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SYNTHESIS, CHARACTERIZATION AND TRACE LEVEL QUANTITATIVE DETERMINATION OF CRITICAL NITROSAMINE IMPURITY IN RIVAROXABAN DRUG SUBSTANCES BY USING LC-MS/MS METHOD

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ABSTRACT

The present research explains the efficient synthetic method and nanogram-level quantification of *N*-Nitroso Rivaroxaban impurity (NNRI) by using LC-MS/MS. The *N*-Nitroso Rivaroxaban Impurity (NNRI) of Rivaroxaban drug substances has been synthesized and a nanogram-level sensitive quantification of the NNRI impurity analytical method was developed. Identification and separation of analyte were achieved in a column having ascents Express C18 (150 mm x 4.6 mm, 2.7 µm). The run time of the developed method was 30 minutes with a flow rate of 0.5 mL/min. This developed method can be useful to determine the N-Nitrosamine impurity (NNRI) of Rivaroxaban drug substances and drug products in commercial-scale batches. The established LCMS method was found to be specific, selective, precise, and accurate.

Keywords: *N*-Nitrosamine Impurity, N-Nitroso Rivaroxaban, API Commercial process, LC-MS/MS, LOD and LOQ

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INTRODUCTION

During the synthesis of pharmaceutical drugs, unwanted compounds or impurities are still found in active pharmaceutical ingredients (APIs). Nori, *et al.* published a Review about Nitrosamine impurity's presence in drug products and they are well-versed in the guidelines of regulatory authorities.[1] In recent years the regulatory evaluation about awareness of undesirable levels of the carcinogenic potential of N-nitrosamine impurities has been a severe nervousness. Nitrosamine chemical compounds probably cause cancer in human after their consumption[2], the quantitative presence of n-nitrosamine unwanted impurities in some approved drug products has increased the risk of their mutagenic and carcinogenic potential.[3] The Bayer Pharmaceutical developed the Rivaroxaban is 5-Chloro-*N*-({(5S)-2-oxo-3-[4-(3-oxo-4-morpholinyl) phenyl]- 1,3-oxazolidin-5-yl}methyl)-2-thiophenecarboxamide. It is an anticoagulant drug (Blood thinner) used to treat and prevent blood clots. Regulatory published reports of *N*-nitrsoamine impurities in drug substances and drug products were reported in the past, however, reports on the detection of *N*-nitrosodimethylamine (NDMA) in Valsartan drug substance documented in June 2018which led to a series of regulatory actions including market recalls and an Article 31 of Directive 2001/83/EC referral procedure in the European Union (EU).[4,5] In line with these reports and given the potential applicability of the identified root causes to other drug substances and drug products, it was marked to conduct a more comprehensive review of *N*-nitrosamine impurities in medicines. The most common and serious problem in the Pharmaceutical industry for the rejection of regulatory approvals of their commercial generic medicines and recalls of approved medicines from the market is because of inadequate discussion/control strategy of possible *N*-Nitrosamine impurities and practicable presence of nitrosamine impurities respectively in the drug substances and drug products. As per the existing commercial route of synthesis and reported degradation pathways of the Rivaroxaban drug substances[6-7],

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it is predicted that the formation of *N*-Nitrosamine Rivaroxaban Impurity (NNRI) during commercial synthesis of Rivaroxaban drug substance is a high possibility. Because our prediction for the practicable presence of NNRI in Rivaroxaban drug substances leads us to synthesize and quantitative determination in the Rivaroxaban drug substance[8-11] and the need for analytical method developed in-line with ICH quality guideline having chapter number ICH-Q2.[12] This gap for assessing quality of Rivaroxaban drug substances has been identified and our research begins from the synthesis of new nitrosamine impurity and its quantitative determination in drug substances by LC-MS method. The chemical structure of Rivaroxaban and *N*-Nitroso Rivaroxaban Impurity (NNRI) as shown in Fig.-1

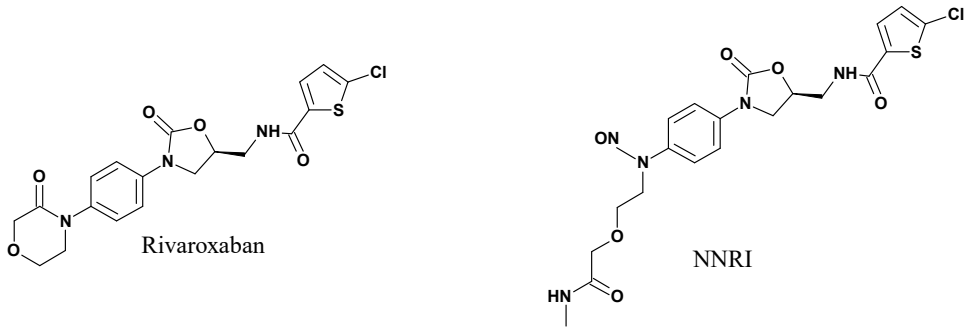

Fig-1: Chemical Structure of Rivaroxaban and N Nitroso Rivroxaban Impurity (NNRI)

EXPERIMENTAL

Materials

All solvents and reagents used in this experiment were of analytical grade. Formic acid, Acetonitrile, and Dimethyl sulfoxide were obtained from Spectrochem Chemicals Pvt. Ltd and Sigma Aldrich (Merck Chemicals). Rivaroxaban Tablet was purchased from the Indian Medical Market.

Synthesis of N-Nitroso Rivaroxaban Impurity (NNRI)

N-Nitroso Rivaroxaban Impurity prepared as per the scheme shown in Fig.-2.

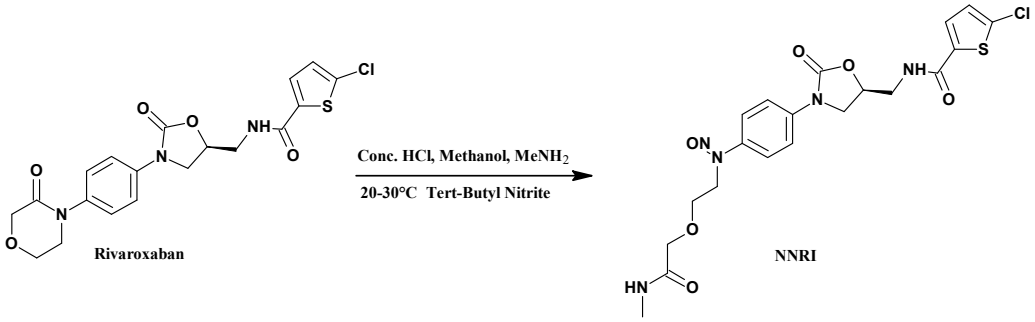

Fig-2: Scheme for the Synthesis of NNRI

Procedure

1.0 g of Rivaroxaban API (Extracted from Tablet) was suspended in Methanol (10.0 mL), Conc. HCl (1.0 g) at 50-55°C for 2-3 hours. Aq. $MeNH_2$ (0.85 g) was added at 20-30°C and maintained at 20-30°C for 6-8 hours. Added (1.1 g) of *tert*-Butyl Nitrate 10-15°C and stirred for 2-3 hours. Upon completion of the reaction by TLC, concentrated and extracted with DCM to get crude isolation followed by column purification produced 0.2 g of NNRI. (Yield: 35. 2%).

Spectroscopic Data

FTIR

The infrared spectrum of NNRI was recorded using a Shimadzu IR Affinity-1 FTIR spectrophotometer over the range of 4000-650 cm^{-1} with a resolution of 4.0 cm-1 using a KBr pellet. The characteristic absorption bands are listed in below Table-1.

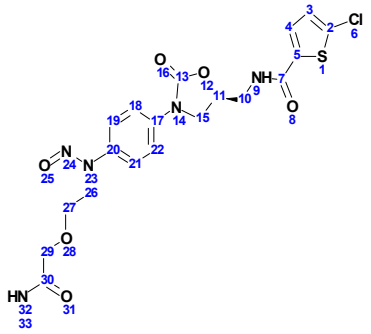

Table-1: Showing the Result of IR Data

Band (cm^{-1})	Assignment
3414 & 3325	-N-H stretching
3084 & 2941	-C-H stretching
1753	-C=O stretching
1657 & 1551	-C=C stretching
1092	-C-O stretching

^{1}H –NMR

The ^{1}H –NMR spectrum of NNRI impurity was recorded using a BRUKER-400 MHz instrument in DMSO-d6. The details are provided in Table-2.

Table-2: Proton-NMR Data

S.No.	Chemical shift (ppm)	No. of Protons	Multiplicity	Assignments
1	2.62-2.63	3	d (J= 4.4 Hz)	33- CH_3
2	3.59-3.62	2	t (J= 5.2 Hz)	10- CH_2
3	3.67-3.70	2	t(J= 5.2 Hz)	26- CH_2
4	3.82	2	s	29- CH_2
5	3.93-3.97	1	dd (J= 9.2 & 6.4 Hz)	15'- CH_2
6	4.27-4.34	3	m	15" & 27-CH_2
7	4.89-4.94	1	m	11-CH
8	7.24-7.25	1	d (J=4.4 Hz)	3-CH
9	7.53	1	br-s	32-NH
10	7.75	5	s	4,18,19,21 & 22-CH
11	9.02-9.05	1	t(J=6.0 Hz)	9-NH

^{13}C NMR data

The ^{13}C –NMR spectrum of NNRI impurity was recorded using a BRUKER-400 MHz instrument in DMSO-d6. The details are provided in Table-3.

Table-3: ^{13}C NMR Data

S.No.	Chemical Shift	Assignment
1	25.10	33-C
2	42.19	10-C
3	43.84	26-C
4	47.39	15-C
5	66.52	27-C
6	69.74	29-C
7	71.75	11-C
8	118.59	18 &22-C
9	121.30	19 & 21-C
10	128.14	3-C
11	128.45	4-C
12	133.27	2-C
13	136.89	17-C

14	137.71	5-C
15	138.44	20-C
16	154.08	13-C
17	160.82	7-C
18	168.99	30-C

Mass Data

ESI mass spectrum of NNRI impurity was recorded using a Thermo Scientific mass spectrometer. The spectral data is presented below in Table-4.

Table-4: Mass Data

Exact mass (g/mol)	Observed mass [m/z]	Assignment
495.10	494.37 $[M-H]^-$	$[C_{20}H_{21}ClN_5O_6S]^-$

Diluent

Mobile Phase A: Transfer 1.0 mL of Formic acid into 1000 mL of water. Shake well to mix and sonicate to degas.

Mobile Phase – B: Acetonitrile

Diluent: Dimethyl sulfoxide; the typical Blank chromatogram is shown in Fig.-3

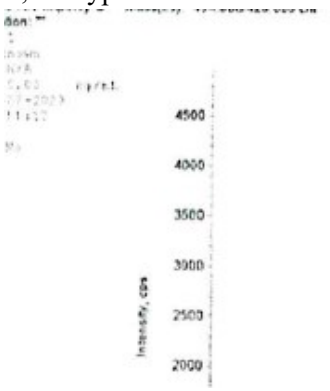

Fig.-3: Typical Blank Chromatogram

Standard Stock Preparation

Transfer 3 mL of NNRI stock solution into a 0.1L analytical volumetric flask and dilute to volume with diluents. Shake well to mix.

Standard Solution Preparation

Transfer 1 mL of standard stock solution into the 10 mL volumetric flask and dilute to volume with diluents. Shake well to mix**.** The typical Standard solution chromatogram is shown in Fig.-4.

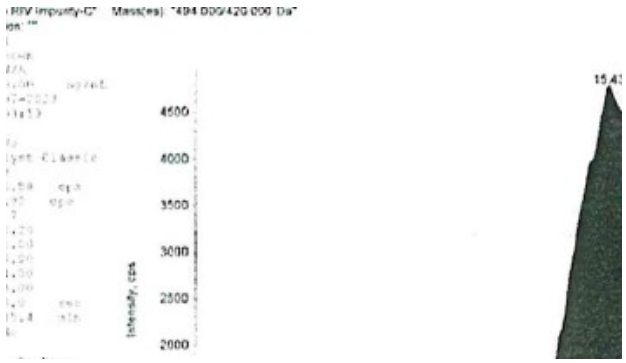

Fig.-4: Typical Standard Solution Chromatogram

Sensitivity Solution
Transfer 1 mL of standard solution into a 10 mL volumetric flask and dilute to volume with diluents. Shake well to mix.

Sample Preparation
Accurately weigh about 0.05 g of sample in duplicate and transfer into two separate 10 mL volumetric flask. Add about 5 mL of diluents to both of the flasks, sonicate to dissolve, and dilute to volume with diluents. Shake well to mix. The typical sample chromatogram is shown in Fig.-5

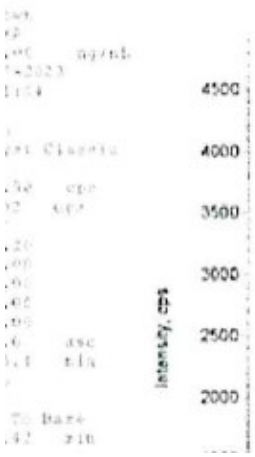

Fig.-5: Typical Sample Solution Chromatogram

RESULTS AND DISCUSSION

Method Development
Before freezing the method, development begins extensively with various stationery phase columns which compromise amino, C14, C8, and C18 and are checked with various mobile phases' mixtures like acetic acid, acetonitrile, Methanol, formic acid, and Ammonium formate. Finally, the concluding chromatographic parameters were obtained in ascents Express C18 (150 mm x 4.6 mm, 2.7 µm) with Acetonitrile along with 30 minute run time. The mass chromatography was optimized with ESI in MRM mode.

Method Validation
This accepted method was validated as per the guidelines described in ICH-Q2.

System Suitability
As a part of the study, NNRI impreciseness standard solution (0.6 ppm) was injected into LC-MS, and the obtained results are tabulated in Table-5.

Table-5: System Suitability Data

Preparation	Response of Analyte	Analyte Retention Time
Standard solution-1	69462	15.43
Standard solution-2	67288	15.45
Standard solution-3	69882	15.43
Standard solution-4	69175	15.44
Standard solution-5	69466	15.44
Standard solution-6	69304	15.46
Average	69096.17	NA
SD	917.2809	
% RSD	1.33	

The percentage relative standard deviation (%RSD) area of the analyte obtained from six standard solutions is within acceptance criteria hence system suitability criteria have been confirmed.

Specificity

The specificity study was carried out by injecting Blank, control, and spiked sample solutions. The outcome of the analysis was no blank interference at the retention time of the NNRI peak in the spiked sample solution.

LOD and LOQ

The calibration curve approach was utilized for the estimation of LOD and LOQ values according to the ICH guidelines of analytical methods validation. For NNRI values of the LOD and LOQ are 0.020 ppm and 0.058 ppm, respectively. The %RSD (relative standard deviation) peak area obtained from three LOD preparations is 3.29 and from six LOQ preparations is 2.88 all the data are concise in Table-6 and Table-7.

Table-6: Limit of Detection (0.02 ppm)

Preparation	Response of Analyte	S/N ratio
LOD Solution-1	2674	63
LOD Solution -2	2745	88
LOD Solution -3	2571	84
Average	2663	NA
SD	87.48905	
% RSD	3.29	

Table-7: Limit of Quantification (0.058 ppm)

Preparation	Response of Analyte	S/N ratio
LOQ Solution-1	7431	175
LOQ Solution-2	6932	155
LOQ Solution-3	7206	107
LOQ Solution-4	7271	242
LOQ Solution-5	6952	212
LOQ Solution-6	7200	285
Average	7165	NA
SD	192.12	
% RSD	2.88	

Precision and Accuracy

The accuracy and precision were established by injecting three replicate preparations of LOQ solution having a concentration of 0.058 ppm of analyte (NNRI). The obtained % RSD values were within the acceptance criteria. The results of accuracy and precision analysis are tabulated in Table-8.

Table-8: Accuracy at LOQ (0.058 ppm)

Preparation	Amount added(ppm)	Amount recovered (ppm)	% Recovery	% RSD of % recovery
LOQ Solution-1	0.0581	0.0649	111.70	2.64
LOQ Solution-2	0.0580	0.0622	107.24	
LOQ Solution-3	0.0581	0.0618	106.37	

Based on the outcome of the method quantification studies, the recommendations of the standard test
The procedure of NNRI impurity in Rivaroxaban drug substances is more concise in Table-9.

Table-9: Method Recommendation for NNRI in Rivaroxaban

LOD (ppm)	LOQ (ppm)	Retention time (min)
0.020	0.058	15.4

Chromatographic Conditions

Column : Ascentis Express C18 (150 mm x 4.6 mm, 27µm)

Flow rate	: 0.5 mL/min
Column oven temperature	: 55°C
Sample cooler temperature	: 25°C
Injection volume	: 10 µL
Run time	: 30 min
Retention time	: Rivaroxaban Drug substance: 12.9 min
	: NNRI Impurity: 15.6 min
Mobile Phase A	: 1 mL of Formic acid in 1L of Water
Mobile Phase B	: Acetonitrile
Diluent	: DMSO

Gradient

: Time Period (min)	0.01	3.0	23.0	23.10	30.0
A	75	75	55	75	75
B	25	25	45	25	25

Mass Parameter for Negative Mode

Polarity: Negative
Ion Source gas-1: 45
Ion Source gas-2: 40
Collision gas (CAD): 6
Temperature (°C): 500
Ion Spray voltage (IS): 4500
Entrance Potential (EP): 8
Collision cell exit potential (CXP): 8
Polarity Dwell time (milli second): 200

Finally, the commercial control sample of Rivaroxaban drug substances was analyzed and the data was found below the quantification limit.

CONCLUSION

The new *N*-Nitrosamine Rivaroxaban Impurity (NNRI) of Rivaroxaban drug substances has been synthesized and the LC-MS/MS trace level quantification method has been developed. Based on the observed results, the method has been specific, precise, and sensitive and shows acceptable accuracy. Hence, this LC-MS/MS method can be used to control the mentioned Nitrosamine impurity (NNRI) and to evaluate the quality of the Rivaroxaban drug substance. This LC-MS quantification method is a practical advantage and will be useful for the generic pharmaceutical industry to maintain the quality and safety of Rivaroxaban drug substances.

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CONFLICT OF INTERESTS

The authors affirm that there is no conflict of interest.

AUTHORS CONTRIBUTION

All the authors contributed significantly to this manuscript, participated in reviewing/editing and approved the final draft for publication. The research profile of the authors can be verified from their ORCID ids, given below:

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