating efficient chromatographic performance. The method demonstrated excellent linearity over the concentration range of 15–90 µg/mL (R^2 = 0.9993), high precision (%RSD <1%), satisfactory accuracy (98.13–101.32% recovery), robustness against small experimental variations, and high sensitivity with low LOD (0.015 µg/mL) and LOQ (0.05 µg/mL). Furthermore, the method successfully resolved Nifurtimox from its degradation products under acidic, alkaline, oxidative, thermal, and photolytic stress conditions, confirming its stability-indicating capability. Compared with conventional HPLC methods reported for Nifurtimox, the developed method combines rapid analysis, excellent sensitivity, reliable validation characteristics, and effective separation of degradation products in a single analytical procedure. These features make the method suitable for routine pharmaceutical quality control, assay of bulk drugs and finished dosage forms, method validation studies, stability testing, forced degradation studies, and quality assurance during formulation development and shelf-life evaluation.

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