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DEVELOPMENT AND VALIDATION OF NITECAPONE IN HUMAN PLASMA USING TOLCAPONE AS INTERNAL STANDARD BY LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

Aswini Kumar Parida [*1], K. Srinivasa Rao [2] and Ajay Kumar Patnaik [3]

S. P. E. R. [1], Berhampur University, Berhampur - 760007, Odisha, India.
Department of Pharmacy [2], Indira Gandhi National Tribal University, Amarkantak - 484887, Madhya Pradesh, India.
Department of Chemistry [3], Ravenshaw University, Cuttack - 753003, Odisha, India.

Keywords:

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Correspondence to Author:
Aswini Kumar Parida

SPER, Berhampur University, Berhampur - 760007, Odisha, India.

E-mail: aswini.parida1810@yahoo.com

ABSTRACT: The present LC-MS/MS method for estimation of Nitecapone in human plasma using Tolcapone as an internal standard is established and validated as per FDA guidelines. A good response was obtained with ZORBAX Eclipse plus C18 column (150 mm × 4.6 mm ID, 5 µm) and mobile phase with a mixture of 0.01 M Ammonium phosphate buffer with pH adjusted to 5.0 with OPA and Acetonitrile (70:30, v/v) at a flow rate of 0.8 mL/min by positive ion mode (API 4000) with an injection volume of 20 µL and a run time of 3.0 min. Detection is performed by atmospheric pressure electrospray ionization (ESI) tandem mass spectrometry in positive ion mode. The precursor to product ion transitions is m/z 266.20 to 156.20 for Nitecapone and m/z 274.20 to 183.10 for Tolcapone (Internal standard) were used for quantization. The retention time of Nitecapone and Tolcapone (Internal standard) were found to be 2.12 min and 2.58 min, respectively. Linearity established for Nitecapone in the range of 50 ng/mL to 2000 ng/mL with correlation coefficient (r = 0.9997), and the overall percentage recovery was 90.39 % for Nitecapone and 92.34 % for Tolcapone (Internal standard) respectively. The CV % values of accuracy and precision for Nitecapone were found to be ≤ 15%, which indicates the accuracy and precision of the proposed method. This method is suitable for routine therapeutic drug monitoring of Nitecapone.

INTRODUCTION: The literature review reveals that very few LC methods were reported for the estimation of Nitecapone individually and combined with other drugs at the time of commencement of work. However, there are very few LC-MS/MS methods were reported for the estimation of Nitecapone in human plasma, and the HPLC methods available are with long runtime, hence time-consuming.

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Upon literature survey [1-6], very few LC-MS/MS methods have been reported for the determination of Nitecapone and Tolcapone. To the best of our knowledge, the simultaneous determination of Nitecapone and Tolcapone in human plasma has not been reported.

Objective of the Research Work: Hence, in the present study, an attempt has been made to develop a new, rapid, and reliable bio-analytical method for the determination of Nitecapone in human plasma with a short time of analysis. The aim of this method is the determination of these COMT inhibitors simultaneously in human plasma with a simple and fast extraction and quantification procedure to allow their therapeutic monitoring when needed in a reasonable time with a low cost.

Besides, fast bio-analytical methods would be beneficial in pharmacokinetic or bioequivalence studies where a high number of samples need fast analysis. Hyphenated techniques refer to the coupling of an independent analytical instrument like MS, FTIR, and NMR to the HPLC system to provide detection. The most common and effective hyphenated technique is LC-MS. MS detection has become the standard detector system for bio-analytical methods and the analysis of pharmaceutical compounds in biological systems (*e.g*., plasma or urine). The LC-MS/MS technique was selected to develop a satisfactory, sensitive, selective method with a desirable time of the chromatographic run.

MATERIALS AND METHOD:

Method and Experimental Design: A LC-MS/MS method was performed on a liquid chromatographic system consist of Shimadzu LC 10, an auto sampler of Shimadzu (SIL-HTc) coupled with an Applied Biosystems SCIEX a triple quadrupole mass spectrometer (API 4000) with electrospray ionization (ESI) used for analysis and Applied Biosystems/ MDS SCIEX Analyst software (version 1.4.2) for processing and data collecting. Zorbax Eclipse Plus C_{18} column (150 mm × 4.6 mm ID, 5 µm) is used as a stationary phase. An ultrasonic bath sonicator (Frontline FS 4, Mumbai, India), semi-micro analytical balance (India), and Whatman filter paper No. 41 are used in the study. The work was executed in the year 2018.

Reagents used: Nitecapone were procured from Yarrow chemicals, Mumbai, India. Tolcapone (Internal Standard) was procured from Mankind Pharma Limited, India. Acetonitrile of HPLC grade was procured from Rankem Ltd., India. The water of HPLC grade was obtained from Merck Specialities Private Limited, Mumbai, India. Ammonium phosphate and orthophosphoric acid of HPLC grade were procured from Merck Specialities Private Limited, Mumbai, India.

Drug Profile of Nitecapone: It has been used by the physician in the treatment of patients with Parkinson's disease. Parkinson's disease is an enduring degenerative disorder of the central nervous system that mostly involves the motor system. The general indications of Parkinson's disease are trembling, severity, slowness of movement, and difficulty with walking. To overcome these problems, an oral treatment of Nitecapone was initiated for the treatment of Parkinson's disease [7]. The structure [7] of Nitecapone is shown in **Fig. 1**.

Chemical Structure:

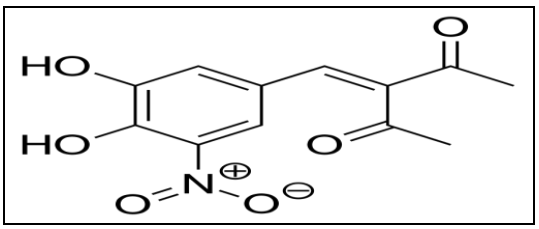


FIG. 1: CHEMICAL STRUCTURE OF NITECAPONE

Chemical Name: 3 - (3, 4 – dihydroxy – 5 - nitro-benzylidene) pentane-2, 4 – dione [7].
Molecular Formula: $C_{12}H_{11}NO6$ [7].
Molecular Weight: 265.221 g/mol [7].
Appearance: Beige powder.
Solubility: Soluble in dimethyl sulfoxide, methanol, dimethylformamide, acetonitrile, and ethanol.
CAS Number: 116313-94-1 [7].

Mechanism of Action: Nitecapone is a selective inhibitor of the enzyme catechol – O - methyl transferase (COMT). Clinical trials for Nitecapone oral therapy indicate that it is safety, efficacy, rapid and intensive control over Parkinson's disease. So, Nitecapone is clinically approved for the treatment of Parkinson's disease [7].

Drug Profile of Tolcapone: It has been used by physician in the treatment of patients with Parkinson's disease [8]. Structure [8] of Tolcapone is shown in **Fig. 2.**

Chemical Structure:

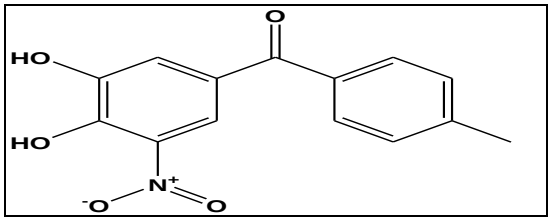


FIG. 2: CHEMICAL STRUCTURE OF TOLCAPONE

Chemical Name: (3, 4 - dihydroxy - 5 - nitrophenyl) - (4 - methylphenyl) methanone [8].

Molecular Formula: $C_{14}H_{11}NO_5$ [8].

Molecular Weight: 273.24 g/mol [8].
Appearance: Yellowish powder.

Solubility: Freely soluble in acetone and tetrahydrofuran. Soluble in methanol, acetonitrile, and ethylacetate. Sparingly soluble in chloroform and dichloromethane. Insoluble in water and n-hexane

CAS Number: 134308-13-7 [8].

Mechanism of Action: Tolcapone is a selective inhibitor of the enzyme catechol - O-methyl transferase (COMT). Tolcapone is clinically approved for the treatment of Parkinson's disease.

Theoretical Analysis and Objective of the Present Work:
Preparation of Mobile Phase: For the preparation of mobile phase [9], an accurately weighed quantity of 1.32 g of Ammonium phosphate was taken into a 1000 mL beaker and diluted to 1000 mL with HPLC grade water and degassed in an ultrasonic water bath for 10 min and filtered through 0.45 µm nylon membrane filter using vacuum filtration gives required buffer concentration of 0.01 M Ammonium phosphate buffer and the pH was adjusted to 5.0 with orthophosphoric acid. This pH 5 buffers mixed with HPLC grade Acetonitrile in the proportion of 70:30, % v/v, and it was filtered through 0.45 µm nylon membrane filter and degassed by ultra-sonication for 10 min.

Bio-analytical Conditions: The chromatographic analysis was performed by using a mobile phase of 0.01 M Ammonium phosphate buffer with pH was adjusted to 5 with orthophosphoric acid were mixed with HPLC grade Acetonitrile in the proportion of 70:30, % v/v with flow rate 0.8 mL/min by positive ion mode (API 4000). The detection was performed by atmospheric pressure electrospray ionization (ESI) tandem mass spectrometry in positive ion mode [10]. The details of the conditions shown in **Table 1**.

MASS SPECTROMETRY CONDITIONS

Acquisition duration	:	3.0 min
Polarity	:	Positive
Scan Time	:	200 milliseconds (for each MRM
Resolution	:	Ql: Unit and Q3: Unit Detection

Q1 Mass Q3 Mass: Nitecapone 266.2, 156.2.

Tolcapone: 274.2283.1

Can Vary by ± 0.5 Mass Units Source/Gas Parameters (Positive Mode): API 4000

Gas 1(GS1): 40.00 (Psi).
Gas 2(GS2): 30.00 (Psi).
Curtain Gas (CUR): 20.00 (Psi).
Collision Gas (CAD): 6.00 (Psi).
Ion Spray Voltage (IS): 4000.00.
Temperature ºC: 500.00.
Interface Heater: Switched on.

TABLE 1: COMPOUND PARAMETERS FOR MASS SPECTROMETRY TUNING OF NITECAPONE

Parameter	Nitecapone
Declustering potential (DP)	71.00 V
Entrance potential (EP)	10.00 V
Collision energy (CE)	32.00 V
Collision cell exit potential (CXP)	6.00 V

Preparation of Standard and Working Solutions for Nitecapone: The Nitecapone stock solution was prepared by dissolving 10 mg of Nitecapone in 0.5 % ammonia solution in acetonitrile and made up the volume with the same in a 10 mL volumetric flask to produce a solution of 1000 µg/mL. This solution was kept in the refrigerator at 2-8 °C. The stock solutions were diluted to suitable concentrations using diluent for spiking into a plasma to obtain calibration curve standards, quality control samples for further use. All other dilutions were made in mobile phase [11].

Preparation of Stock solution for Tolcapone (Internal Standard): A stock solution of Tolcapone (Internal standard) was prepared by dissolving 10 mg of Tolcapone in diluent (a mixture of HPLC grade acetonitrile and water in a ratio (60:40, v/v) and made up the volume with the same in a 10 mL volumetric flask to produce a solution of 1000 µg/mL. This solution was kept in the refrigerator at 2-8 °C. Working is solutions were prepared by suitably diluting the above-mentioned stock solution afresh before use [11].

Preparation of Calibration Curve Standards and Quality Control (QC) Samples: Calibration curve standard consisting of a set of eight non-zero concentrations ranging from 50 ng/mL to 2000 ng/mL of Nitecapone was prepared. Prepared

quality control samples consisted of concentrations of 50 ng/mL (lower limit of quantification quality control sample), 150 ng/mL (Lower quality control sample), 1000 ng/mL (middle quality control sample), and 1700 ng/mL (higher quality control sample) for Nitecapone. These samples were stored at -70 °C ± 10 °C until use. Twelve sets of LQC and HQC samples were stored at -20 °C ± 5 °C to check stability.

Preparation of Plasma Samples: For the preparation of plasma samples, human blood samples were collected into polypropylene tubes containing K_2-EDTA. Each tube was centrifuged for 15 min at 8500 rpm and the supernatant was collected in another tube. To the supernatant 1 mL of acetonitrile was added and kept for 10 min for the plasma proteins to precipitate, and then the supernatant was collected for further use [11].

Procedure for Spiked Human Plasma: Liquid-liquid extraction was used to isolate Nitecapone, and Tolcapone IS from human plasma. For this, aliquots of 20 µL of internal standard and 100 µL of plasma sample were added into labeled polypropylene tubes and vortexed briefly. Followed by the addition of 20 µL of diluent and vortexed.

Then 20 µL of 2% orthophosphoric acid buffer was added to it and vortexed. Followed by addition of 5 mL of ammonium phosphate and shaken for 20 min on a reciprocating shaker at 300 rpm. Samples were centrifuged at 5000 rpm for 5 min at 5 °C.

Then supernatant organic layer (5.0 mL) was transferred to pre-labeled glass dry test tubes and evaporated to dryness in turboVap at 40 °C. The samples were reconstituted in 1000 µL of the mobile phase, which contains 0.01M ammonium phosphate buffer: acetonitrile (70:30; v/v), and 20 µL of the sample were injected to HPLC with MS-MS detection [11].

Preparation of Sample Solution: After bulk spiking, aliquots of 100 µL for calibration curves and 100 µL for quality controls of spiked plasma samples were pipetted out into a pre-labeled polypropylene microcentrifuge tubes, and then all the bulk spiked samples were stored to deep freezer at -70 °C ± 10 °C, except twelve replicates each of LQC and HQC, which were stored in -20 °C ± 5 °C for generation of stability data. The thawed samples were vortexes to ensure complete mixing of the contents.

Methodology:

Parameters: The details of the chromatographic and mass spectroscopic parameters[9] are as follows:

Equipment: Shimadzu LC 10, an auto sampler of Shimadzu (SIL-HTc) coupled with an Applied Biosystems SCIEX a triple quadrupole mass spectrometer (API 4000) with electrospray ionization (ESI) and Applied Biosystems/MDS SCIEX Analyst software (version 1.4.2).

Column: ZORBAX Eclipse plus C_{18} column (150 mm × 4.6 mm id, 5 µm particle size).

Mobile Phase: 0.01 M Ammonium phosphate buffer with pH was adjusted to 5 with ortho-phosphoric acid were mixed with HPLC grade acetonitrile in the proportion of 70:30, v/v.

Flow Rate: 0.8 mL/min.
Run time: 3 min.
Splitness: 25:75.
Column oven temperature: 35 ± 2 ºC.
Auto sampler temperature: 10 ºC.
Injection volume: 20 µL.
Retention time of Nitecapone: 2.12 min.
Retention time of Tolcapone : 2.58 min.

RESULTS AND DISCUSSION:

Method Optimisation: For the optimisation[10]of LC-MS/MS method, several parameters and mobile phase compositions were tried. A satisfactory separation and good peak symmetry for Nitecapone were obtained with ZORBAX Eclipse plus C_{18} column (150 mm × 4.6 mm id, 5 µm particle size) and mobile phase containing a mixture of 0.01M Ammonium phosphate buffer with pH was adjusted to 5 with orthophosphoric acid, and Acetonitrile in the proportion of (70:30, v/v) was delivered at a flow rate of 0.8 mL/min by positive ion mode (API 4000) with an injection volume of 20 µL and a run time of 3 min. Detection is performed by atmospheric pressure electrospray ionization (ESI) tandem mass spectrometry in positive ion mode. The precursor to product ion transitions is *m/z* 266.20 to 156.20 for Nitecapone and *m/z* 274.20 to 183.10 for Tolcapone (Internal standard) were used for quantization was shown in **Fig. 3, 4, 5** and **6**.

The retention time of Nitecapone and Tolcapone (Internal standard) was found to be 2.12 min and 2.58 min, respectively. A typical chromatogram of blank plasma, mobile phase, Nitecapone, and Tolcapone (Internal standard) is shown in **Fig. 7, 8, 9,** and **10**. The structures of precursor (Q1) and product (Q3) ions of Nitecapone and Tolcapone IS are shown in **Fig. 5** and **6** below.

Chromatogram of blank plasma, mobile phase, Nitecapone and Tolcapone (Internal standard) as shown in **Fig. 7, 8, 9,** and **10** below:

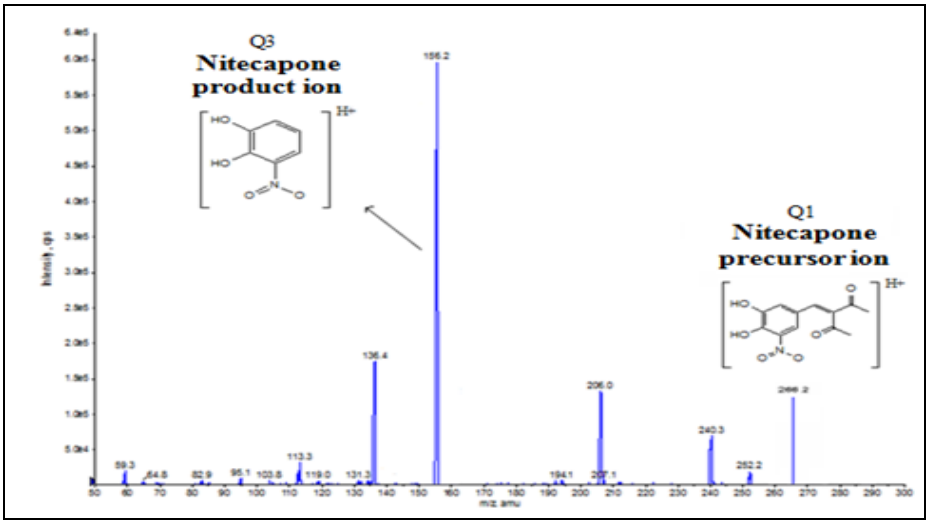


FIG. 3: MASS SPECTRA OF NITECAPONE FOR PRECURSOR AND PRODUCT ION MASSES

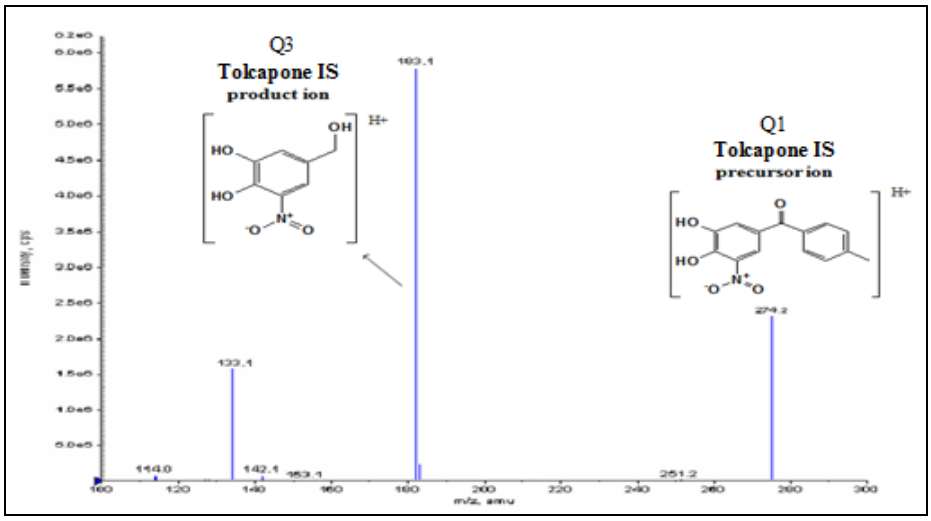
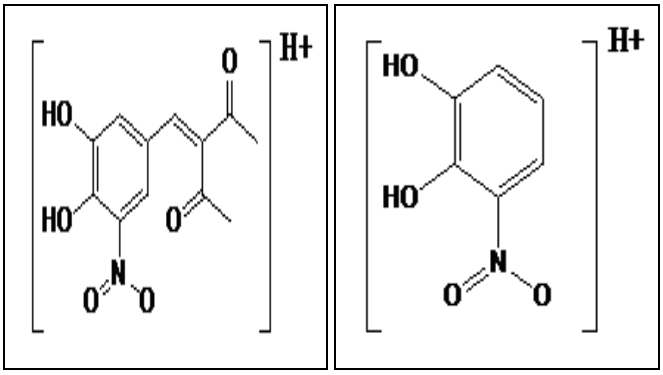
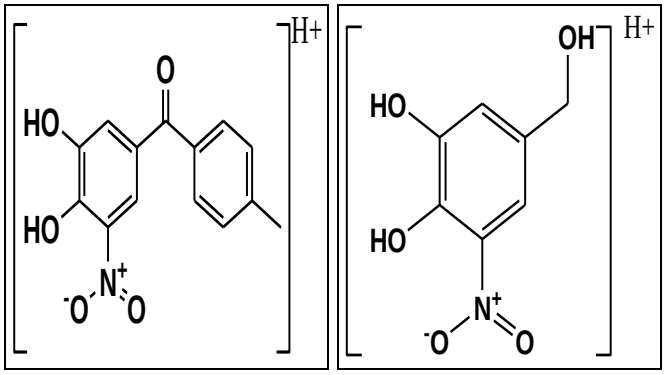


FIG. 4: MASS SPECTRA OF TOLCAPONE IS FOR PRECURSOR AND PRODUCT ION MASSES



/z = 266.2 (Q1) m/z = 156.2 (Q3)

FIG. 5: STRUCTURES OF PRECURSOR (Q1) AND PRODUCT (Q3) IONS OF NITECAPONE



m/z =274.2 (Q1) m/z =183.1(Q3)

FIG. 6: STRUCTURES OF PRECURSOR (Q1) AND PRODUCT (Q3) IONS OF TOLCAPONE IS

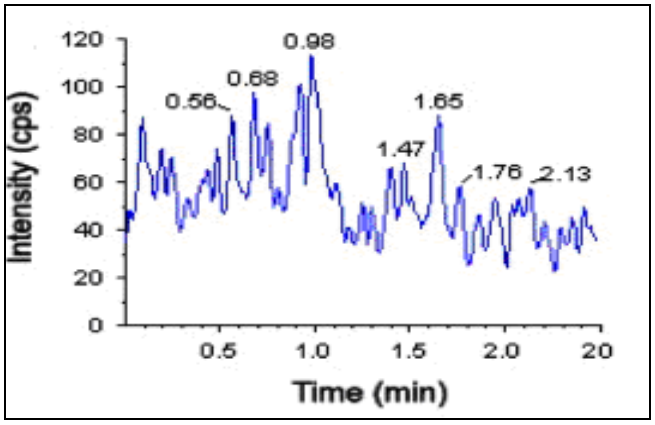


FIG. 7: CHROMATOGRAM OF BLANK

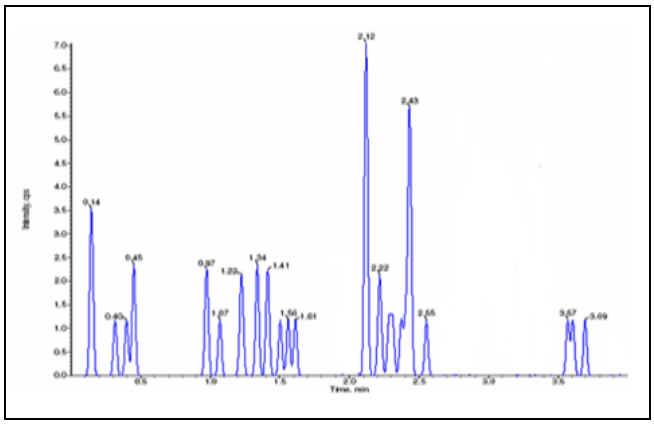


FIG. 8: CHROMATOGRAM OF MOBILE PHASE

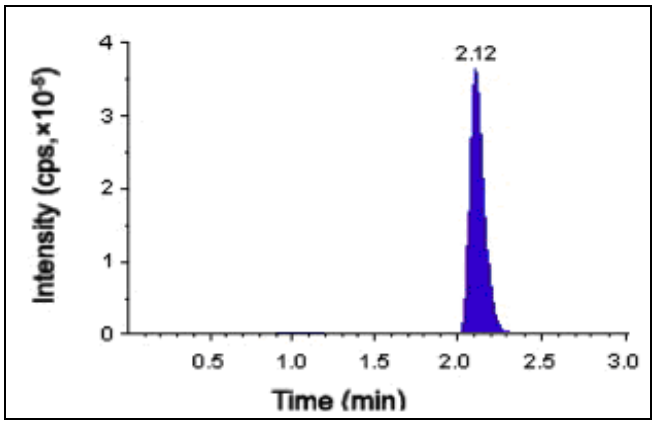


FIG. 9: CHROMATOGRAM OF NITECAPONE

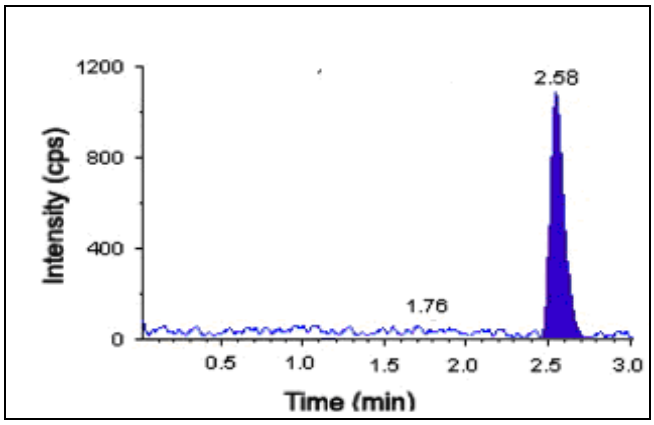


FIG. 10: CHROMATOGRAM OF TOLCAPONE (INTERNAL STANDARD)

Method Validation: The established LC-MS/MS method is validated for selectivity, specificity, sensitivity, linearity, accuracy, precision, recovery, stability, and carry over test according to the principles of the FDA guidelines [12-15].

Screening of Plasma Lots and Specificity: The selectivity of the present method was evaluated by screening six different lots of blank plasma. All of them were found to have no significant endogenous interferences at the retention times of the analyte and the internal standard.

The same human EDTA plasma lots free of interfering substances were used to prepare the calibration curve standards, and the quality control samples for the validation study is cited in **Table 2.**

TABLE 2: PERCENT INTERFERENCES AT THE RETENTION TIMES OF NITECAPONE AND THE TOLCAPONE (INTERNAL STANDARD)

Plasma Lot no.	Nitecapone			Tolcapone (IS)		
	Area at Analyte RT in blank	Analyte Area in LLOQ	Interference Area (%) at Analyte RT	is Area in Blank	is Area in LLOQ	Interference Area (%) at is RT
Blank-1	0	3863	0	0	381648	0
Blank-2	0	3759	0	0	385681	0
Blank-3	0	3892	0	0	380523	0
Blank-4	0	3679	0	0	387227	0
Blank-5	0	3543	0	0	379122	0
Blank-6	0	3887	0	0	389743	0

Sensitivity: The lowest limit of reliable quantification (LLOQ) for Nitecapone was set at the concentration of 50 ng/mL. The precision and accuracy of Nitecapone at this concentration were estimated.

Linearity: The linearity of Nitecapone was assessed at six concentration levels in the range of 50, 150, 450, 1000, 1700, and 2000 ng/mL in plasma samples.

Peak area ratios for each solution against its corresponding concentration were measured and the calibration curve was obtained.

Extraction Recovery: Twenty-four blank matrix samples were processed, and six sets of each blanks samples were reconstituted with the aqueous quality control dilutions at low, middle, and high concentration without an internal standard, which represents 100% extraction of analyte(s) (non-extracted samples). Six blanks were reconstituted with the internal standard solution, which represents a 100 % extraction of internal standard (Non-extracted sample). The non-extracted samples were injected. The recovery comparison samples of Nitecapone were compared against extracted samples of LQC, MQC, and HQC of PA Batch-I (Precision and accuracy). The recovery comparison samples of internal standard were compared against the response of internal standard in MQC level.

Extraction recoverry (R%) = Psbe / Psae × 100

Where R is extraction recovery, Psbe is the mean value of the peak area responses obtained from plasma samples spiked with analyte before extraction, and Psae is the mean value of the peak area responses obtained from plasma samples spiked with analyte after extraction.

Accuracy and Precision: Intra assay precision and accuracy were determined by analyzing six replicates at four different quality control levels in two runs on the same day. Inter-assay precision and accuracy were determined by analyzing six replicates at four different quality control levels on five different runs. The acceptance criteria included accuracy within ≤ 15% deviation (SD) from the nominal values, except LLOQ quality control, where it should be ≤ 20% and a precision of ≤ 15% relative standard deviation (RSD), except for LLOQ quality control, where it should be < 20%.

Stability: The stability of Nitecapone in plasma was performed using six replicates of two quality control samples at low and high levels. Samples were prepared by spiking drug-free plasma with appropriate volumes of Nitecapone standard solutions. The stability was evaluated with different studies such as room temperature stock solution stability, refrigerated stock solution stability, room temperature spiking solution stability, refrigerated spiking solution stability, freeze-thaw, short term stability, benchtop stability, *etc*. Stability tests were conducted to evaluate the analyte stability in stock solutions and in plasma samples under different conditions. The stock solution stability at room temperature and refrigerated conditions (2-8 °C) was performed by comparing the area response of the analytes (stability samples) with the response of the sample prepared from the fresh stock solution. Bench top stability (6 h), processed sample stability (auto sampler stability for 32 h), freeze-thaw stability (four cycles), reinjection stability (24 h), wet extract stability (30 h) and plasma samples stability at -20 °C were performed at LQC and HQC levels using six replicates at each level. Samples were considered to be stable if assay values were within the acceptable limits of accuracy (≤15% SD) and precision (≤15% RSD).

Matrix Effect Test of Nitecapone: Two sets of extracted blank plasma samples each containing six tubes (plasma taken from six different lots) are taken. One set of tubes are reconstituted with an equivalent aqueous concentration of LQC and the other set of tubes are reconstituted with equivalent aqueous concentration of HQC. These samples are known as post spiked samples. These samples are analyzed along with equivalent aqueous LQC and HQC samples. The matrix effect is evaluated by determining the % response ratio using the formula.

Response ratio (%) = Mean area ratio of post spiked samples / Mean area ratio of equivalent aqueous samples × 100

LC-MS/MS Analysis: A binary mixture of 0.01 M Ammonium phosphate buffer with pH was adjusted to 5 with orthophosphoric acid, and Acetonitrile in the proportion of (70:30, v/v) was proved to be the most suitable mobile phase of all the combinations since the chromatographic peaks obtained were well defined and resolved and free from tailing. A mobile phase flow rate of 0.8 mL/min with a splitless of 25/75 was found to be suitable in the study range of 0.3-1.0 mL/min.

Detection of the ions was performed by multiple reaction monitoring (MRM) of the transitions *m/z* 266.20 to 156.20 for Nitecapone and *m/z* 274.20 to 183.10 for Tolcapone (Internal standard). The retention time of Nitecapone and Tolcapone (Internal standard) was found to be 2.12 min and 2.58 min, respectively.

Linearity: The calibration curve was linear in the range of 50 ng/mL to 2000 ng/mL of the Nitecapone, as shown in **Table 3**. A straight line fit made through the data points by the least square regression analysis showed a constant proportionality with minimal data scattering. The correlation coefficient (R^2) is 0.9997 for Nitecapone, as shown in **Table 3** and **Fig. 11**.

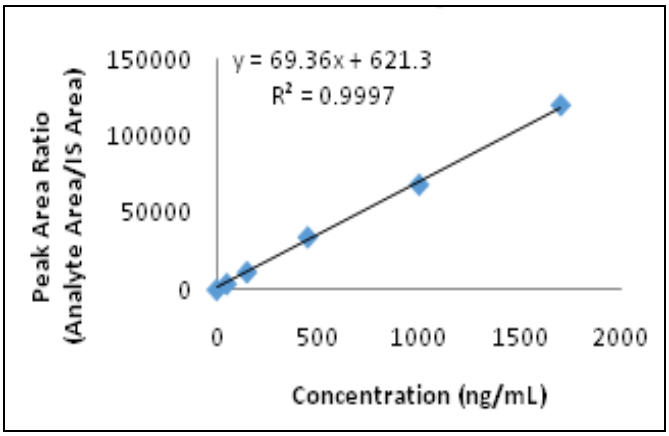


FIG. 11: CALIBRATION CURVE OF NITECAPONE

TABLE 3: LINEARITY OF NITECAPONE

Concentration of Nitecapone (ng/mL)	Peak Area Ratio (Analyte Area/IS Area)
50	3771
150	11312
450	33935
1000	67869
1700	119218
2000	135738

Selectivity: There was no significant interference from endogenous components observed at the mass transitions of Nitecapone and Tolcapone (internal standard).

Recovery of the Nitecapone and Tolcapone Internal Standard: Recovery for Nitecapone was found to be in the range of 80.22% to 95.95%, and the mean recovery for Nitecapone was 90.39%. While for Tolcapone (Internal standard) the mean recovery was 92.34%. The recovery results of Nitecapone and Tolcapone (Internal standard) are shown in **Tables 4** and **5,** respectively.

TABLE 4: RECOVERY OF NITECAPONE FROM HUMAN PLASMA

	LQC Response		MQC Response		HQC Response	
	Extracted Quality Control	Non Extracted Quality Control	Extracted Quality Control	Non Extracted Quality Control	Extracted Quality Control	Non Extracted Quality Control
Sample ID	LQC (07-12)	LQC (1-6)	MQC (07-12)	MQC (1-6)	HQC (07-12)	HQC (1-6)
1	11312	14291	68754	71734	118744	124025
2	11454	14277	68212	70926	119848	123329
3	11598	14268	68209	72182	119817	124498
4	11392	14171	67918	71892	119221	124802
5	11323	14108	67972	72018	119710	125191
6	11314	14144	67858	71665	119616	125397
Mean	11398.8	14209.8	68153.8	71736.2	119493	124540
SD	112.47	78.35	329.49	439.07	430.86	769.47
CV%	0.99	0.55	0.48	0.61	0.36	0.62
N	6	6	6	6	6	6
Recovery%	80.22		95.01		95.95	
Overall recovery%			90.39			

TABLE 5: RECOVERY OF TOLCAPONE (INTERNAL STANDARD) FROM HUMAN PLASMA

Extracted Quality Control ID	IS Response in Extracted Samples (Area)	Non-Extracted Quality Control ID	IS Response in Non-Extracted Samples (Area)
MQC-7	421129	non extracted-MQC-1	457293
MQC-8	430101	non extracted-MQC-2	458100
MQC-9	418822	non extracted-MQC-3	455183
MQC-10	417182	non extracted-MQC-4	457189
MQC-11	421973	non extracted-MQC-5	451334
MQC-12	415627	non extracted-MQC-6	455144
Mean	420806	Mean	455707
SD	5133.321	SD	2455.72
CV%	1.22	CV%	0.54
N	6	N	6
Recovery %		92.34	

Within-Batch Precision and Accuracy: Within-batch precision for LLOQ quality control ranged from 0.22% to 0.28%, and for LQC, MQC, and HQC ranged from 0.005% to 0.196%. Within-batch accuracy ranged for LLOQ quality control ranged from 100.24% to 100.28%, and for LQC, MQC and HQC ranged from 99.99% to 100.17%. The results of within-batch precision and accuracy for Nitecaponeare represented in **Table 6**.

TABLE 6: WITHIN-BATCH PRECISION AND ACCURACY FOR NITECAPONE

Quality Control	Concentration (ng/mL)			
	LLOQ QC	LQC	MQC	HQC
	50	150	1000	1700
1	50.04	150.02	1000.07	1700.02
2	50.11	150.1	1000.03	1700.05
3	50.07	150.01	1000.1	1700.13
4	50.4	150.24	1000.21	1700.25
5	50.03	150.03	1000.18	1700.11
6	50.08	150.01	1000.08	1700.22
Mean	50.12	150.07	1000.11	1700.13
SD	0.139	0.091	0.069	0.091
CV%	0.28	0.060	0.007	0.005
Nominal %	100.24	100.05	100.01	100.01
N	6	6	6	6
7	50.01	150.03	1001.05	1700.04
8	50.12	150.19	1003.07	1700.02
9	50.06	150.2	1000.18	1700.19
10	50.32	150.14	1000.19	1700.27
11	50.13	150.02	1000.6	1700.08
12	50.19	150.07	1005.04	1700.28
Mean	50.14	150.11	1001.69	1700.15
SD	0.108	0.079	1.962	0.116
CV%	0.22	0.053	0.196	0.007
Nominal %	100.28	100.07	100.17	100.01
N	6	6	6	6
13	50.3	150.13	1000.01	1698.4
14	50.17	150.04	1000.04	1700.18
15	50.03	150.28	1000.3	1700.45
16	50.2	150.22	1000.15	1700.18
17	50.08	150.18	1000.1	1700.01
18	50.01	150.11	1000.8	1700.18
Mean	50.13	150.16	1000.23	1699.90
SD	0.112	0.085	0.296	0.748
CV%	0.22	0.057	0.030	0.044
Nominal %	100.26	100.11	100.02	99.99
N	6	6	6	6

Intra-Day Precision and Accuracy: Intra-day precision for LLOQ quality control was 0.417%, and for LQC, MQC and HQC ranged from 0.036% to 0.092%. Intra-day accuracy for LLOQ quality control was 100.33%, and for LQC, MQC, and HQC ranged from 100.02% to 100.07%. The results of intra-day precision and accuracy for Nitecaponeare represented in **Table 7**.

Between Batch / Inter-Day Precision and Accuracy: Between batch precision for LLOQ quality control was 0.142%, and for LQC, MQC and HQC ranged from 0.023% to 0.092%. Between batch accuracy for LLOQ quality control was 100.04% and for LQC, MQC, and HQC ranged from 100.02% to 100.06%. The results of between batch/inter-day precision and accuracy for Nitecapone are represented in **Table 8**.

Stability: The processing and storage conditions of clinical samples need to maintain the integrity of a drug or at least keep the variation of pre-analysis as minimal as possible. For this reason, stability studies play an important role in bioanalytical method development. In this study, the stability was assessed by considering different studies such

as room temperature stock solution stability, refrigerated stock solution stability, room temperature spiking solution stability, refrigerated spiking solution stability, benchtop stability, autosampler stability, freeze-thaw stability, re-injection stability andwet-extract stability. The results show that Nitecapone is stable under the studied conditions since in all cases, the international acceptance criteria (variation values for area smaller than 15 %) were met.

TABLE 7: INTRA-DAY PRECISION AND ACCURACY FOR NITECAPONE

Quality control	Concentration (ng/mL)			
	LLOQ QC	LQC	MQC	HQC
	50	150	1000	1700
1	50.8	150.4	1000.18	1700.01
2	50.07	150.07	1001.1	1700.28
3	50.12	150.01	1003.18	1699.97
4	50.14	150.22	1000.1	1700..45
5	50.02	150.14	1000.2	1702.18
6	50.04	150.13	1000.5	1700.14
7	50.03	150.18	999.98	1700.21
8	50.09	150.1	999.87	1700.24
9	50.17	149.98	1000.12	1700.03
10	50.22	149.87	1000.09	1700.5
11	50.12	150.16	1000.02	1699.80
12	50.18	150.07	1000.09	1700.07
Mean	50.2	150.1	1000.5	1700.3
SD	0.2	0.1	0.9	0.6
CV%	0.417	0.088	0.092	0.036
Nominal %	100.33	100.07	100.05	100.02
N	12	12	12	12

TABLE 8: BETWEEN BATCH/INTER-DAY PRECISION AND ACCURACY FOR NITECAPONE

Quality control	Concentration (ng/mL)			
	LLOQ QC	LQC	MQC	HQC
	50	150	1000	1700
1	50.05	149.95	1000.04	1700.11
2	49.97	150.02	1000.18	1700.8
3	50.03	150.04	1000.12	1700.54
4	50.17	150.17	1000.3	1700.41
5	49.94	150.12	1000.14	1702.12
6	50.06	150.03	1000.17	1700.63
7	49.98	150.12	1000.02	1700.18
8	49.96	150.03	1000.05	1700.44
9	49.94	150.45	1000.1	1700.08
10	50.01	149.97	1000.8	1700.58
11	49.92	150.04	1000.30	1699.99
12	50.12	150.02	1000.20	1700.88
13	49.99	150.02	1000.01	1700.12
14	50.11	150.06	1000.03	1700.11
15	49.99	150.4	1000.14	1700.13
16	50.12	150.01	1000.76	1700.16
17	50.01	150.23	1000.34	1699.94
18	50.03	150.04	1000.18	1700.04
Mean	50.02	150.10	1000.22	1700.40
SD	0.07	0.14	0.23	0.51
CV%	0.142	0.092	0.023	0.030
Nominal %	100.04	100.06	100.02	100.02
N	18	18	18	18

Room Temperature Stock Solution Stability: The stability was found to be 99.3% for Nitecapone with the precision ranged from 0.4 % to 0.9%. The stability was found to be 100.6% for Tolcapone (Internal standard) with the precision ranged from 0.8% to 1.3%.

The results of room temperature stock solution stability are shown in **Table 9**.

Refrigerated Stock Solution Stability (at 2-8 °C): The stock solution was found to be stable for four days. The four days stock solution stability of Nitecapone and Tolcapone (Internal standard) was found to be 99.5% and 100.2%, respectively and are shown in **Table 10**.

Room Temperature Spiking Solution Stability: The stability was found to be 99.2% for Nitecapone with the precision ranged from 0.79% to 0.8%. The stability was found to be 99.8% for Tolcapone (Internal standard), with the precision ranged from 0.6% to 1.4%. The results of room temperature spiking solution stability are shown in table [11].

Refrigerated Spiking Solution Stability of Nitecapone (at 2-8 °C): The spiking solutions were found to be stable for three days. The three days spiking solution stability of Nitecapone at LQC level was found to be 99.01% and is shown in **Table 12**.

Bench Top Stability: Nitecapone was found to be stable up to 6 h as per the acceptance criteria. The percent mean nominal ranged from 100.02% to 100.06%, and the precision ranged from 0.013% to 0.063%. Results of benchtop stability are shown in table 13.

Auto-sampler Stability: The results demonstrate that the processed samples were stable for 32 h. The percent nominal at 32 h ranged from 100.05% to 100.1%, and precision ranged from 0.046 % to 0.063%. The result of autosampler stability is shown in **Table 14**.

Freeze-thaw Stability: Freeze-thaw stability of Nitecapone is shown in **Table 15**. The percent nominal ranged from 99.98% to 100.01%, and the precision ranged from 0.058 % to 0.060% for four freeze-thaw cycles.

Re-injection Stability: The results demonstrate that the reinjected samples were stable for 24 h. The percent stability at 24 h ranged from 99.84% to 100.01%, and the precision ranged from 0.01% to 0.27% for 24 h. Results of rejection stability of Nitecapone are shown in **Table 16.**

Wet-extract Stability: Wet extract stability results are shown in **Table 17**. The results demonstrate that the processed samples were stable for 30 hours. The percent nominal at 30 h ranged from 100.02% to 100.03%, and the precision ranged from 0.053% to 0.068%.

TABLE 9: ROOM TEMPERATURE STOCK SOLUTION STABILITY OF NITECAPONE AND TOLCAPONE (INTERNAL STANDARD) FOR 0 AND 6 H

S. no.	Nitecapone Peak Area		Tolcapone (Internal standard) Peak Area	
	0 h	6 h	0 h	6 h
1	68883	68328	412447	420348
2	68627	68437	411346	413476
3	68827	68867	423891	417466
4	68380	67506	410137	417187
5	68286	67808	412120	411288
6	68272	67348	410127	414874
Mean	68546	68049	413345	415773
SD	271.78	590.23	5256.77	3224.78
CV %	0.4	0.9	1.3	0.8
Stability %	99.3		100.6	

TABLE 10: REFRIGERATED STOCK SOLUTION STABILITY OF NITECAPONE AND TOLCAPONE (INTERNAL STANDARD) AT 2-8 °C FOR 4 DAYS

S. no.	Nitecapone		Tolcapone (Internal Standard)	
	Stability Standard Stock	Comparison Standard Stock	Stability Standard Stock	Comparison Standard Stock
	Peak Area	Peak Area	Peak Area	Peak Area
1	67992	68717	415487	420438
2	68413	68281	415650	419342
3	67932	68863	420485	417822
4	68334	68235	413453	417562

5	68835	67866	413392	419928
6	69865	67329	413882	403466
Mean	68562	68215	415392	416426
SD	716.70	562.75	2685.30	6450.80
CV %	1.0	0.8	0.6	1.5
N	6	6	6	6
Mean response of standard stock	68562		415392	
Mean standard response	68215		416426	
Response %	99.5		100.2	

TABLE 11: ROOM TEMPERATURE SPIKING SOLUTION STABILITY OF NITECAPONE AND TOLCAPONE (INTERNAL STANDARD) FOR 0 AND 6 H

S. no.	Nitecapone Peak Area		Tolcapone (Internal standard) Peak Area	
	0 h	6 h	0 h	6 h
1	68647	68568	415856	421983
2	68856	68338	415887	418466
3	67945	68221	411927	416563
4	68348	68568	418365	414367
5	68511	67234	418345	417265
6	69543	67666	429388	415692
Mean	68642	68099	418295	417389
SD	537.96	538.08	5922.17	2644.80
CV %	0.8	0.79	1.4	0.6
Stability %	99.2		99.8	

TABLE 12: REFRIGERATED SPIKING SOLUTION STABILITY OF NITECAPONE AT LQC LEVEL AT 2-8 °C FOR 3 DAYS

S. no.	Stability Standard Spiking Solution (LQC)	Comparison Standard Spiking Solution (LQC)
	150 ng/mL	152 ng/mL
1	150.1	151.12
2	149.98	151.93
3	150.11	152.21
4	150.01	151.43
5	150.09	151.91
6	150.12	150.81
Mean	150.07	151.57
SD	0.06	0.54
CV %	0.039	0.356
N	6	6
Response %	99.01	

TABLE 13: BENCH TOP STABILITY OF NITECAPONE FOR 6 h

S. no.	Concentration (ng/mL)	
	LQC	HQC
	150.0	1700.0
1	150.01	1700.21
2	150.2	1700.23
3	149.99	1700.26
4	150.03	1700.08
5	150.1	1700.31
6	150.2	1700.73
Mean	150.09	1700.30
SD	0.09	0.22
CV %	0.063	0.013
Nominal %	100.06	100.02
N	6	6

TABLE 14: AUTOSAMPLER STABILITY OF NITECAPONE IN PROCESSED HUMAN PLASMA SAMPLES FOR 32 h

S. No.	Concentration (ng/mL)	
	LQC	HQC
	150	1700
1	150.11	1701.21
2	150.14	1700.03
3	149.97	1701.26
4	150.13	1703.18
5	150.01	1702.11
6	150.05	1702.03
Mean	150.07	1701.64
SD	0.07	1.06
CV %	0.046	0.063
Nominal %	100.05	100.10
N	6	6

TABLE 15: FREEZE-THAW STABILITY (FT– IV CYCLE) OF NITECAPONE

S. no.	Concentration (ng/mL)	
	LQC	HQC
	150	1700
1	149.87	1699.21
2	150.1	1701.03
3	149.94	1699.26
4	150.01	1701.01
5	150.02	1699.01
6	149.89	1701.02
Mean	149.97	1700.09
SD	0.09	1.02
CV %	0.058	0.060
Nominal %	99.98	100.01
N	6	6

TABLE 16: RE-INJECTION STABILITY OF NITECAPONE FOR 0 H AND 24 h

S. no.	Re-injection stability of Nitecapone for 0 hours		Re-injection stability of Nitecapone for 24 hours	
	Concentration (ng/mL)			
	LQC	HQC	LQC	HQC
	150	1700	150	1700
1	150.02	1700.3	150.02	1700.01
2	149.22	1699.94	149.94	1699.12
3	149.98	1700.12	150.02	1700.02
4	150.1	1700.34	149.03	1699.09
5	150.22	1699.98	150.01	1700.52
6	149.78	1700.04	149.57	1701.01
Mean	149.89	1700.12	149.77	1699.96
SD	0.36	0.17	0.40	0.76
CV %	0.24	0.01	0.27	0.04
Nominal %	99.92	100.01	99.84	100.00
N	6	6	6	6
	Stability %		99.92	99.99

TABLE 17: WET-EXTRACT STABILITY OF NITE-CAPONE FOR 30 h

S. no.	Concentration (ng/mL)	
	LQC	HQC
	150	1700
1	149.97	1699.71
2	150.12	1699.33
3	149.96	1700.16
4	150.11	1701.31
5	150.12	1699.71
6	149.99	1702.32
Mean	150.05	1700.42
SD	0.08	1.15
CV %	0.053	0.068
Nominal %	100.03	100.02
N	6	6

TABLE 18: MATRIX EFFECT OF NITECAPONE

Plasma (Batch no.)	LQC (150.0 ng/mL) Mean				HQC (1700.0 ng/mL)			Mean
	1	2	3		1	2	3	
1	150.1	150.3	149.92	150.11	1700.01	1700.21	1700.01	1700.08
2	149.98	150.12	150.1	150.07	1701.01	1700.13	1700.03	1700.39
3	150.12	149.97	150.3	150.13	1700.02	1700.3	1699.82	1700.05
4	150.03	150.8	149.01	149.95	1700.01	1700.2	1700.23	1700.15
5	150.2	150.03	150.02	150.08	1700.04	1699.93	1700.2	1700.06
6	150.04	149.99	149.99	150.01	1699.98	1699.67	1700.31	1699.99
(Lipemic)	150.23	150.16	150.3	150.23	1700.03	1699.83	1700.12	1699.99
(Hemolytic)	150.13	150.14	150.12	150.13	1700.22	1700.64	1699.43	1700.1
	Mean			150.09		Mean		1700.10

SD	0.09	SD	0.13
CV %	0.1	CV %	0.01
Nominal	100.1	Nominal	100.01
N	8	N	8

Matrix Effect: No significant matrix effect was observed in all the eight batches, including hemolysis and lipemic plasma for Nitecapone at low (LQC) and high (HQC) concentrations. The precision and accuracy for Nitecapone at LQC concentration were found to be 0.1% and 100.1%, respectively, and at HQC concentration was found to be 0.01% and 100.01 %, respectively and shown in **Table 18.**

SUMMARY AND CONCLUSION: The present LC-MS/MS method for the estimation of Nitecapone in human plasma by using Tolcapone as an internal standard is a simple and inexpensive liquid-liquid extraction procedure and an isocratic chromate-graphy condition using a reversed-phase column suitable for real-time analysis. This is a rapid and accurate method for the estimation of Nitecapone in human plasma. Peaks were well separated and without any interference. The best response was obtained with Zorbax Eclipse plus C_{18} column (150 mm × 4.6 mm ID, 5 µm) and mobile phase containing a mixture of 0.01 M Ammonium phosphate buffer with pH was adjusted to 5.0 with ortho-phosphoric acid and acetonitrile in the proportion of (70:30, v/v %) was delivered at a flow rate of 0.8 mL/min by positive ion mode (API 4000) with injection volume of 20 µL and a run time of 3 min.

Detection is performed by atmospheric pressure electrospray ionization (ESI) tandem mass spectrometry in positive ion mode. The precursor to product ion transitions is *m/z* 266.20 to 156.20 for Nitecapone and *m/z* 274.20 to 183.10 for Tolcapone (Internal standard) were used for quantization. The retention time of Nitecapone and Tolcapone (Internal standard) was found to be 2.12 min and 2.58 min, respectively. Linearity was established for Nitecapone in the range of 50 ng/mL to 2000 ng/mL with correlation coefficient (r = 0.9997), and the overall percentage recovery was 90.39% for Nitecapone and 92.34% for Tolcapone (Internal standard) respectively. The CV % values of accuracy and precision for Nitecapone were found to be ≤ 15%, which indicates the accuracy and precision of the proposed method.

The CV % values of accuracy and precision of Nitecapone for stability studies were found to be ≤ 15%, which indicates the stability of the proposed method. The LC-MS/MS method for the estimation of Nitecapone in human plasma by using Tolcapone as an internal standard exhibited excellent performance in terms of selectivity, linearity, accuracy, precision, recovery, stability, and matrix effect test. In addition, the reported method has a short analysis run time, an advantage over previously reported methods. Therefore, this method is suitable for the therapeutic drug monitoring of Nitecapone.

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