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Determination of Nifuratel in Human Plasma by Using Liquid Chromatography-Tandem Mass Spectrometry and Its Application to a Pharmacokinetic Study

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Abstract

In this study, a rapid, straightforward, and sensitive method was developed and validated for the quantification of nifuratel (NIF) in human plasma using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Plasma samples were processed using liquid-liquid extraction with nifuratel 13C D3 as the internal standard (IS). Chromatographic separation was achieved on a Waters ACQUITY UPLC BEH C18 (1.7 μm, 2.1 × 50 mm) column using a mobile phase consisting of formic acid, water, and acetonitrile at a flow rate of 0.60 mL/min. NIF was identified and quantified via mass spectrometry with electrospray ionization (ESI) in positive ion mode and multiple reaction monitoring (MRM). The method showed linearity in the range of 0.25 - 150 ng/mL for NIF. The developed and validated method was applied to the bioequivalence study in human plasma samples.

Keywords

Nifuratel, Nifuratel 13C D3, Human Plasma, LC-MS/MS, Bioequivalence

1. Introduction

Nifuratel is a nitrofuran derivative with a broad antimicrobial spectrum with the chemical name [5-([methylthio] methyl)-3-([5-nitrofurfurylidene] amino)-2-oxazolidinone (**Figure 1**). This compound is effective against Trichomonas vaginalis and exhibits antibacterial properties comparable to nitrofurantoin, along with some antifungal effects against *Candida albicans* [1].

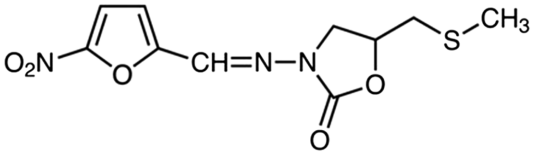

Figure 1. Chemical structure of nifuratel.

Nifuratel's activity is particularly pronounced against bacterial, protozoal, and fungal pathogens affecting the female genital tract. It is primarily eliminated through renal excretion, offering strong antibacterial effects within the urinary tract. The drug is commonly prescribed for conditions such as urethritis, cystitis, pyelitis, and other urinary infections, as well as pre-surgical preparations for urinary tract surgeries [2].

Several analytical methods for determining NIF in human plasma have been reported, most notably using high-performance liquid chromatography (HPLC) coupled with UV detection [1] [3]. However, to the best of our knowledge, no liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods employing liquid-liquid extraction have been reported.

In recent years, LC-MS/MS has been widely used for the quantification of pharmaceutical compounds in biological matrices due to its superior sensitivity, specificity, and lower sample volume requirements compared to HPLC-UV methods [4]-[6]. Numerous studies have successfully demonstrated the determination of drug concentrations in human plasma using LC-MS/MS, such as antibiotics [4], antivirals [5], and antifungals [6], highlighting the advantages of this technique in bioanalytical applications.

Compared with previously reported HPLC-UV methods, the developed LC-MS/MS method provides higher sensitivity, shorter analysis time (3 minutes), and selectivity with minimal matrix interference, making it highly suitable for high-throughput analysis in large-scale clinical pharmacokinetic and bioequivalence studies.

Liquid-liquid extraction (LLE) was selected for sample preparation due to its simplicity, cost-effectiveness, and its proven ability to minimize matrix effects in the analysis of pharmaceutical compounds in plasma. Furthermore, compared to more complex techniques such as solid-phase extraction (SPE), LLE offered sufficient recovery and selectivity for the intended pharmacokinetic study, making it an ideal choice for the development of a rapid and reliable method.

This study aimed to develop a simple, cost-effective, and highly sensitive LC-MS/MS method for the quantification of nifuratel in human plasma, employing liquid-liquid extraction, and to validate this method using clinical trial samples.

2. Experimental

2.1. Chemicals

Nifuratel (100%) was provided by Chongqing Southwest Pharmaceutical Factory Co., Ltd. (China), and Nifuratel 13C D3 (98.6%) (internal standard, IS) was ob-

tained from Toronto Research Chemicals (Toronto, Canada). Acetonitrile, diethyl ether, and formic acid (HPLC-grade) were purchased from Merck (Darmstadt, Germany). Blank human plasma (K2EDTA) was sourced from Bioivt (UK), and the water used for the mobile phase preparation was purified using a Millipore MilliQ water purification system (USA).

2.2. Stock Solutions, Calibration Standards and QCs

Stock solutions of Nifuratel (1 mg/mL) and Nifuratel 13C D3 (IS) (0.2 mg/mL) were prepared in acetonitrile. Working solutions for NIF were obtained by diluting the stock solutions in acetonitrile. The internal standard working solution was prepared in acetonitrile at 10 ng/mL. All stock and working solutions were kept in the dark, covered with aluminum foil, and stored at −20˚C when not in use.

Calibration standards and QC samples were prepared by spiking known amounts of the working solutions into blank plasma. Calibration standards were set at concentrations of 0.25, 0.5, 4, 20, 45, 90, 135, and 150 ng/mL. The QC samples were prepared at 0.25 ng/mL (LLOQ), 0.75 ng/mL (QC Low), 6 ng/mL (QC Medium 1), 60 ng/mL (QC Medium 2), and 120 ng/mL (QC High). The QC samples were stored at −70˚C until needed.

2.3. Instrumentation

LC-MS/MS analysis was carried out using an ACQUITY UPLC I-Class/Xevo TQ-S micro IVD System, controlled by MassLynx Software, with data processing through the TargetLynx Application Manager. Separation was conducted on an ACQUITY UPLC BEH C18 column (1.7 µm, 2.1 × 50 mm) at 40˚C. The mobile phase consisted of solvent A (water with 0.01% aqueous formic acid) and solvent B (acetonitrile with 0.01% aqueous formic acid) at a flow rate of 0.6 mL/min. The gradient program ran as follows: from 0 to 0.3 min, isocratic at 80% A; from 0.4 to 1.5 min, linear gradient from 80% A to 50% A; from 1.5 to 2 min, linear gradient from 50% A to 10% A; from 2 to 2.2 min, constant at 10% A; from 2.2 to 3 min, linear gradient from 10% A to 90% A. The total runtime was 3 minutes. A 4 µL sample was injected into the system, and the autosampler was set at 10˚C.

The MRM transitions were m/z 286.1 → 241.97 for NIF and m/z 290.1 → 246.04 for IS. The mass spectrometer was optimized for maximum ion intensity, with the source parameters summarized in Table 1.

Table 1. LC/MS/MS source parameters.

Interface	ESI	**Desolvation temperature (˚C)**	350
Capillary voltage (kV)	0.8	**Cone voltage (V)**	55
Desolvation gas flow (L/Hr)	650	**Collision voltage(V)**	9
Cone gas flow (L/Hr)	50	**Dwell (s)**	0.06
Source block temperature (˚C)	150	**Desolvation temperature (˚C)**	350

2.3. Sample Preparation

Plasma samples (200 µL) were mixed with 50 µL of IS (100 ng/mL) in a 10 mL centrifuge tube and vortexed for 5 seconds. Diethyl ether (3 mL) was added, followed by vortexing for 30 seconds. The samples were centrifuged at 4600 rpm for 10 minutes, and 2.5 mL of the organic layer was transferred to a test tube and evaporated under nitrogen in a 40˚C water bath using a Zymark Turbovap system. The residue was reconstituted with 200 µL of diluent (70:30 MPA: MPB, v/v), and a 4 µL aliquot was injected into the chromatographic system.

2.4. Pharmocokinetic Study

28 healthy volunteers were randomised in a pharmacokinetic study which has been reviewed and approved by the Erciyes University Bioavaibility-Bioequivalence Trial Ethical Committee (05.10.2022-2022/244). Written informed consents were collected from all participants. Blood samples were taken by a short intravenous catheter and were collected into tubes using K_2 EDTA as anti-coagulating agent. Blood samples were collected at pre-dose and 0.25, 0.50, 1.00, 1.33, 1.66, 2.00, 2.33, 2.66, 3.00, 3.50, 4.00, 5.00, 6.00, 8.00, 10.00, 12.00, 16.00 and 24.00 hours after drug administration. After centrifugation at 3000 rpm for 10 min, the separated plasma from each sample was transferred into transparent, polypropylene tubes and all the aliquoted plasma samples were flash freezed immediately. The flash frozen samples (aliquoted plasma samples) were transferred to a deep-freezer and stored at −70˚C.

3. Results and Discussion

3.1. Method Validation

The method was validated according to the guidelines of the US-FDA Bioanalytical Method Validation Guidance [7] and the European Medicines Agency's guidelines for bioanalytical method validation [8]. The validation included assessments of selectivity, carry-over, linearity, calibration curve accuracy, precision, recovery, matrix effects, and stability.

3.1.1. Selectivity and Carry-Over

Selectivity was evaluated by analyzing blank plasma samples from eight different sources, including hemolyzed and lipemic samples, to detect any interference at the retention times of NIF and the internal standard. No interferences were observed, confirming the method's selectivity (**Figure 2**). The injection carry-over test was conducted to assess the degree of analyte carry-over between samples in each run. A blank sample, which was extracted, was inserted into the injection sequence following the highest calibration standard (ULOQ). The injection volume for the blank was the same as that of the other samples. No carry-over was observed.

3.1.2. Linearity

A calibration curve was constructed from a blank sample (a plasma sample pro-

cessed without the IS), a zero sample (a plasma processed with the IS) and eight non-zero samples covering the total range 0.25 - 150 ng/mL, including the LLOQ. The calibration curves were generated using the analyte to IS peak area ratios by weighted ($1/x^2$) least-squares linear regression on consecutive days. The acceptance criterion for a calibration curve was a correlation coefficient (r) of 0.99 or greater, and that each back-calculated standard concentration must be within 15% deviation from the nominal value except at the LLOQ, for which the maximum acceptable deviation was set at 20%. At least 75% of the eight non-zero samples must meet the exception criteria, which include acceptable LLOQ and ULOQ [4] [5].

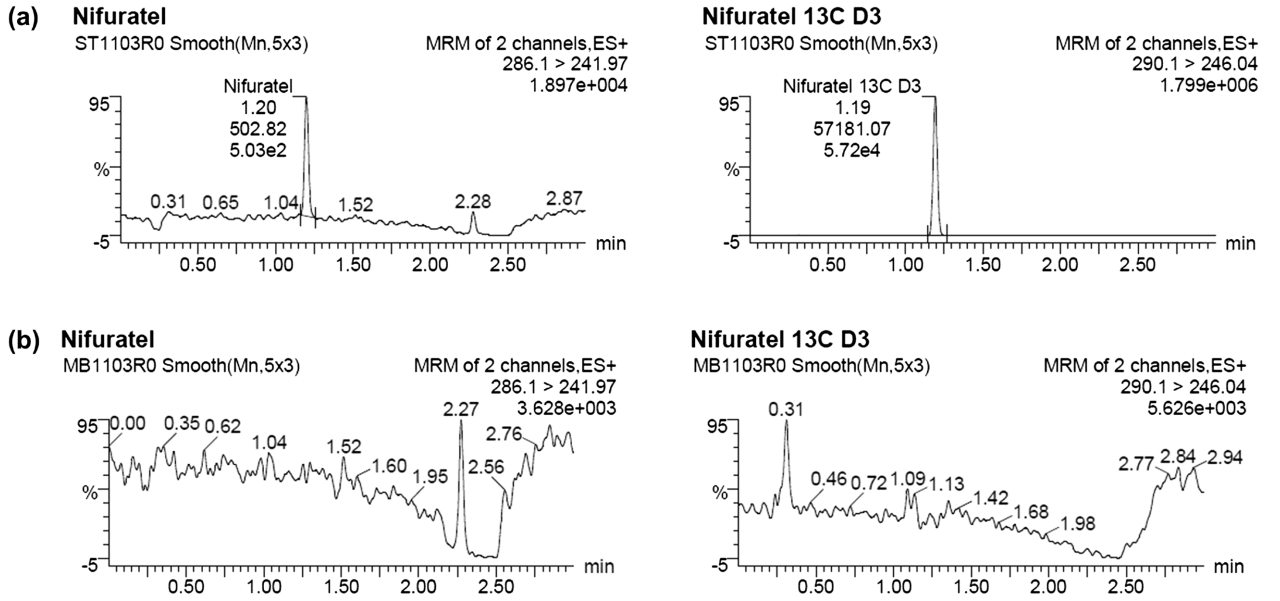

Figure 2. MRM chromatograms of 0.25 ng/mL NIF spiked with internal standard (a) and human blank plasma (b).

3.1.3. Accuracy and Precision

The within-batch precision and accuracy were evaluated by analyzing QC samples at five different concentration levels (0.25 ng/mL (LLOQ), 0.75 ng/mL (QC Low), 6 ng/mL (QC Medium), 60 ng/mL (QC High) and 120 ng/mL (ULLOQ)) with six replicates in a batch. Both within-batch and between-batch precision and accuracy were assessed with QC samples at five concentration levels. The precision values were within acceptable limits of ≤15% for all QC levels, except for the LLOQ, where the deviation did not exceed 20%. The data on within- and between-batch precision and accuracy of the method were summarized in **Table 2**.

3.1.4. Matrix Effect

Matrix effects were evaluated using plasma from six different sources, including hemolytic and lipemic samples. The results showed that no significant matrix effects were observed for NIF at both low and high QC concentrations, with acceptable precision (%CV) of ≤ 15% [8]. The precision (%CV) of QC2 and QC4 were 3.009% and 2.957%, respectively.

Table 2. Within-batch precision and accuracy of the method for determining NIF in plasma samples.

Intra-batch (n = 18)				Inter-batch (n = 3)			
Sample	Concentration (mean ± SD; ng/mL)	RE (%)	CV (%)	Sample	Conc. Found (mean ± SD; ng/mL)	RE (%)	CV (%)
LLOQ (1)	0.253 ± 0.008	1.267	3.318				
LLOQ (2)	0.243 ± 0.013	−2.733	5.314	LLOQ	0.245 ± 0.0112	−2.000	4.552
LLOQ (3)	0.239 ± 0.007	−4.533	3.052				
QC Low (1)	0.825 ± 0.063	10.044	7.640				
QC Low (2)	0.793 ± 0.024	5.667	3.034	QC Low	0.803 ± 0.041	7.089	5.076
QC Low (3)	0.7927 ± 0.015	5.556	1.843				
QC Medium (1)	6.170 ± 0.075	2.825	1.209				
QC Medium (2)	6.167 ± 0.131	2.783	2.132	QC Medium	6.140 ± 0.149	2.339	2.418
QC Medium (3)	6.085 ± 0.216	1.408	3.544				
QC High (1)	60.465 ± 1.166	0.776	1.928				
QC High (2)	58.732 ± 1.093	−2.113	1.861	QC High	59.188 ± 1.477	−1.353	2.496
QC High (3)	58.367 ± 1.360	−2.722	2.330				
ULLOQ (1)	115.120 ± 2.018	−4.066	1.753				
ULLOQ (2)	114.951 ± 3.040	−4.207	2.645	ULLOQ	116.358 ± 3.273	−3.035	2.813
ULLOQ (3)	119.001 ± 3.242	−0.832	2.724				

SD: standard deviation; RE: relative error; CV: coefficient of variation; LLOQ: (0.25 ng·mL^{-1}); QC Low: (0.75 ng·mL^{-1}); QC Medium: (6 ng·mL^{-1}); QC High: (60 ng·mL^{-1}), ULLOQ: (120 ng·mL^{-1}).

3.1.5. Recovery

The recovery of NIF was assessed by comparing the analyte responses from six extracted plasma samples at low, medium, and high quality control concentrations (0.75, 60, and 120 ng/mL) to those of six properly diluted standard solutions. The overall mean recovery for NIF was found to be 98.90% ± 1.64%.

For the internal standard (IS), the responses of six extracted samples at the medium QC concentration (60 ng/mL) were compared to those of six appropriately diluted IS solutions. The mean recovery for the internal standard was 99.24%.

3.1.6. Stability and Dilution

The following tests assessed the stability of the analyte under conditions that are typically encountered during real sample handling and analysis.

The stability of spiked plasma samples was assessed under varying temperature and time conditions, in addition to evaluating the stability of the stock standard solution. NIF demonstrated stability in human plasma at room temperature for up to 5 hours and was also stable for 2 hours in whole blood under the same con-

ditions. No significant degradation occurred when processed plasma samples were stored in the autosampler tray of the instrument at 10˚C for 24 hours. For long-term stability, low and high QC samples were stored frozen at both -20˚C and −70˚C and remained stable for 11 days. The stability results were within the acceptance criteria of ±15% of the nominal concentration, as summarized in Table 3. Stock solution stability at room temperature was evaluated for short-term storage and found to remain stable after 6 hours, while long-term storage at −20˚C for 13 days also preserved analyte stability. Stability of the internal standard (IS) was confirmed over a 13-day period.

Dilution integrity was assessed by spiking blank plasma with NIF concentrations above the upper limit of quantification (ULOQ), followed by dilution with blank plasma at dilution factors ranging from 2 to 20 times. A minimum of six replicates were tested for each dilution factor. The accuracy and precision of the method were evaluated, and the results met the required criteria, with deviations within ±15%. This demonstrates that the method can reliably quantify NIF even at concentrations exceeding the ULOQ when properly diluted.

Table 3. Results of stability of NIF in human plasma under different storage conditions.

NIFURATEL				
Storage condition	Nominal Conc. (ng/mL)	Conc. Found mean ± SD (ng/mL)	CV (%)	RE (%)
Autosampler stability[a]	0.75	0.792 ± 0.024	2.979	5.556
	120	117.798 ± 1.775	1.507	−1.835
Short-term plasma stability[b]	0.75	0.804 ± 0.023	32.856	7.178
	120	118.111 ± 2.911	2.465	−1.574
Long-term plasma stability (−70˚C)	0.75	0.799 ± 0.0206	2.577	6.578
	120	120.523 ± 1.419	1.177	0.436
Long-term plasma stability (−20˚C)	0.75	0.812 ± 0.0202	2.491	8.267
	120	117.845 ± 1.836	1.558	−1.795
Freeze-thaw stability[c]	0.75	0.788 ± 0.0276	3.511	5.000
	120	120.700 ± 2.422	2.007	0.584
Whole Blood Stability[b]	0.75	0.787 ± 0.009	1.164	4.889
	120	118.615 ± 1.146	0.966	−1.154

RE: Relative error (Accuracy), CV: Coefficient of Variation (Precision), SD: Standard Deviation; [a]Kept at autosampler temperature, 10˚C; [b]Stored at room temperature; [c]Stored at −70˚C.

3.2. Application To Bioequivalence Study

The validated method was successfully applied to a bioequivalence study. 28 subjects were planned and randomised. There has been one drop-out. As a result, 27 subjects completed the clinical phase of the study. The plasma samples obtained from 27 healthy human volunteers following oral administration of nifuratel 200

mg test and reference formulations, were analysed. In this single-dose, two-period, cross-over, randomised study nifuratel tablets were administered after standardised high-fat and high-calorie breakfast. Serial blood samples were collected throughout 24 hours. The mean plasma concentration-time profile is shown in Figure 3. The mean ± sd of maximum plasma concentration (Cmax) for the test and reference were found to be 33.215 ± 12.523 ng/mL and 30.864 ± 9.477 ng/mL, respectively. The time to reach peak plasma concentration (tmax) for the test and reference was found to be 2.66 h and 3.50 h respectively. The mean ± sd of area under the plasma concentration-time curve from zero to tlast (AUC0-t) for the test and reference was found to be 93.940 ± 34.763 ng h/mL and 96.564 ± 30.711 ng h/mL, respectively. The mean ± sd of area under the plasma concentration-time curve from zero to infinity (AUC0-∞) was 95.108 ± 34.830 ng h/mL for the test and 98.458 ± 31.437 ng h/mL for the reference. The elimination half-life (t½) was 1.15 h for the test and 1.26 h for the reference. T/R mean ratios for Cmax and AUC0-t were 104.5826 % and 96.3590 %, respectively. The 94.12% confidence intervals for Cmax and AUC0-t were found to be 94.28 - 116.01 and 87.68 - 105.89, respectively, which were within the acceptable range of 0.80 - 1.25. Based on these statistical considerations, it was concluded that the two nifuratel formulations (T and R) were bioequivalent.

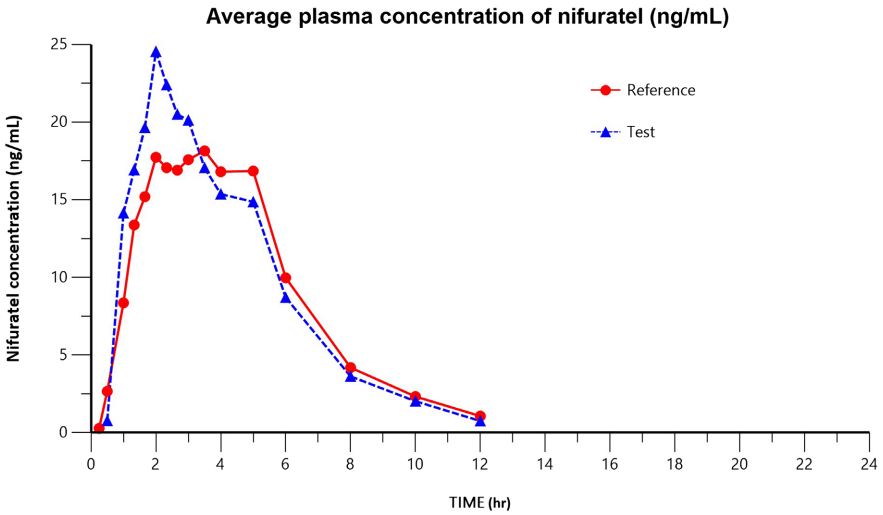

Figure 3. The mean plasma concentration-time profile.

4. Conclusion

In this study, a robust and sensitive LC-MS/MS method was successfully developed and validated for the quantification of nifuratel (NIF) in human plasma. The method demonstrated high precision, accuracy, and linearity within the concentration range of 0.25 - 150 ng/mL, making it suitable for routine analysis in clinical studies. The method showed no significant carry-over, matrix effects, or interferences, ensuring reliable and reproducible results. The recovery of NIF was excellent, with a mean recovery of 98.90%, and the internal standard recovery was sim-

ilarly high at 99.24%. Stability evaluations confirmed that the analyte remained stable under various storage and handling conditions, including during freeze-thaw cycles and autosampler conditions. The method has been effectively utilized in a bioequivalence study to assess the pharmacokinetics of NIF following the oral administration of nifuratel tablets.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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