

BIOANALYTICAL LC-MS METHOD FOR DETECTION AND QUANTIFICATION OF NIFURTIMOX IN HUMAN PLASMA

İNSAN PLAZMASINDA NİFURTİMOKS'UN TESPİTİ VE MİKTARININ BELİRLENMESİ İÇİN BİYOANALİTİK LC-MS YÖNTEMİ

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ABSTRACT

Objective: Nifurtimox is a type of nitrofurantoin derivative employed for treating sleeping sickness as well as Chagas disease. The aim is to develop and validate a precise and specific liquid chromatography mass spectrometric technique for the estimation of Nifurtimox in human plasma.

Material and Method: Chromatographic separation had been accomplished on reverse-phase C18 (4.6 mm × 100 mm, 3.5 µm) column employing benznidazole as an internal standard along mobile phase made up of methanol and 5 mM aqueous ammonium acetate in a 75:25 (v/v) ratio at a flow rate of 0.8 ml/min in isocratic mode. A quadrupole mass spectrometer running in positive mode and with numerous reaction monitoring settings was employed to quantify the procedure.

Result and Discussion: The developed analytical method exhibited good recovery and was validated over a calibration range of 0.5–400 ng/ml. In the ESI spectra, the major ion peaks for Nifurtimox and the internal standard, benznidazole, were observed at m/z 288 and m/z 261, respectively, corresponding to their [M+H]⁺ ions. The method exhibited LOD, LOQ, and LLOQ of 0.2 ng/ml, 1.0 ng/mL and 0.5 ng/ml respectively. Precision at intra and interday and accuracy were evaluated at low (1 ng/ml), medium (50 ng/ml), and high (400 ng/ml) quality control levels, showing a %RSD below 2. The percentage recoveries exceeded 95%, with %RSD values under 2. Overall, the validated method is suitable for routine quantification of Nifurtimox in biological samples.

Keywords: Bioanalytical method, human plasma, LC-MS, method validation, nifurtimox

ÖZ

Amaç: Nifurtimoks, Chagas hastalığının yanı sıra uyku hastalığının tedavisinde kullanılan bir tür nitrofuran türevidir. Bu çalışmada Amaç, insan plazmasında Nifurtimox'ın belirlenmesi için hassas ve seçici bir sıvı kromatografisi kütle spektrometrik yöntemi geliştirmek ve doğrulamaktır.

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Gereç ve Yöntem: Kromatografik ayırım, ters faz C18 (4,6 mm × 100 mm, 3,5 µm) kolonunda, benznidazol iç standart olarak kullanılarak gerçekleştirilmiştir. Mobil faz, metanol ile 5 mM sulu amonyum asetatın 75:25 (h/h) oranındaki karışımından oluşturulmuş ve izokratik modda 0.8 ml/dk akış hızında kullanılmıştır. Analitin kantitatif tayini, pozitif iyonizasyon modunda ve çoklu reaksiyon izleme (MRM, Multiple Reaction Monitoring) ayarlarında çalışan bir kuadropol kütle spektrometresi kullanılarak gerçekleştirilmiştir.

Sonuç ve Tartışma: Geliştirilen analitik yöntem, iyi geri kazanım değerleri göstermiş ve 0.5–400 ng/ml kalibrasyon aralığında valide edilmiştir. ESI spektrumlarında, Nifurtimoks ve iç standart olarak kullanılan benznidazol için başlıca iyon pikleri sırasıyla m/z 288 ve m/z 261 değerlerinde gözlenmiş olup, bu pikler bileşiklerin $[M+H]^+$ iyonlarına karşılık gelmektedir. Yöntemin tespit limiti (LOD), tayin limiti (LOQ) ve alt kantitasyon limiti (LLOQ) sırasıyla 0.2 ng/ml, 1.0 ng/ml ve 0.5 ng/ml olarak belirlenmiştir. Gün içi ve günler arası kesinlik ile doğruluk; düşük (1 ng/ml), orta (50 ng/ml) ve yüksek (400 ng/ml) kalite kontrol düzeylerinde değerlendirilmiş ve tüm düzeylerde %RSD değerlerinin 2'nin altında olduğu gösterilmiştir. Geri kazanım değerleri %95'in üzerinde olup, bunlara ait %RSD değerleri de 2'nin altında bulunmuştur. Sonuç olarak, valide edilen yöntem biyolojik örneklerde Nifurtimoks'un rutin kantitatif tayini için uygundur.

Anahtar Kelimeler: Biyoanalitik yöntem, insan plazması, LC-MS, nifurtimoks, yöntem doğrulaması

INTRODUCTION

Nifurtimox (NFT) is a type of nitrofur derivative employed for treating sleeping sickness (African trypanosomiasis) as well as Chagas disease [1]. African trypanosomiasis, results from infection with *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense* [2]. The disease, spread through the bite of the tsetse fly, is almost invariably fatal if left untreated [3]. For *T. b. gambiense* infection, the combination of eflornithine and nifurtimox is now widely adopted as the preferred first-line therapy [4,5]. Chagas disease is a long-lasting systemic parasitic infection caused by the protozoan *Trypanosoma cruzi* [6-8]. After the acute phase subsides, a significant proportion of patients may progress to chronic disease, marked by cardiomyopathy, arrhythmias, enlargement of the digestive organs, and, less commonly, polyneuropathy or stroke [9]. The most severe outcome is dilated cardiomyopathy, which can result in heart failure, rhythm disturbances, conduction blocks, thromboembolic events, and sudden death [10]. Its global spread is being accelerated by urbanization, international migration, and emerging transmission routes such as oral and vertical transmission [11-12].

Nifurtimox, used in Chagas disease and African trypanosomiasis, acts through reductive activation by trypanosomal type I nitroreductases. This process generates cytotoxic open-chain nitrile metabolites, independent of oxygen, that inhibit both parasite and mammalian cells [13]. The drug's selectivity likely depends on the parasite-specific expression of type I nitroreductase, highlighting its central role in nifurtimox action [14]. Prolonged NFT use may result in adverse reactions, which might include neurological and gastrointestinal problems [15]. To mitigate variability during sample processing and detection, benznidazole (BNZ) was employed as an internal standard. Figure 1 displays the chemical structures of the NFT [16] and BNZ [17].

As electrospray ionization (ESI) has advanced, LC-MS has become a standard approach that offers a simple interface that may be employed with a variety of biological substances [18-21]. The use of internal standards allows sensitivity and accuracy and minimizes ion suppression effect. Development of bioanalytical methods is important throughout the procedure of drug discovery as well as development and supports pharmacokinetic, toxicokinetic, bioavailability, and bioequivalence studies. The review of literature on NFT reveals that only pharmacokinetic studies [22-24] have used LC-MS/MS techniques. However, the technique employed in their study was not clearly described, and no one has reported bioanalytical method in human plasma. Objective of present research is for developing a bioanalytical LC/MS method for quantitative determination of NFT in blood plasma.

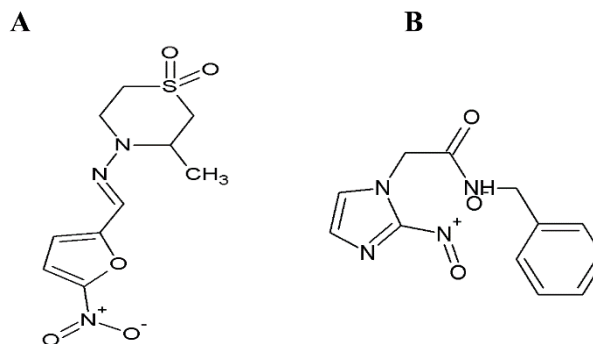


Figure 1. Chemical structure of NFT (A) and BNZ internal standard (B)

MATERIAL AND METHOD

Reagents

Merck Chemicals Private Limited, located in Mumbai, was the supplier of the HPLC-grade solvents. Thermo-Fisher Scientific India PVT LTD, located in Mumbai, supplied analytical-grade chemicals such as sodium acetate, ammonium acetate, and dihydrogen phosphate.

Instrumentation

Method development and validation were conducted “utilizing the Waters 2695 Alliance HPLC instrument (Waters Corp., USA), which includes a four solvent pump, auto injector, temperature-controlled column oven, and online degasser along Waters 487 UV detector. A mass spectrometer connected to an electrospray ionization source running in positive mode is interfaced with the HPLC device. Micromass ZQ mass detector model LAA 1369 (Micromass Ltd., UK) had been used for mass spectrometric analysis, and Mass Lynx software was used to interpret the results.

Preparation of Calibration Standards and Quality Control Samples

NFT standard drug, Human plasma, as well as matching internal standard (IS) BNZ (spiked samples) were combined to provide a range of standard working solutions (0.5, 10, 25, 50, 100, 250, and 400 ng/ml). In order to represent low quality control (LQC), middle quality control (MQC), moreover high-quality control (HQC), respectively, suggested method was validated employing 1, 50, and 400 ng/ml.

Sample Extraction

A liquid - liquid extraction (LLE) technique was used to isolate nifurtimox from human plasma. Based on optimization comparing various organic solvents (acetonitrile, methanol, ethyl acetate, and hexane), a 65:35 (v/v) ethanol : diethyl ether mixture was selected due to its superior extraction efficiency, reduced matrix interference, and enhanced recovery of nifurtimox. To each 1 ml aliquot of frozen plasma in polypropylene tubes, 50 μ l of nifurtimox working solution and 50 μ l of internal standard (IS) solution (500 ng/ml) were added and vortexed for 5 min to ensure adequate mixing. Subsequently, 100 μ l of the extraction solvent was added, followed by vortexing for 10 min to facilitate partitioning of the analyte into the organic layer. The samples were centrifuged at 4000 rpm for 5 min at room temperature, and the organic supernatant was carefully transferred into labeled tubes. The collected extract was evaporated to dryness under a gentle stream of nitrogen at 40°C. The dried residue was reconstituted in methanol, vortexed for 1 min, and transferred for LC - MS analysis. Quality control (QC) samples were processed using the same procedure.

Optimization of Sample Extraction Procedure

To obtain consistent and accurate analyte estimation, extraction optimization, chromatographic conditions, and mass spectrometric conditions are equally important. The LLE approach was used for the extraction process in order to prevent unwanted matrix effects. In addition to keeping the samples below 10°C during sample processing and analysis, the elution solution was kept on the acidic side to prevent interconversion of the mobile phase's pH.

Optimization of Mass Spectrographic Parameters

When compared to atmospheric pressure chemical ionization (APCI) mode, electro spray ionization (ESI) offered the highest response. When the analyte is being infused into continuous flow of the mobile phase to the electrospray ion source, the mass instrument is tuned to achieve sensitivity and signal stability. The electrospray ionization approach was used to tune all analytes in both positive as well as negative ionization modes. Positive ionization mode was chosen for the NFT and IS based on the analyte's sensitivity. The mass parameters that were optimized were source temperature at 300 °C, nitrogen gas flowing at 300 pressure, extractor voltage at 3V, capillarity voltage at 3.0V, along with cone voltage at 40V. To determine the optimal ionization state of fragmentation, the device was run in multiple reactions monitoring (MRM) scan mode.

Optimization of Liquid Chromatographic Parameters

Optimization of liquid chromatographic conditions focused on achieving adequate resolution and sharp peak shapes for both NFT and the IS. Various stationary phases (C8 and C18) and columns from multiple manufacturers were initially evaluated to improve peak symmetry and retention behavior. Among these, the Zorbax SB-C18 column (4.6 mm × 100 mm, 3.5 µm) showed superior performance in terms of retention, theoretical plates, and separation efficiency compared to the other tested columns. Using BNZ as the IS, optimal chromatographic separation was achieved with a mobile phase of methanol: 5 mM ammonium acetate (75:25, v/v) at a flow rate of 0.8 mL/min and a column temperature of 27°C. These conditions provided well resolved, symmetric peaks with consistent response and minimal matrix interference. Therefore, the Zorbax SB-C18 column was selected as the final choice for all further analyses.

Method Validation

In compliance with ICH regulations and reported literature [25-31], method's sensitivity, accuracy, stability, and recovery were validated. Three LQC, MQC, HQC, and lower limit of quantification (LLQC) quality control samples have been examined. Chromatograms from blank and spiked samples were compared in order to evaluate the method's sensitivity. Six distinct batches of blank plasma solutions were infused to test the method's specificity and ensure no endogenous substances interfered with NFT and BNZ IS. Eight distinct concentrations - 0.5, 1, 10, 25, 50, 100, 250, and 400 ng/ml - were used to prove linearity. Six duplicates of QC samples (1, 50, and 400 ng/ml) were injected to assess intraday and interday precisions. Paralleling the mean peak regions of extracted and unextracted samples at LQC, MQC, as well as HQC standard levels allowed for recovery.

RESULT AND DISCUSSION

Selectivity

A sensitive as well as selective bioanalytical technique is essential for the successful completion of bioequivalence/pharmacokinetic studies. So far, no proper published methods are available for determining NFT in human plasma with LC-MS. Selectivity of technique in human plasma was evaluated in individual matrix. Peak response in the blank samples was compared against those of spiked LLOQ; moreover, negligible interference was noted at time of retention of analytes as well as IS. The Blank and standard chromatograms was presented in Figure 2 and Figure 3 respectively.

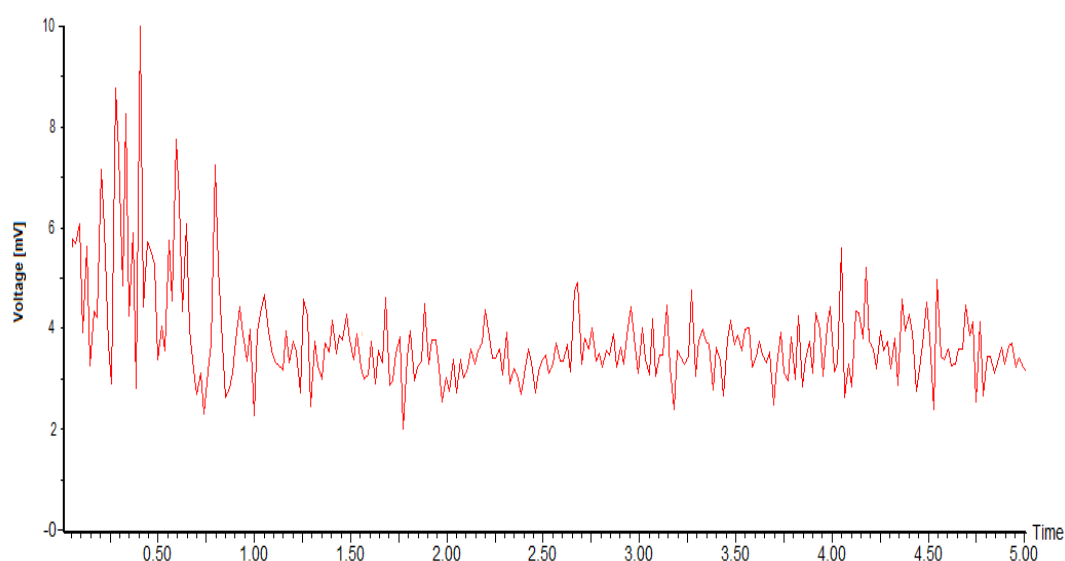


Figure 2. Blank chromatogram

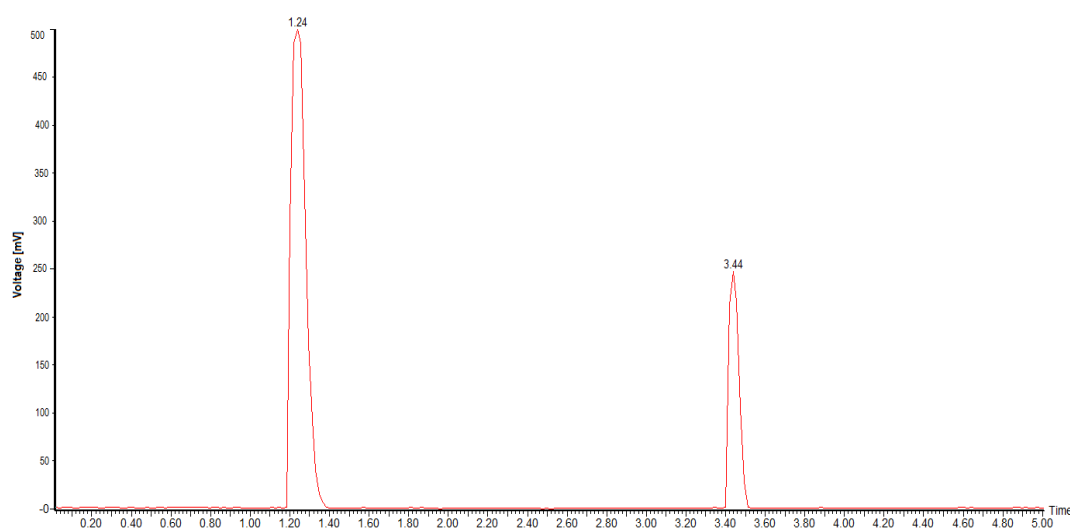


Figure 3. Standard Chromatogram of NFT and IS under proposed conditions

Mass Analysis

For quantitation purposes, one mass/charge (m/z) transition per analyte, i.e. m/z 288 $\rightarrow$ m/z 86 for NFT and m/z 261 $\rightarrow$ m/z 113 for IS, was monitored. Predominant peaks in ESI spectra of NFT and IS correspond to $[M+H]^+$ ions at m/z 288 and m/z 261. The mass spectra of NFT standard and IS were presented in Figure 4 and Figure 5. With total run duration of five minutes, which might encompass washing phase, the retention times for NFT and IS were around 1.24 and 3.44 minutes, respectively.

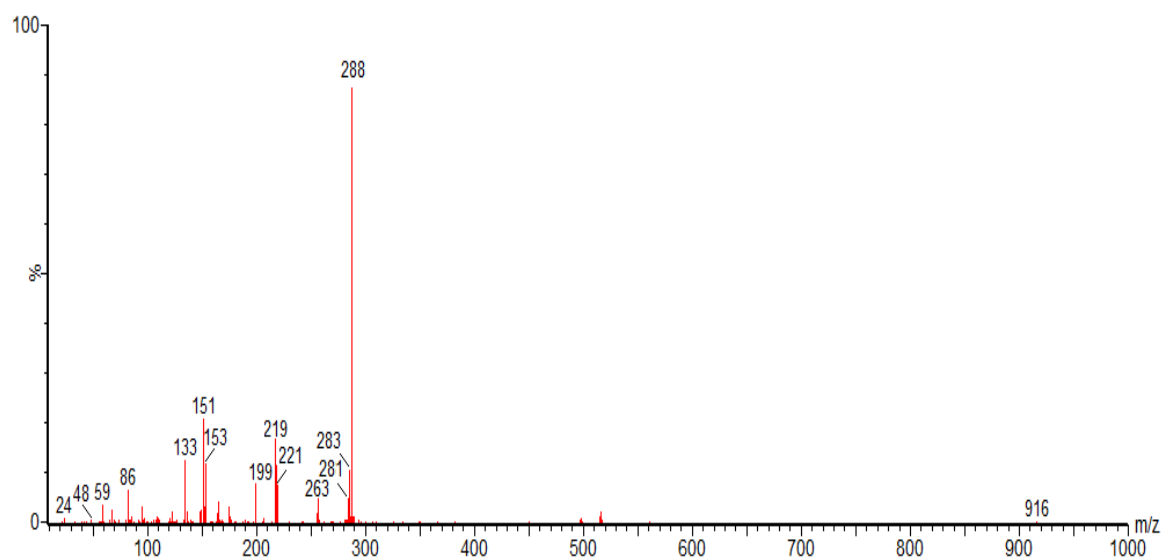


Figure 4. Mass spectra of Nifurtimox (NFT) standard

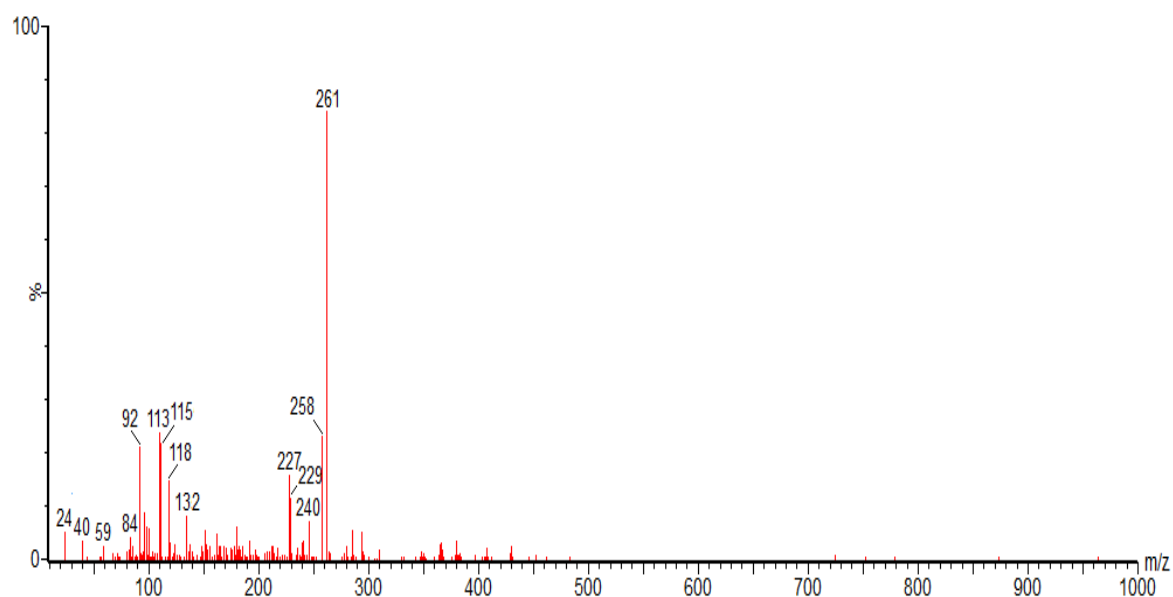


Figure 5. Mass spectra of Benznidazole internal standard

Sensitivity and Linearity

Plotting peak area ratio (y) of analyte to IS against nominal concentration of analyte allowed for study of linearity of each calibration solution. The findings are shown in Figure 6 and Table 1. Sensitivity of method had been established by determining limit of detection (LOD) moreover limit of quantification (LOQ) under chromatographic conditions, had been found to 0.2 ng/ml and 1.0 ng/ml, respectively, and LLOQ for determination of analyte was found to 0.5 ng/ml.

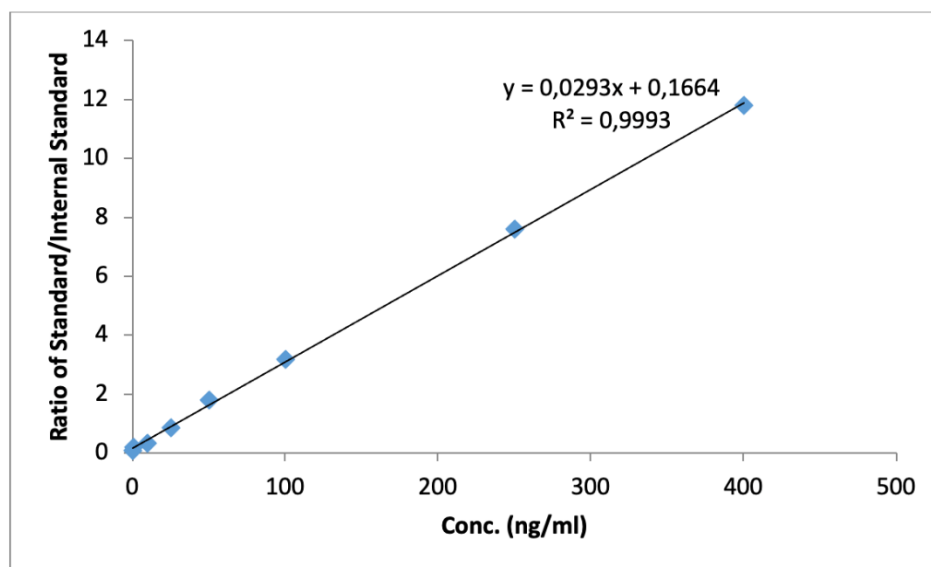


Figure 6. Linearity graph of peak area ratio of NFT and IS

Table 1. Results of linearity study

S No	Concentration ng/ml	Peak Area		Ratio of Standard/IS
		Nifurtimox - Standard	Benznidazole – IS	
1	0.5	7849.0	98967.1	0.079
2	1	19584.7	98662.5	0.199
3	10	32629.5	98457.1	0.331
4	25	84757.6	98556.9	0.860
5	50	178458.2	98457.2	1.813
6	100	315746.1	98932.7	3.192
7	250	747515.9	98475.2	7.591
8	400	1163261.8	98564.8	11.802

IS: Internal Standard

Precision

In order to assess precision, intraday and interday average runs at the LQC, MQC, as well as HQC levels had been carried out. Each mean run included six replicas of the quality control samples, and findings are represented in Tables 2 and Table 3. Six separate runs were analyzed again to assess accuracy, and low %RSD readings suggested that the procedure was accurate.

Table 2. Results of intraday precision studies (n=6)

QC Level	Peak Area Ratio of Standard to IS (Mean ± SD)	%RSD
HQC	11.70 ± 0.125	1.06
MQC	1.80 ± 0.004	0.20
LQC	0.20 ± 0.002	0.86

SD: Standard deviation, %RSD: % Relative standard deviation

Table 3. Results of interday precision studies (n=6)

QC Level	Peak Area Ratio of Standard to IS (Mean $\pm$ SD)	%RSD
HQC	11.85 $\pm$ 0.106	0.91
MQC	1.81 $\pm$ 0.006	0.35
LQC	0.20 $\pm$ 0.001	0.70

SD: Standard deviation, %RSD: % Relative standard deviation

Accuracy

The results of the recovery study conducted using quality control samples at the HQC, MQC, and LQC levels are presented in Table 4. For each concentration level, six samples were analyzed to determine the percentage recovery. The average extraction at HQC was 381.27 ng/ml with a % recovery of 94.51. The average extraction at HQC was 381.27 ng/ml with a % recovery of 95.31. The average extraction at MQC was 47.60 ng/ml with a % recovery of 95.20. The average extraction at LQC was 47.60 ng/ml with a % recovery of 95.20. The average extraction at LQC was 0.97 ng/ml with a % recovery of 96.69. The extraction recovery at HQC, MQC, and LQC levels exceeded 95%, with %RSD values below 2%, indicating efficient and consistent analyte extraction.

Table 4. Recovery study results at different QC levels (n=6)

QC Level	Nominal Concentration (ng/ml)	Peak Area Ratio of Standard to IS (Mean $\pm$ SD)	% Recovery (Mean $\pm$ SD)	%RSD (Recovery)
HQC	400	11.87 $\pm$ 0.267	95.31 $\pm$ 1.158	1.23
MQC	50	1.80 $\pm$ 0.019	95.20 $\pm$ 0.861	0.90
LQC	1	0.20 $\pm$ 0.002	96.69 $\pm$ 1.290	1.33

n: Number of injections, SD: Standard deviation, %RSD: % Relative standard deviation

Freeze Thaw Stability

Freeze thaw stability of NFT was evaluated at LQC and HQC levels over three freeze thaw cycles. Samples were frozen at -20°C for at least 24 h and thawed at room temperature before each cycle. Post cycle measured concentrations were compared with freshly prepared QC samples. Percentage deviation was within $\pm 15\%$, indicating that nifurtimox remains stable during repeated freezing and thawing. The results are presented in Table 5.

Table 5. Freeze thaw stability results

QC Level (n = 3)	Nominal Concentration (ng/ml)	Measured Concentration (Mean $\pm$ SD)	% Stability	% RSD
LQC	1	0.98 $\pm$ 0.04	98.0	4.08
HQC	400	392.5 $\pm$ 8.20	98.1	2.09

Carry over Test

Carry-over was assessed by injecting a blank sample immediately after the highest calibration standard (ULOQ of 400 ng/ml). The response in the blank sample was compared with the LLOQ and must be $\leq 20\%$ of LLOQ for analyte and $\leq 5\%$ for IS. The results confirmed negligible carry-over under the established chromatographic conditions. The results are presented in Table 6.

Table 6. Carry over evaluation

Parameter	Acceptance Criterion	Observed Value	Complies
Analyte signal in blank after ULOQ	$\leq 20\%$ of LLOQ response	7.8%	Yes
IS signal in blank after ULOQ	$\leq 5\%$ of IS response in LLOQ sample	3.1%	Yes

Conclusion

The objective of this work was to develop a rapid, sensitive, and selective LC - MS method for the quantitative determination of NFT in human plasma. The method was successfully optimized using a simple liquid - liquid extraction approach, providing high extraction recovery with minimal matrix interference. Method validation was performed in accordance with regulatory guidelines, indicating good linearity, precision, accuracy, and stability across the validated concentration range. The established LLOQ enables the reliable detection of NFT at clinically relevant plasma levels. The method also showed robust performance during bench top and freeze thaw confirming its suitability for routine analysis. Due to its simplicity, reproducibility, and good analytical performance, this validated bioanalytical method is well suited for application in pharmacokinetic studies, therapeutic drug monitoring, and bioequivalence evaluations of nifurtimox in diverse clinical settings.

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AUTHOR CONTRIBUTIONS

Conception: C.R.N.G., V.D., S.R.Y.; Design: J.K.B., J.A., C.R.N.G., V.D., S.R.Y.; Supervision: C.R.N.G., S.R.Y.; Resources: J.K.B., J.A., C.R.N.G., V.D., S.R.Y.; Materials: J.K.B., J.A., C.R.N.G., V.D., S.R.Y.; Data Collection and Processing: J.K.B., J.A., C.R.N.G., S.R.Y.; Analysis and Interpretation: C.R.N.G., V.D., S.R.Y.; Literature Search: C.R.N.G., V.D., S.R.Y.; Writing Manuscript: V.D.; Critical Review: C.R.N.G., S.R.Y.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

REFERENCES

1. Murray, R.J. (2017). Nifurtimox. In Kucers' The Use of Antibiotics (pp. 3211-3221). CRC Press. [\[CrossRef\]](#)
2. Cox, F.E. (2004). History of sleeping sickness (African trypanosomiasis). Infectious Disease Clinics, 18(2), 231-245. [\[CrossRef\]](#)
3. Kennedy, P.G. (2019). Update on human African trypanosomiasis (sleeping sickness). Journal of Neurology, 266(9), 2334-2337. [\[CrossRef\]](#)
4. Kennedy, P.G. (2013). Clinical features, diagnosis, and treatment of human African trypanosomiasis (sleeping sickness). The Lancet Neurology, 12(2), 186-194. [\[CrossRef\]](#)
5. Kennedy, P.G. (2008). The continuing problem of human African trypanosomiasis (sleeping sickness). Annals of Neurology: Official Journal of the American Neurological Association and the Child Neurology Society, 64(2), 116-126. [\[CrossRef\]](#)
6. Rassi, A., Marin-Neto, J.A. (2010). Chagas disease. The Lancet, 375(9723), 1388-1402. [\[CrossRef\]](#)

7. Lindner, A.K., Lejon, V., Chappuis, F., et al. (2020). New WHO guidelines for treatment of gambiense human African trypanosomiasis including fexinidazole: Substantial changes for clinical practice. *The Lancet Infectious Diseases*, 20(2), e38-e46. [\[CrossRef\]](#)
8. Stass, H., Just, S., Weimann, B., et al. (2021). Clinical investigation of the biopharmaceutical characteristics of nifurtimox tablets-implications for quality control and application. *European Journal of Pharmaceutical Sciences*, 166, 105940. [\[CrossRef\]](#)
9. Pérez-Molina, J.A., Molina, I. (2018). Chagas disease. *The Lancet*, 391(10115), 82-94. [\[CrossRef\]](#)
10. Nunes, M.C.P., Dones, W., Morillo, C.A., et al. (2013). Chagas disease: an overview of clinical and epidemiological aspects. *Journal of the American College of Cardiology*, 62(9), 767-776. [\[CrossRef\]](#)
11. De Sousa, A.S., Vermeij, D., Ramos, A.N., et al. (2024). Chagas disease. *The Lancet*, 403(10422), 203-218. [\[CrossRef\]](#)
12. Schofield, C.J., Jannin, J., Salvatella, R. (2006). The future of Chagas disease control. *Trends in parasitology*, 22(12), 583-588. [\[CrossRef\]](#)
13. Docampo, R., Moreno, S.N., Stoppani, A.O., et al. (1981). Mechanism of nifurtimox toxicity in different forms of *Trypanosoma cruzi*. *Biochemical Pharmacology*, 30(14), 1947-1951. [\[CrossRef\]](#)
14. Cerecetto, H., González, M. (2011). Antiparasitic prodrug nifurtimox: Revisiting its activation mechanism. *Future microbiology*, 6(8), 847-850. [\[CrossRef\]](#)
15. Lang, D., Schulz, S.I., Piel, I., et al. (2022). Structural and mechanistic investigation of the unusual metabolism of nifurtimox. *Chemical Research in Toxicology*, 35(11), 2037-2048. [\[CrossRef\]](#)
16. National Center for Biotechnology Information. (2026). <https://pubchem.ncbi.nlm.nih.gov/compound/6842999>, Nifurtimox. Accessed date: 18.08.2026.
17. National Center for Biotechnology Information. (2026). <https://pubchem.ncbi.nlm.nih.gov/compound/Benznidazole>. Accessed date: 18.08.2026.
18. Moroni, A.B., Calvo, N.L., Kaufman, T.S. (2023). Selected aspects of the analytical and pharmaceutical profiles of nifurtimox. *Journal of Pharmaceutical Sciences*, 112(6), 1523-1538. [\[CrossRef\]](#)
19. Bedogni, G., Azcárate, F., Seremeta, K., et al. (2024). Development of a fast RP-HPLC method for the separation of nifurtimox and its forced degradation products through a design of experiment approach. *Journal of Molecular Liquids*, 399, 124293. [\[CrossRef\]](#)
20. Roach, J.A., Musser, S.M., Morehouse, K., et al. (2006). Determination of usnic acid in lichen toxic to elk by liquid chromatography with ultraviolet and tandem mass spectrometry detection. *Journal of Agricultural and Food Chemistry*, 54(7), 2484-2490. [\[CrossRef\]](#)
21. Vincent, I.M., Creek, D.J., Burgess, K., et al. (2012). Untargeted metabolomics reveals a lack of synergy between nifurtimox and eflornithine against *Trypanosoma brucei*. *PLoS neglected tropical diseases*, 6(5), e1618. [\[CrossRef\]](#)
22. Coura, J.R., De Castro, S.L. (2002). A critical review on Chagas disease chemotherapy. *Memórias do Instituto Oswaldo Cruz*, 97, 3-24. [\[CrossRef\]](#)
23. Bern, C., Montgomery, S.P., Herwaldt, B.L., et al. (2007). Evaluation and treatment of Chagas disease in the United States: A systematic review. *Jama*, 298(18), 2171-2181. [\[CrossRef\]](#)
24. Jackson, Y., Alirol, E., Getaz, L., et al. (2010). Tolerance and safety of nifurtimox in patients with chronic chagas disease. *Clinical Infectious Diseases*, 51(10), e69-e75. [\[CrossRef\]](#)
25. Guideline, I.H.T. (2005). Validation of analytical procedures: text and methodology. Q2 (R1), 1(20), 05.
26. Koppisetty, B.R.B., Prasad, Y.R., Amgoth, K.M.P., et al. (2023). Utility of quality by design approach in rp-hplc method development for quantification of lamivudine and efavirenz in combination formulation. *Journal of Faculty of Pharmacy of Ankara University*, 47(2), 625-636. [\[CrossRef\]](#)
27. Koppisetty, B.R.B., Tatapudi, H.K., Dadi, V., et al. (2023). QbD based RP-HPLC method for simultaneous determination of a emtricitabine, tenofovir diproxil fumarate and efavirenz in tablet dosage form—an application to stability indicating assay. *Analytical Chemistry Letters*, 1-19. [\[CrossRef\]](#)
28. Koppisetty, B.R.B., Kollabathula, V.R., Challa, G.N., et al. (2024). Quality by design-assisted RP-HPLC method for determination of ritonavir and darunavir in pharmaceutical formulation using central composite design. *Separation Science Plus*, e202300210. [\[CrossRef\]](#)
29. Dadi, V., Medapati, S., Baratam, J.K., et al. (2024). Quality by design assisted rp-hplc method for estimation of teriflunomide and its process impurities in drug substance. *Journal of Faculty of Pharmacy of Ankara University*, 48(3), 18-18. [\[CrossRef\]](#)
30. Panchumarthy, R., Bathula, S., Koppisetty, B.R.B., et al. (2025). A Stability indicating RP-HPLC method for determination of foslevodopa and foscarnidopa in bulk and formulation. *Separation Science Plus*, 8(5), e70063. [\[CrossRef\]](#)

31. Vasudha, D., Naidu, C.G., Koppisetty, B.R.B., et al. (2024). A quality by design assisted RP-HPLC method utilizing central composite design for the assay of eliglustat and its organic impurities in drug. *Analytical Chemistry Letters*, 14(5), 755-769. [\[CrossRef\]](#)