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Research Article

A Cost-Effective RP-HPLC Bioanalytical Technique for Simultaneous Estimation of Nintedanib and Naringenin in Mice Plasma: Development, Validation, and Stability Assessment

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Abstract

Nintedanib (NTB), a first-line tyrosine kinase inhibitor used for Pulmonary Idiopathic fibrosis and lung cancer, found promising effects when combined with bioenhancer Naringenin (NGN) for increased bioavailability. The purpose of the study is to developed a bioanalytical technique for concurrently estimating NTB and NGN in mice plasma using RP- HPLC using Alectinib (ALB) as internal standard. The Gradient elution technique was developed to separate NTB, NGN, and ALB within 15 minutes of total run time. The mobile phase consisted of acetate buffer pH 5.2 and Acetonitrile in a Linear gradient pattern. The flow rate of 1.0 mL/min, and the injection volume of 50 µL. Detection was achieved at a wavelength of 290 nm, and the retention times for NTB, NGN, and ALB were found to be 4.5, 6.2, and 10.2 minutes, respectively. Mice Plasma samples were processed using a protein precipitation technique to extract the analytes. The method demonstrated linearity with R^2 of 0.9953 for NTB and 0.9978 for NGN, over a concentration of 50-1000 ng/mL and 75-1050 ng/mL, respectively. The results of all the validation parameters performed as per ICH guidelines M10 were within the accepted limits with less than 3 %RSD. The % recovery from plasma was more than 95%. Freeze- thaw, Bench-top, short-term, and long-term stability studies were also achieved at LQC, MQC, and HQC levels. The established bioanalytical technique can simultaneously estimate the NTB and NGN in plasma samples and is suitable for further pharmacokinetic studies.

Keywords: Nintedanib; Naringenin; Simultaneous Estimation Bioanalytical Method; RP-HPLC; Mice Plasma; Stability Studies; Bio-analytical Method Validation

Abbreviations

RP-HPLC: Reversed Phase High Performance Liquid Chromatography; NTB: Nintedanib; NGN: Naringenin; ALB: Alectinib; IS: Internal Standard; MF: Marketed Formulation; NTB-NCs: Nintedanib Nano-Crystals; ICH: International Council for Harmonization; AUC: Area Under Curve; Cmax: Concentration Maximum; Tmax: Time Maximum; MRT: Mean Residence Time; LQC: Lower Quality Control; MQC: Mid Quality Control; HQC: High Quality Control; ULOQ: Upper Limit of Quantification; LLOQ: Lower Limit of Quantification; CPCSEA: Committee for the Purpose of Control and Supervision of Experiments on Animals; P&A: Precision and Accuracy; MF: Marketed Formulation; NCs: Nano-Crystals and NTB-NCs: Nintedanib Nano-crystals

Introduction

Cancer is one of the leading causes of mortality, affecting the health of many humans. Modernization and changing lifestyles of humans lead to increased cancer cases [1,2]. Lung cancer and Idiopathic Lung fibrosis are among the most occurring known diseases worldwide. "Nintedanib", a tyrosine kinase inhibitor, is a first-line treatment choice and therapy maintenance. The chemical structure of nintedanib is depicted in Figure 1(a). Nintedanib belongs to BCS Class IV; therefore, they have low solubility, low bioavailability, and high protein binding. To counter the disadvantages associated with this drug, a need to increase the bioavailability of the nintedanib with minimal side effects occurs.

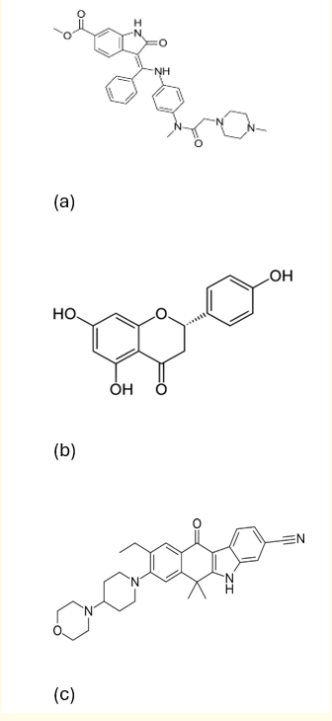

Figure 1: Figure 1(a): Chemical structure of Nintedanib [3]. Figure 1(b): Chemical structure of Naringenin [4]. Figure 1(c): Chemical structure of Alectinib [5].

Therefore, to address this issue, bioenhancers in combination with nintedanib can be used. In this research article, one such attempt was made to increase Nintedanib's bioavailability by using Naringenin as a bioenhancer. The chemical structure of Naringenin is shown in Figure 1(b).

An attempt was made to develop a simple, precise, and robust method for simultaneous estimation of nintedanib and bioenhancer with the utmost precision and accuracy in a shorter duration of time at a very minute quantity. The developed method should be capable of quantifying even subtle levels in the marketed formulation and combination with Naringenin.

The literature review examines various hyphenated analytical practices, including Liquid Chromatography - Mass Spectroscopy (LC-MS) and UHPLC-ESI (+)-MS/MS, for quantifying nintedanib [6-8] and Naringenin [9-11] individually in the formulations.

According to a literature review, several attempts have been made to develop an individual bio-analytical method for nintedanib and Naringenin. Still, none have tried to develop a technique simultaneously capable of estimating nintedanib and Naringenin in less time with the most precise and accurate data from the mice's plasma.

The current article aims to develop an HPLC-UV bioanalytical method that can quickly, precisely, and robustly estimate nintedanib and Naringenin simultaneously in APIs with standard and non-standard formulations from the mice plasma for the pharmacokinetic assessment and validate the method according to ICH guidelines ICH M10 [12]. The effectiveness and practicality of the suggested method were assessed with a focus on quality control research.

Materials and Methods

Chemicals and reagents

All solvents used for the mobile phase were HPLC grade, Nintedanib was obtained from Sun Pharma as a gift sample, and Naringenin was purchased from Sigma Aldrich.

Instruments and apparatus

A HPLC system (Agilent 1220 Infinity II), a compact Binary solvent delivery pump module, and a manual rheodyne injector with a 50 µL fixed loop with UV/Visible detector. Microsoft Excel (PK Solver) was used for statistical calculations using bioanalytical method validation.

Isosbestic point determination for NTB and NGN (λ)

10-50 µg/mL and 20-100 µg/mL of NTB and NGN working solutions were prepared to determine the wavelength. The scan was performed in the range of 200-400 nm. A detection wavelength of 290 nm was selected for further analysis of NTB and NGN. The scan for wavelength determination of the NTB and NGN standard solution is depicted in Figure 2.

Chromatographic conditions

The chromatographic conditions were optimized by different means (different buffers and different organic phases). Early chromatographic work was performed stepwise with various combinations of buffer phase with pH ranging from 5.00 to 5.20 and organic

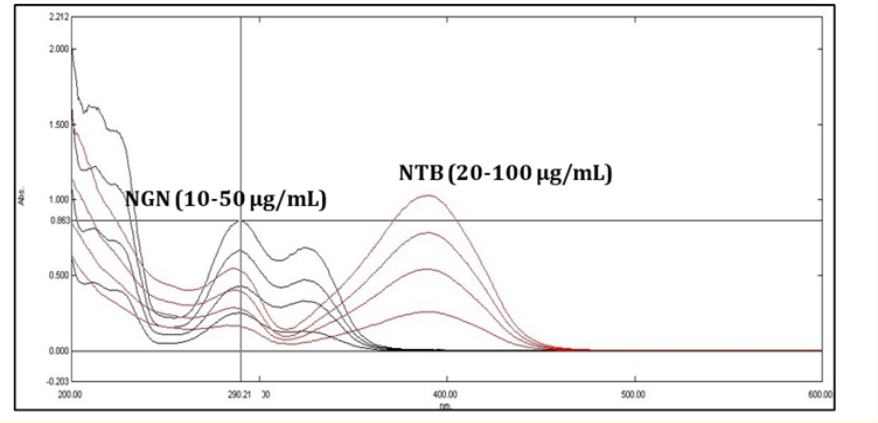

Figure 2: Overlay UV spectrum of NTB (10- 50 µg/mL) and NGN standard (20-100 µg/mL) with isosbestic point (Optimal Wavelength) for both the drugs at 290 nm.

phases (acetonitrile (ACN) and/or methanol). The wavelength for monitoring the eluent was selected by scanning a standard solution of NTB and NGN within 200 to 400 nm using a double- beam UV/Vis spectrophotometer (Shimadzu Spectrophotometer UV 1800, Japan).

Trials were initiated to achieve the optimum separation by varying the concentrations of buffer agent on different peak parameters was evaluated, viz. mobile phase Ammonium Acetate (pH 5.00 to 5.20) in a range 5- 20 mM concentration with ACN at 1.0 mL/min flow rate in a buffer to organic ratio noted from isocratic elution to Linear gradient elution. However, optimum effective peak symmetry was obtained in Linear gradient elution. Moreover, the effects of different levels of all these factors were systematically addressed on system suitability parameters such as %RSD of peak area, retention time, capacity factor, asymmetry, resolution, and peak width. All noted measurements were performed with an injection volume of 50 µL and UV detection at 290 nm of samples dissolved in a diluent of water and Acetonitrile in the ratio of 1:1, respectively.

Preparation of standard and resolution solution

Diluted standard solutions of each analyte representing 10 µg/mL concentration were prepared with diluent. Alectinib (ALB, Figure 1c) was used as an internal standard (IS) for NTB and NGN. Resolution solution containing 4µg/mL and 2 µg/mL each of NTB and NGN and 2 µg/mL of ALB was prepared from respective stock solutions. For optimization purposes, 50 µL of resolution solution was injected into the chromatograph and system suitability parameters. %RSD of peak area for six injections of all analytes, %RSD of retention time for six injections of all analytes, and peak asymmetry factor at 10% peak height and resolution were studied.

Sample preparation and extraction

The protein Precipitation method was employed to separate NTB and NGN from the plasma matrix. 5.0 mg each of NTB, NGN, and Alectinib (ALB) were weighed, transferred into a 10-mL volumetric flask, dissolved, and made up to the mark using a diluent (500 µg/mL). 5 µg/mL was made from the solutions mentioned above. 500 ng/mL of ALB was used as the internal standard in mice plasma. 700 ng/mL of NTB and 800 ng/mL of NGN were added. 0.8 mL of mice plasma was added to a 2 mL Eppendorf. After that, acetonitrile was used to bring the volume up to 2 mL for plasma precipitation. To separate the proteins from the prepared samples, they were vortexed for 5 minutes and centrifuged at 10000 RPM for 15 minutes at 4 ± 5 °C. The supernatant was carefully pipetted out and injected into the chromatographic system.

Bioanalytical method validation [12]

The developed HPLC conditions were validated according to ICH M10 guidelines for bioanalytical method validation. The specificity of the method was performed by injecting blank plasma, spiked plasma samples, and plasma samples spiked with frequently prescribed medication, which were analyzed. Selectivity of the method was performed by injecting six samples at the LLOQ level along with six blank plasma samples, which were tested for interference by comparing the mean peak response obtained by injecting blank plasma samples to the mean peak response of LLOQ (50 ng/mL NTB and 75 ng/mL NGN). The representative chromatograms were generated to show no interference of the plasma components or sample matrix in the presence of the main analyte peak. Dilution integrity and carry over effect were also accessed to establish specificity of the developed method.

Calibration curve of NTB and NGN

The standard curve was determined on each day of the six-day validation period; the slope, intercept, and correlation coefficient were determined. Each run consisted of a double control, system suitability sample, blank samples (a plasma sample processed without an IS), a control sample (a plasma processed with IS), and a calibration curve consisting of twelve non-zero samples covering the total range (LLOQ to 50 ng/mL for NTB and 75 ng/mL NGN) and QC samples at three concentrations (n=6, at each concentration). Such runs were generated on six consecutive days. Calibration samples were analyzed from low to high at the beginning of each run, and other samples were distributed randomly throughout the run. For the calculation of the standard curve, plots of peak area ratios against concentration were used.

Sensitivity

The sensitivity (LLOQ) was determined by signal-to-noise ratio. The resolution solution was serially diluted and spiked to the rat plasma, and injections were made to obtain a chromatogram. Similarly, blank plasma samples were also processed and injected into chromatographs. The LLOQ was expressed for the analyte concentration having a response at least 5 times more than a blank response.

Precision and accuracy (P&A)

P&A for the developed method for NTB were employed at LQC (112.5ng/mL), MQC (350 ng/mL), HQC (920.5 ng/mL), and ULOQ (1000 ng/mL).

P&A for NGN were employed at LQC (112.5ng/mL), MQC (400 ng/mL), HQC (960.5 ng/mL), and ULOQ (1050.5 ng/mL).

All were performed in triplicate and analyzed using the HPLC method. Precision was expressed as the coefficient of variation (%CV). Precision and accuracy values (%CV) less than or equal to 15% for QC samples, whereas less than or equal to 20% for LLOQ and ULOQ were acceptable.

Recovery studies

Recovery was executed by injecting 5 replicates of aqueous LQC and 3 replicates of extracted QC samples at the LQC level. The extracted and unextracted areas of analyte and IS were injected, and % recovery was calculated.

Stability studies

The stabilities studies were performed for the following stability studies mentioned below

Freeze–thaw stability [12]

Freeze thaw stability (3 cycles) was performed by injecting six freshly prepared and six stability samples at LQC and HQC levels for NTB and NGN.

Bench-top stability study [12]

The spiked plasma sample at HQC Level for NTB and NGN was performed at room temperature (Bench-top) for 8 hours by injecting 6 sample sets at the MQC level.

Short-term stability and long-term stability [12]

The short-term stability (8 hr. Room Temperature) and long-term stability (7 days at -20°C) were performed for NTB and NGN by injecting 6 sample sets at the MQC level.

Matrix effect [12]

The matrix effect was performed by the post-extraction addition method. This method was performed at two different concentration levels, i.e., LQC and HQC level samples.

The LQC and HQC samples in each blank biological matrix source were injected by external spiking of the extracted blank matrix.

In-Vivo plasma drug concentration [13]

Male Balb/c mice weighing around 25-30 g andaged 5-6 weeks were used for the study. For the pharmacokinetic investigation, rats were randomly divided into 4 groups of 6 animals per group. The oral administration was carried out using an oral feeding needle (16 gauge), and the (Drug) NTB-NCs Mini- tablet with NGN (Bioenhancer) was given orally. A homogeneous suspension of NTB and NGN was prepared in the vehicle with water.

Blood was collected from the animal's retro-orbital plexus (up to 0.5 mL) in Eppendorf tubes with 50 µL of disodium EDTA solution at predetermined intervals (0.5, 1, 2, 4, 6, 8, 10, 12, 24, 36, and 48 hours). The plasma was separated at 10°C for 15 minutes at 10,000 rpm and then kept at -20°C for later investigation. The samples were processed according to the protein precipitation protocol. The study was carried out per CPCSEA guidelines and was approved by the Institutional Ethics Committee with the protocol number MSU/IAEC/2022-23/1940 to control the supervision of animal experiments.

Pharmacokinetic parameter for the analysis

Non-compartmental analysis with PK Solver (Microsoft Excel) was used to determine various pharmacokinetic parameters, including Cmax (maximum concentration in plasma), Tmax (time required to reach maximum concentration in plasma), Area Under the Curve (AUC), Half-life (t1/2), Mean Residential Time (MRT), and so on. Each group had a graph of plasma drug concentration versus time. The relative bioavailability of NTB-NCs and NGN Mini pills concerning the marketed formulation.

Results and Discussion

Wavelength selection and optimization trials

The present HPLC method claims to quantify the analytes at low plasma concentrations precisely. To avoid plasma interference, the retention factor of the first analyte was kept at more than 4 minutes. The optimal wavelength of 290 nm was selected for the analysis when NTB and NGN were scanned at 200-400 nm (Figure 2). The optimum response for NTB and NGN was selected for working standard and sample at 290 nm. For the effective separation of NTB and NGN with IS, various trials for mobile phase optimization with ACN and ammonium acetate (5.20 ± 0.05) as buffer were designed simultaneously, as mentioned below in Table1.

The optimized chromatographic conditions with gradient elution mode are mentioned in Table 2, with SST parameters for resolution solution is noted in Table 3.

Table No.1: Mobile phase optimization trials for estimating NTB and NGN in Blood samples.

Sr. No.	Mobile Phase	Elution Method	Observation	Result
1	Methanol – 10 mM Potassium dihydrogen orthophosphate (50:50, v/v)	Isocratic	Good Elution Strength: Analytes were eluted at nearly the same retention time and had a non-symmetrical peak shape.	Rejected
2	ACN – 10 mM Potassium dihydrogen orthophosphate (50:50, v/v)	Isocratic	Good Elution Strength: Analytes were eluted at nearly the same retention time and had a non-symmetrical peak shape.	Rejected
3	ACN – 10 mM Ammonium acetate (80:20, v/v)	Isocratic	Good Elution Strength, Analytes were eluted at nearly the same retention time.	Rejected
4	ACN – 10 mM Ammonium acetate	Gradient	Good Elution Strength, less resolution between the two peaks.	Rejected
5	ACN – 10 mM Ammonium acetate	Gradient	Good Elution Strength, Resolution of 12.5, spectral pure peak, and symmetrical peak shape.	Accepted

Table 2: Optimized chromatographic condition for NTB and NGN Estimation.

Column	**Agilent Zorbax C18 column (250 × 4.6 mm) 5 μm i.d.**							
Mobile Phase	Buffer: 10 mM ammonium acetate pH 5.2 ± 0.05 and Acetonitrile MP MP A (%v/v): Buffer: ACN:80:20 MP B (%v/v): Buffer: ACN: 20:80							
Gradient Program	As mentioned below							
	Time (min)		2.50	6.50	9.00	13.00	15.00	20.00
	0.00 MP A (%v/v)		35	35	12	12	72	72
		72	65	65	88	88	28	28
	MP B (%v/v)	28						
Mobile phase flow rate	1mL/min							
Column oven temperature	40°C							
Sample temperature	Ambient							
Injection volume	50 **μL**							
Run time	20 min							
NTB, NGN and ALB RT	5.25 ± 0.5 for NTB	7.65 **± 0.5** for NGN			10.35 ± 0.5 for ALB (IS)			
Wavelength (nm)	290 nm							

Table 3: System Suitability Studies for Resolution Solution.

SST Parameters	**ICH Limits**	**NTB**	**NGN**
Retention time	NA	5.25 ± 0.5	7.65 ± 0.5
Resolution	≥ 2	NA	12.05 ± 0.06
Tailing Factor	≤ 2	1.12 ± 0.02	1.19 ± 1.12
Theoretical Plates	≥ 2000	113511.00 ± 1268.37	32106.33 ± 225.41

To select an internal standard (IS), ALB was chosen due to its similar structure and formula ratio. ALB was eluted in the optimized chromatographic HPLC conditions developed for NTB and NGN. The resolution between NTB and NGN was found to be 12.05, and that of plasma protein was found to be 2.54. A representative system suitability chromatogram without plasma is given in Figure 3.

The protein Precipitation method was preferred for extracting NTB and NGN from the mice plasma because of minimum, easy, and reproducible extraction steps. The technique used cold ACN and MeOH. Trials were attempted to minimize the matrix effect and increase the extraction rate. The recovery of NTB and NGN with IS was near about 60% and had a non- symmetrical peak shape with MeOH; therefore, ACN was chosen as a precipitating agent as using ACN did not alter the peak shape and had maximum recovery with minimal matrix effect. Figure 4 represents the plasma spiked chromatogram with sample and blank plasma. In the last precipitation step, the supernatant was mixed with diluent (80: 20: Water: ACN). The % mean recoveries for all the analytes ranged from 85-98% in the currently developed method.

Validation parameters

The developed bioanalytical HPLC method was validated according to ICH M10 guidelines for its specificity and Selectivity, Precision and Accuracy, Calibration range, Recovery studies, Matrix effect, and Stability.

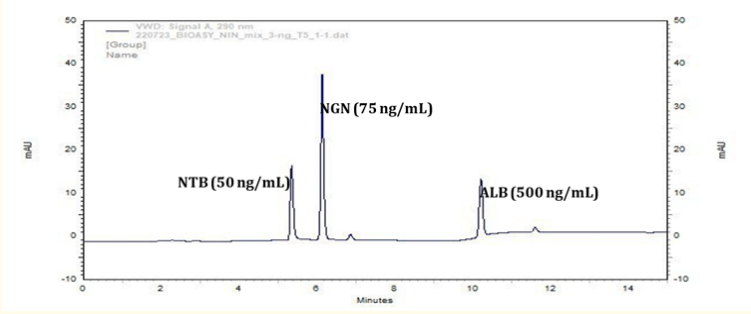

Figure 3: SST Chromatogram: SST Chromatogram representing NTB, NGN, and ALB with optimized chromatogram without plasma.

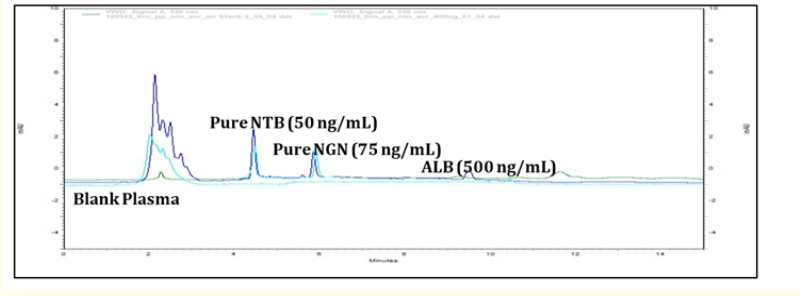

Figure 4: Chromatograms of Blank and Spiked Samples with Plasma at LLOQ Level Chromatogram representing Processed plasma Spiked with NTB, NGN and ALB (Specificity).

Specificity and selectivity

Selectivity of the method was performed at LLOQ level, i.e., for NTB (50ng/mL) and NGN (75ng/mL), the maximum percentage interference for analyte was found to be 0.12% and 0.20% for NTB and NGN respectively. In contrast, for IS, it was 0.06%.

The analyte signal at LLOQ was more than five times the noise level and was well above the limit of acceptance (Table No.4). No interference was observed at the analyte and IS retention time in the chromatogram at the LLOQ level for NTB and NGN (Figure 4). The peak purity plot for NTB (Figure 5(a)), NGN (Figure 5(b)), and ALB (Figure 5(c)) reveals that no interaction was observed between analytes, IS, and Plasma, indicating that peaks are spectrally pure reveling the specificity of the developed method.

Table 4: Selectivity of the method for NTB and NGN.

Analyte (n = 6)	Sequence	% interference for analyte	% interference for IS
NTB	Plasma Blank	0.12 ± 0.009	0.1 ± 0.006
	LLOQ (50 ng/mL)		
NGN	Plasma Blank	0.20 ± 0.01	
	LLOQ (75 ng/mL)		

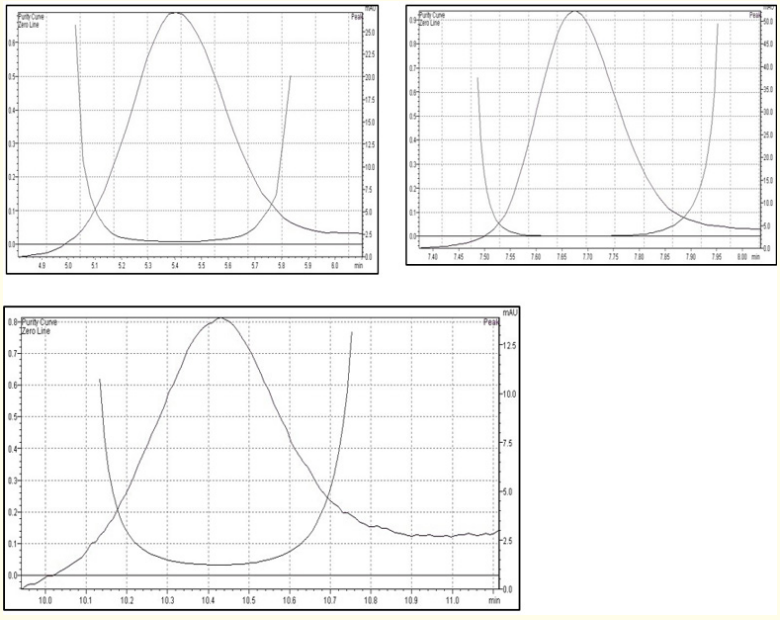

Figure 5: Figure 5(a): Bioanalytical Peak Purity Plot of NTB. Figure 5(b): Bioanalytical Peak Purity Plot of NGN. Figure 5(c): Bioanalytical Peak Purity Plot of ALB.

Precision and accuracy

The intraday Precision and accuracy (Table No.5(a)) for NTB and NGN were found in the range of 86.28% to 105.23% and 89.12% to 101.79%, respectively. The precision (%CV) value for intraday was between 1.41-5.38 and 2.33-3.69 for NTB and NGN, respectively. The Interday precision and accuracy (Table No.5(b)) for NTB and NGN were 86.53% to 101.91% and 82.69% to 99.08%, respectively. The (%CV) value for Interday precision was between 3.45-4.95 and 4.19-6.02 for NTB and NGN, respectively. All the accuracy and precision values met the acceptance criteria according to ICH guideline M10.

Table .5(a): Intraday Precision and Accuracy for NTB and NGN.

Analyte (n = 12)	Quality Control	Mean Conc Found	SD	% CV	% Accuracy
NTB	LQC (112.5 ng/mL)	107.49	5.78	5.38	95.54
	MQC (350 ng/mL)	340.19	10.63	3.12	97.20
	HQC (920.5 ng/mL)	904.43	12.89	1.42	98.25
	ULOQ (1000 ng/mL)	940.82	30.52	3.24	94.03
NGN	LQC (112.5 ng/mL)	107.34	2.82	2.63	95.41
	MQC (400 ng/mL)	379.315	13.99	3.69	94.83
	HQC (960.5 ng/mL)	911.55	27.16	2.98	94.90
	ULOQ (1050.5 ng/mL)	1000.53	23.28	2.33	95.24

Table 5(b): Interday Precision and Accuracy for NTB and NGN.

Analyte (n = 12)	Quality Control	Mean Conc Found	SD	% CV	% Accuracy
NTB	LQC (112.5 ng/mL)	106.31	5.26	4.95	94.50
	MQC (350 ng/mL)	332.86	13.30	4.00	95.10
	HQC (920.5 ng/mL)	863.19	37.23	4.31	93.77
	ULOQ (1000 ng/mL)	936.38	32.33	3.45	93.59
NGN	LQC (112.5 ng/mL)	102.94	6.77	6.02	91.50
	MQC (400 ng/mL)	374.54	15.68	4.19	93.63
	HQC (960.5 ng/mL)	888.14	41.31	4.65	92.79
	ULOQ (1050.5 ng/mL)	980.35	41.25	4.21	93.32

Calibration curve and linearity

The 8-point calibration curve and linearity (Figure 6) were prepared by spiking appropriate amounts of working solution into the blank Plasma to get final concentrations of 50-1000 ng/mL (Table No.6) for the NTB, 75-1050 ng/mL for NGN (Table No.6), and 500 ng/mL for ALB, respectively. The calibration curve was prepared by plotting the peak area ratio of the transition pair of NTB (Figure 7(a)) and NGN (Figure 7(b)) to that of IS (ALB) against the nominal concentration of calibration standards. The % recovery from linearity of NTB and NGN was found in the range of 88.33-105.92% (Table No.7(a)) and 91.41-105.67% (Table No.7(b)), respectively.

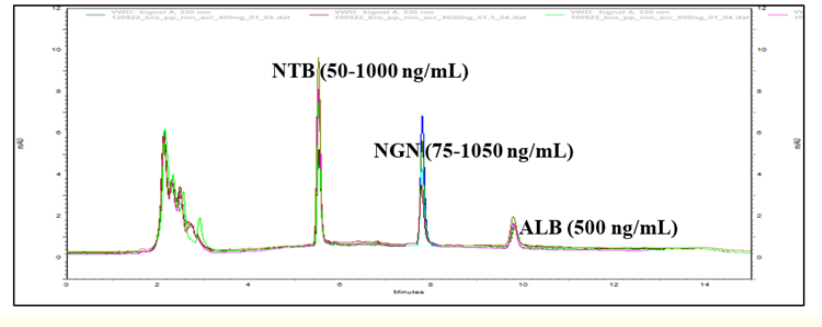

Figure 6: Bioanalytical Overlay plot for NTB (50-1000 ng/mL) and QRN (75-1050 ng/mL) with IS ALB (500 ng/mL).

Table 6: Bioanalytical Linearity of NTB and NGN.

NTB (50-1000 ng/mL)			NGN (75-1050 ng/mL)		
Conc (ng/mL)	Avg Area Ratio ± SD (n = 6)	RSD	Conc (ng/mL)	Avg Area Ratio ± SD (n = 6)	RSD
50	0.065 ± 0.000	0.46	75	0.381 ± 0.011	2.76
75	0.100 ± 0.001	0.88	150	0.759 ± 0.026	3.44
100	0.131 ± 0.002	1.61	300	1.513 ± 0042	2.76
200	0.273 ± 0.014	5.20	450	2.248 ± 0.094	4.18
400	0.533 ± 0.013	2.36	600	3.067 ± 0.061	2.00
600	0.798 ± 0.024	3.01	750	3.750 ± 0.068	1.82
800	0.954 ± 0.024	2.54	900	4.534 ± 0.032	0.71
1000	1.306 ± 0.048	3.67	1050	5.014 ± 0.017	0.34

Table 7(a): Calibration curve and Linearity of NTB.

Calibration curve for NTB																
Sequence No.	Cali. Set-1		Cali. Set-2		Cali. Set-3		Cali. Set-4		Cali. Set-5		Cali. Set-6		Cali. Set-7		Cali. Set-8	
	50 ng/mL		75 ng/mL		100 ng/mL		200 ng/mL		400 ng/mL		600 ng/mL		800 ng/mL		1000 ng/mL	
	Area Ratio	Actual Concentration	Area Ratio	Actual Concentration	Area Ratio	Actual Concentration	Area Ratio	Actual Concentration	Area Ratio	Actual Concentration	Area Ratio	Actual Concentration	Area Ratio	Actual Concentration	Area Ratio	Actual Concentration
PA-1	0.066	44.17	0.099	70.62	0.130	95.24	0.261	198.83	0.525	407.29	0.791	616.79	0.963	752.90	1.351	1059.15
PA-2	0.065	43.74	0.101	71.79	0.130	95.07	0.263	200.38	0.522	404.87	0.826	645.08	0.975	762.29	1.360	1066.60
PA-3	0.066	44.19	0.100	71.31	0.130	94.80	0.273	207.72	0.529	410.00	0.819	639.38	0.975	762.73	1.284	1006.44
PA-4	0.065	43.74	0.100	71.55	0.130	95.32	0.270	205.78	0.536	415.76	0.770	600.29	0.927	724.62	1.247	977.41
PA-5	0.065	43.78	0.102	72.54	0.135	98.81	0.297	226.84	0.554	429.80	0.784	611.50	0.928	725.69	1.288	1009.98
Mean	0.07	43.92	0.10	71.56	0.13	95.85	0.27	207.91	0.53	413.54	0.80	622.61	0.95	745.65	1.31	1023.92
SD	0.00	0.23	0.00	0.70	0.00	1.67	0.01	11.20	0.01	9.95	0.02	18.98	0.02	19.12	0.05	37.83
%CV	0.46	0.53	0.88	0.98	1.61	1.74	5.20	5.39	2.36	2.41	3.01	3.05	2.54	2.56	3.67	3.70
% Accuracy	88.33%		94.16%		95.24%		99.41%		101.82%		102.80%		94.11%		105.92%	

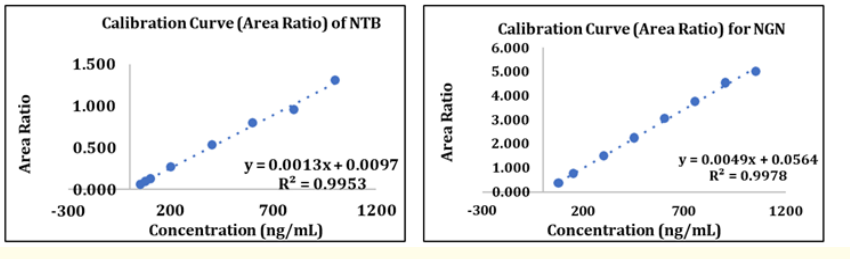

Figure 7: Figure 7(a): Bioanalytical Calibration curve for NTB. Figure 7 (b): Bioanalytical Calibration curve for NGN.

% recovery studies

The % recovery performed at the LQC level by preparing six sample sets: NTB (Table No.8(a)) was found to be 89.49% for recovery in solvent (without Plasma) and 87.13% in Plasma. NGN (Table No.8(b)) was found to be 88.76% for recovery in solvent (without Plasma) and 86.80% in Plasma. The results indicate that the plasma extraction procedure developed for NTB and NGN is acceptable. Nearly a 1% difference from the extraction procedure from solvent to Plasma suggests that the developed extraction procedure can be used for analytical purposes.

Matrix effect

The matrix effect was performed by the post-extraction addition method. This method was performed at LQC level samples by injecting 6 sample sets of NTB and NGN (Table No. 9).

The LQC samples in each blank biological matrix source were injected by external spiking of the extracted blank matrix.

Table 8(a): Recovery data for NTB.

Set Sequence	Absolute recovery–In solvent		Relative recovery-In Plasma	
	Analyte LQC-A (50 ng/mL)		Analyte LQC-A (50 ng/mL)	
	Unextracted sample	Extracted Sample	Unextracted sample	Extracted Sample
Set-1	101358	91352	104586	91352
Set-2	101538	91510	104397	91510
Set-3	99434	91003	105823	91003
Set-4	105382	90766	101737	90766
Set-5	102958	92367	107929	92367
Mean	102134.00	91399.60	104894.40	91399.60
SD	2206.57	614.22	2257.37	614.22
% CV	2.16	0.67	2.15	0.67
% Recovery	89.49		87.13	

Table 8(b): Recovery data for NGN.

Set Sequence	Absolute recovery– In solvent		Relative recovery- In Plasma	
	Analyte LQC-A (75 ng/mL)		Analyte LQC-A (75 ng/mL)	
	Unextracted sample	Extracted Sample	Unextracted sample	Extracted Sample
Set-1	582356	543340	625839	543340
Set-2	590293	534839	619527	534839
Set-3	600231	510291	610934	510291
Set-4	620635	555686	638349	555686
Set-5	623048	533480.2	590274	533480
Mean	603312.60	535527.24	616984.60	535527.24
SD	18081.32	16650.43	17967.80	16650.43
% CV	3.00	3.11	2.91	3.11
% Recovery	88.76		86.80	

Table 9: Matrix effect of NTB and NGN.

Sequence	LQC MF Factor		IS MF Factor	IS Normalized Matrix Factor	
	NTB	NGN		NTB	NGN
Set-1	1.0909	1.1115	1.1932	0.9142	0.9315
Set-2	1.1023	1.1511	1.1373	0.9692	1.0121
Set-3	1.0834	1.0893	1.1225	0.9651	0.9704
Set-4	1.0504	1.1553	1.1469	0.9159	1.0074
Set-5	1.1157	1.1605	1.1821	0.9438	0.9817
Set-6	1.0448	1.1784	1.1335	0.9217	1.0396
Mean	1.08	1.141	1.15	0.94	0.990
SD	0.03	0.034	0.03	0.02	0.038
% CV	2.62	2.94	2.47	2.64	3.81

Stability studies

Stability studies were performed for freeze- thaw and Benchtop at LQC and HQC levels for NTB and NGN. Long- and short-term stability was performed at MQC levels for NTB and NGN, respectively. Table No.10(a) and Table No.10(b) depict NTB and NGN's stability data at different conditions.

Pharmacokinetic Parameter for the analysis of NTB tablet-in-capsule technology

The proposed HPLC method enables the rapid analysis of NTB and NGN from mice plasma simultaneously with no interference. Thus, the developed method allows us to analyze NTB and NGN in tablet-in-capsule technology which was developed and optimized in the lab of Faculty of Pharmacy, The Maharaja Sayajirao University of Baroda, Vadodara for pharmacokinetics study. The mean plasma concentration-time profile of NTB and NGN is shown in Figure 8 below. The data were analyzed by non-compartmental pharmacokinetics via the Microsoft Excel PK Solver®. The Pk findings of NTB-NCs mini tablets with NGN, or tablets in capsule technology (Table No. 11) indicated 856.54 ± 23.34 ng/mL of Cmax and 11134.47 ± 156.23 ng*hr/mL of AUC. The NTB-NCs + NGN mini tablet significantly increased bioavailability by 1.00 and 1.92 times ($p < 0.05$) compared to the NTB and commercial formulations. The nanosized crystals, along with Bioenhancer (NGN), might limit the metabolism of UGT enzymes, increasing the bioavailability of NTB. The increased bioavailability suggests the potential for a dosage dose decrease.

Table 10(a): Solution Stability data for NTB.

Stability (n = 6)	Quality Control	Mean (ng/mL) ± SD	% CV	% Accuracy ± SD
Fresh LQC (112.5 ng/mL)	LQC	111.24 **± 0.98**	0.88	98.88 ± 0.87
Fresh HQC (920.5 ng/mL)	HQC	924.16 ± 24.77	2.68	110.40 ± 2.67
Freeze-thaw (3 Cycle)	LQC	106.64 ± 2.23	2.15	94.79 ± 2.04
	HQC	884.30 ± 20.36	2.30	96.07 ± 2.21
Benchtop Stability (8 hr Room Temp)	LQC	102.68 ± 0.85	0.82	91.27 ± 0.75
	HQC	867.76 ± 16.00	1.84	94.27 ± 1.74
Short-term Stability (MQC = 350 ng/mL)	MQC (Fresh)	349.76 ± 5.49	1.54	99.93 ± 1.57
	MQC	337.00 ± 2.50	1.78	96.29 ± 0.72
Long-term Stability (7 days at -20˚C) (MQC = 350 ng/mL)	MQC (Fresh)	349.76 ± 5.49	1.54	99.93 ± 1.57
	MQC	326.40 ± 10.80	3.31	93.26 ± 3.09

Table 10(b): Solution Stability data for NGN.

Stability (n = 6)	Quality Control	Mean (ng/mL) ± SD	% CV	% Accuracy ± SD
Fresh LQC (112.5 ng/mL)	LQC	109.05 ± 2.51	2.30	96.94 ± 2.23
Fresh HQC (960.5 ng/mL)	HQC	920.52 ± 33.39	3.63	95.84 ± 3.48
Freeze-thaw (3 Cycle)	LQC	107.28 ± 2.94	2.74	95.36 ± 2.62
	HQC	894.17 ± 28.13	3.15	93.09 ± 2.93
Benchtop Stability (8 hr Room Temp)	LQC	101.51 ± 3.16	3.11	90.23 ± 2.80
	HQC	853.69 ± 35.05	4.11	88.88 ± 3.65
Short term Stability (MQC = 400 ng/mL)	MQC (Fresh)	385.84 ± 13.16	3.41	96.46 ± 3.29
	MQC	339.14 ± 16.57	4.89	88.79 ± 4.14
Long-term Stability (7 days at -20˚C) (MQC = 400ng/mL)	MQC (Fresh)	385.84 ± 13.16	3.41	96.46 ± 3.29
	MQC	353.14 ± 6.51	1.84	89.28 ± 1.63

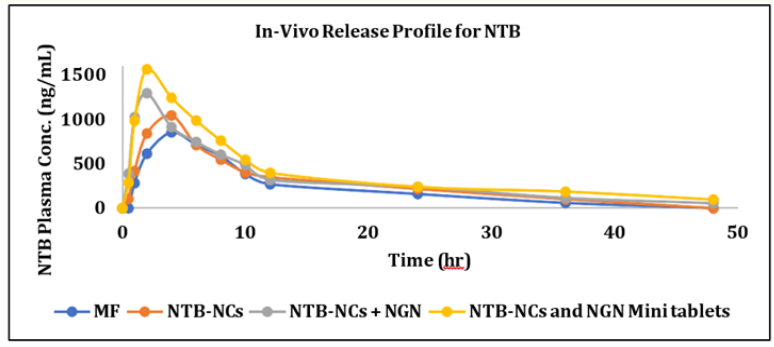

Figure 8: *In-Vivo* Release Profile for NTB-NCs mini tablet with NGN mini tablet.

Table 11: *In Vivo* Pharmacokinetics Parameter of NTB-NCs + NGN Mini Tablets.

Parameters	MF ± SD	NTB-NCs ± SD	NTB-NCs + NGN ± SD	NTB-NCs and NGN Mini tablets ± SD
C_{max} (ng/mL)	856.54 ± 23.34	1045.5 ± 18.98	1298.43 ± 21.76	1568.7 ± 10.65
T_{max} (Hr)	4.00 ± 0.00	4.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00
AUC (ng*Hr/mL)	1134.47 ± 156.23	14594.71 ± 1369.65	15953.53 ± 905.76	21395.18 ± 2076.32
$T_{1/2}$ (Hr)	10.43 ± **1.12**	13.519 ± 2.32	11.34 ± 1.11	18.57 ± 2.76
MRT (Hr)	14.89 ± 0.06	18.371 ± 12.23	16.453 ± 12.23	22.11 ± **3.32**
Relative Bioavailability (Folds)	1.00	1.31	1.43	1.92

Conclusion

The method developed is a simple, rapid, accurate, and reliable procedure for analyzing Nintedanib and Naringenin in mice plasma, meeting all requirements for validating an analytical methodology according to ICH guideline M10. The pk findings for NTB-NCs with NGN as tablet in capsule technology indicated almost 1.92 times increase in bioavailability compared to the marketed formulation, indicating a relative increase in the bioavailability of NTB.

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Conflicts of Interest

There are no conflicts of interest.

Ethical Approval

The *in-vivo* experiments followed rules from the Committee for Control and Supervision of Experiments on Animals (CPCSEA), the Ministry of Social Justice and Empowerment, and the Government of India. The Institutional Animal Ethics Committee of the Faculty of Pharmacy at Maharaja Sayajirao University of Baroda in Vadodara, India, approved the pharmacokinetic study, protocol No.: MSU/IAEC/2022-23/1940.

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Summary

The current research work aimed for the development and validation for the simultaneous estimation of nintedanib and naringenin by RP-HPLC in a mixture and the in-house formulation prepared at the lab from the mice plasma.

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