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Original Article

A Comprehensive LC-MS/MS Method for Detecting Genotoxic Nitrosamine Impurities in Favipiravir API

Swetha Sri Remidicherla[1, 2], Guntupalli Chakravarthi[1,*], Narender Malothu[1], Alavala Rajasekhar Reddy[3]

[1]Department of Pharmaceutical Analysis, KL College of Pharmacy, Koneru Lakshmaiah Education Foundation, Vaddeswaram, Guntur, Andhra Pradesh, INDIA.
[2]Department of Pharmaceutical Analysis, Sarojini Naidu Vanita Pharmacy Maha Vidyalaya, Tarnaka, Secunderabad, Telangana, INDIA.
[3]Department of Pharmaceutical Chemistry, Shobhaben Pratapbhai Patel School of Pharmacy and Technology Management, NMIMS (Deemed to be University), Mumbai, Maharashtra, INDIA.

ABSTRACT

Background: Favipiravir, a purine nucleic acid analogue (T-705), was used to treat SARS-CoV-2 patients. Existing methods often target specific nitrosamines in various matrices but not necessarily Favipiravir. These findings highlight the lack of a comprehensive method for detecting all seven potential nitrosamine impurities in Favipiravir API. **Materials and Methods:** The current approach discusses the trace-level quantification of nitrosamine impurities (NDEA, NDIPA, NEIPA, NMBA, NMPA, NDMA and NDBA) in Favipiravir API. The set of nitrosamine impurities were separated on gradient elution mode (1.0%v/v formic acid and 100.0% methanol) throughout a 20 min run period using Symmetry C18 (150X4.6 mm, 5 µm) with a flow rate of 0.8 mL/min. The Column oven was saturated to attain a temperature of 40º±1.0ºC, where the auto sampler was maintained at 5 . A rinse volume of 1200 µL was employed before and after aspiration, with a dip time of 5 seconds. All the nitrosamine impurities were quantified and ionized in positive polarity mode of Electron Spray Ionization (ESI) using Multiple Reaction Monitoring (MRM). **Results:** The retention times of the impurities NDEA, NDIPA, NEIPA, NMBA, NMPA, NDMA, NDBA and Favipiravir were found to be 8.51, 10.54, 9.60, 6.45, 11.06, 5.41, 12.67 and 6.96 min respectively. % Individual impurity in un spiked test solution has not been detected with any of the impurities. 1.29-1.98 was the %RSD for precision with 6-time repetitions Linearity was tested for specific impurities at six levels (5-100 ng/mL), with correlation coefficients (r^2) ranging from 0.995-0.999. The percentage recovery of each impurity at LOQ level was observed to be 83.7-107.2, whereas 91.1-101.8 at three levels of accuracy (50%, 100%, 200%) injected in triplicate. **Conclusion:** This novel LC-MS/MS method effectively separates and quantifies seven nitrosamine impurities in Favipiravir API, offering a reliable tool for quality control. This study validates an LC-MS/MS method for detecting nitrosamine impurities in Favipiravir API according to International Conference on Harmonization (ICH) guidelines.

Keywords: NEIPA, NDIPA, NMPA, Favipiravir API, NDBA, Genotoxic impurities, ESI(+*ve*).

Correspondence:
Dr. Guntupalli Chakravarthi
Department of Pharmaceutical Analysis, KL College of Pharmacy, Koneru Lakshmaiah Education Foundation, Vaddeswaram, Guntur, Andhra Pradesh, INDIA.
Email: chakra_varthi123@kluniversity.in

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INTRODUCTION

In 2018, NDMA contamination in the production of valsartan was detected by a Chinese Active Pharmaceutical Ingredient (API) manufacturing company. A probe revealed that nitrosamine impurities first appeared after July 2012, when the manufacturing process underwent changes in order to enhance yields and decrease waste. While some Active Pharmaceutical Ingredients (API), may be acceptable for having genotoxicity and carcinogenic properties, for cases of cytotoxic chemotherapy for cancer, impurities in drug substances and drug products have few beneficial effects and mainly possess risk without much corresponding benefit from it.[1,2] This was subsequently followed by a review of the danger of nitrosamine presence in all human medications within the EU and since then, the top regulatory agencies (EMEA and FDA) have mandated, for some angiotensin II receptor blockers (sartans) and other drugs such as metformin, histamine antagonists and nitroglycerin among others, that strict control and regulation over the levels of nitrosamine impurities present in medications be in place and followed thoroughly.[3] An important safety concern related to the use of solvents in medicine manufacture was brought to light by the NDMA contamination event involving valsartan. Sodium nitrite, which creates nitrous acid in an acidic media, was used to remove excess sodium azide, resulting in the nitrosation of Dimethylamine (DMA) contamination in dimethyl formamide to yield NDMA. The FDA and EMA believe that one of the major

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sources of nitrosamine impurities is the use of solvents during the drug manufacturing process and require manufacturers to keep an eye on nitrosamine levels in solvents.[4] While nitrosamine impurities possess the main risk of "potential genotoxicity," it refers to the lack of information on human toxicity and the availability of reliable data on genotoxicity from animal research.[5] The following are the safety levels: NMBA: 96 ng/day, NDMA: 96 ng/day, NDEA: 26.5 ng/day and NEIPA:26.5 ng/day.[5,6] Favipiravir as shown in Figure 1, is a pyrazine carboxamide derivative which was discovered by Toyama Chemical Co., Ltd. in Japan in 2014 and later became a generic drug in 2019. It is a pyrazine modified with aminocarbonyl, hydroxy and fluoro groups at positions 2, 3 and 6, respectively. It is known to have antiviral properties against RNA viruses. It inhibits the RNA-dependent RNA polymerase of numerous RNA viruses and is licensed for the treatment of influenza in Japan.[7,8] For the treatment of deadly infections such the COVID-19 and Lassa viruses as well as the Ebola and Lassa viruses, Favipiravir has been extensively investigated.[9,10] The mass spectrum can be used to determine the analytes' mass, elemental and isotopic composition, or to understand the sample's chemical structure. Charged particles striking a detector and generating current serve as the basis for detection.[11] Through literature revealed, methods pertaining to detection of Favipiravir in human serum by LC-MS/MS,[12,13] Nitrosamine impurities in Sartans,[14,15] Nitrosamine impurities in drinking water,[16-19] swab sampling method in the manufacturing area.[20,21] After reviewing these articles, it has been concluded that these investigations provide useful insights into the development and application of analytical methodologies for the quantification of Favipiravir alone as a component and in combination but no method has been reported which can detect presence of all 7 nitrosamine impurities in API as well as marketed products of Favipiravir. This research describes an LC-MS/MS method for detecting nitrosamine impurities in Favipiravir API. The developed method was validated following the guidelines.[22-24]

Chemicals and Apparatus

The Active pharmaceutical ingredient standards and samples were supplied by Hetero Drugs Pvt. Ltd., Hyderabad, India. HPLC-grade Formic acid and methanol were procured from Merck. The Milli-Q water for the experiment was obtained from Millipore, Milli-Q Plus water purification system. Clean chem Laboratories LLP provided impurity working standards such as N-Nitrosodiethylamine (NDEA), N-Nitroso Dimethyl amine (NDMA), N-Nitroso-N-Methyl-4-Aminobutyric Acid (NMBA), N-Methyl-N-Nitroso Isopropyl Amine (NMIPA), N-Nitrosodiisopropylamine (NDIPA) and N-Nitroso Ethyl Isopropyl Amine (NEIPA). Shimadzu-AUW 220D was employed to weigh the materials.

Sample Preparation

API Solution

Favipiravir API was weighed accurately and then dissolved in HPLC grade methanol to obtain 500 ppm concentration (S1). Serial dilutions D1 and D2 were done to obtain a 5 ppm solution. To prepare dilution no.1 at 50 ppm, 0.2 mL was taken from S1 and was made up to 2 mL using Methanol (D1). To prepare dilution no.2 of 5 ppm, 0.2 mL solution was taken from D1 and was made up to 2 mL using methanol. Which was the final sample of Favipiravir (D2).

Impurity Solutions

It is important to make sure that all of the solutions were made according to Table 1 for typical low temperature storage.

Mobile Phase Preparation

1% v/v Formic acid: added 1 mL of 100 % formic acid to a 100 mL graduated cylinder using a volumetric pipette and made up the volume to 100 mL with de-ionized water, made to pass through 0.45 µm membrane filter and mixed well by inversion.

Diluent

Utilized HPLC grade Methanol which was readily available.

Methodology

Post to the trials, well resolved peaks were retained with a mix of 1% v/v Formic acid (A)+100% HPLC grade Methanol (B)

Table 1: Preparation of stock sample of nitrosamine impurities.

Sl. No.	Name of impurity	Weight taken in 50 mL	Volume Pipetted (mL)	Volume made up to (mL)	Obtained concentration (ppm)
1.	NEIPA	3.3 mg	0.15	2	5
2.	NDIPA	3.3 mg	0.15	2	5
3.	NDMA	2.4 mg/20 mL	0.083	2	5
4.	NMPA	3.3 mg	0.15	2	5
5.	NMBA	2.4 mg/20 mL	0.2	2	5
6.	NDEA	3.3 mg	0.15	2	5
7.	NDBA	3.3 mg	0.15	2	5

at 0.8 mL/min. Elution was made in gradient mode as given in Table 2. Where a Symmetry C_{18} (150 mmx4.6 µm), 5 µm particle size was kept as a stationary phase. The column oven temperature was preserved at 40°C±1.0°C. Prepared impurity solutions were injected into Shimadzu Liquid Chromatography Mass Spectrometer LCMS-8040 equipped with quaternary pump, auto-sampler with carousal, thermostatic column oven whereas detection was done by photodiode array. An electrospray ionization for bombarding the impurity, Argon for collision, Nitrogen gas for nebulization were used to obtain transitions of precursor ions. The Mass analyzer employed in the system was Triple quadrapole. Later the nitrosamine impurities were subjected to Multiple Reactions Monitoring (MRM)[25] to obtain fragment ions. *m/z* ranging between 50-100 was chosen for acquisition of ions. With the help of Lab solutions Ver 5.75 SP2, mass spectra for each transition was recorded and integrated. The ion source used in the optimized MS parameters was ESI. The capillary voltages in positive and negative ionization modes were 4.5 and -3.5 kV, respectively. The cone voltage was 40 volts. The source temperature was 120°C. The nebulizing liquid flow velocity was 3 L per minute. The temperatures for the desolvation line and heat block were 250°C and 400°C, respectively. The drying gas flow velocity was 15 L/min. The auto sampler was maintained to attain a temperature of 20°C±0.5°C. It was determined that each nitrosamine impurity's peak shape and resolution were satisfactory.

Table 2: Gradient profile for elution.

Time (in min)	A (1% Formic acid)	B (Methanol)
0.20	0	0
0.30	98	2
10.00	10	90
17.00	10	90
18.00	98	2
20.00	0	0

Validation Parameters

Specificity

Assessed by injecting each individual impurity for presence of foreign peaks, also performed to confirm the retention time respectively.

Precision

A particular concentration of each impurity was tested for its repeatability and reproducibility (*n*=6) on the day of performance as well as its succeeding day performed by different analyst. Peak area and retention time was analyzed using the statistical parameter of relative standard deviation.

Linearity and Range

Spiked solution containing seven nitrosamine impurities in various concentrations were looked for meeting the guideline of regression (r^2) value.

LOD and LOQ

The experiment was carried out using solutions with concentrations ranging from 5 to 100 ng/mL to determine the limit.

Accuracy

Respective concentration of each individual's impurity were injected to find out the recovery of them out of spiked solution.

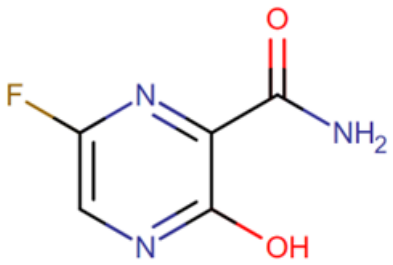

Figure 1: Structure of Favipiravir.

Table 3: Column and rows depicting Peak Area.#

Injection No.	NDEA	NDIPA	NEIPA	NMPA	NDBA	NDMA	NMBA
1	81254	161239	124561	67204	274823	19235	54870
2	80451	160125	126894	67425	275649	19874	54238
3	79823	162326	128216	66853	268914	19647	55829
4	83654	161234	130287	68467	265438	19879	54892
5	80359	156271	129212	65284	274156	19570	53937
6	80245	161680	125531	65023	279851	19873	54821
AVG	80964	160479	127450	66709	273139	19680	54765
STD	1397.73	2183.30	2194.45	1322.29	5145.43	255.23	652.03
RSD	1.726	1.360	1.722	1.982	1.884	1.297	1.191

Method precision corresponding to respective impurity when n=6.

RESULTS AND DISCUSSION

Specificity

The target compound (Favipiravir API) is separated from other components (impurities) in the sample. This allows for accurate analysis and quantification of the target compound. The difference in retention times between Favipiravir API and each impurity is significant. This which allows for clear identification and quantification of Favipiravir API without interference from the impurities. In this case, the separation window ranges from 3.58 min (Favipiravir API vs. NDIPA) to 5.71 min (Favipiravir API vs. NDBA). Nitrosamine impurities and Favipiravir API were found to be at following retention times in MRM chromatogram shown in Figure 2. The analysis identified the retention times of impurities as NDMA eluted the earliest at 5.414 min, followed by NMBA, NDEA and NEIPA within a short time frame. NDIPA and NMPA had slightly longer retention times around 10-11 min. The final two eluting peaks belonged to NDBA at 12.674 min and the Favipiravir API itself at 6.96 min.

Precision

Upon performing the repetitive injections (6) for the prepared impurity solutions (40 ng/mL), on the same day as well as succeeding day represented in Tables 3-5, statistical measures for peak area and retention time were found to between 1.2997-1.982 and 0.149-1.542 respectively.

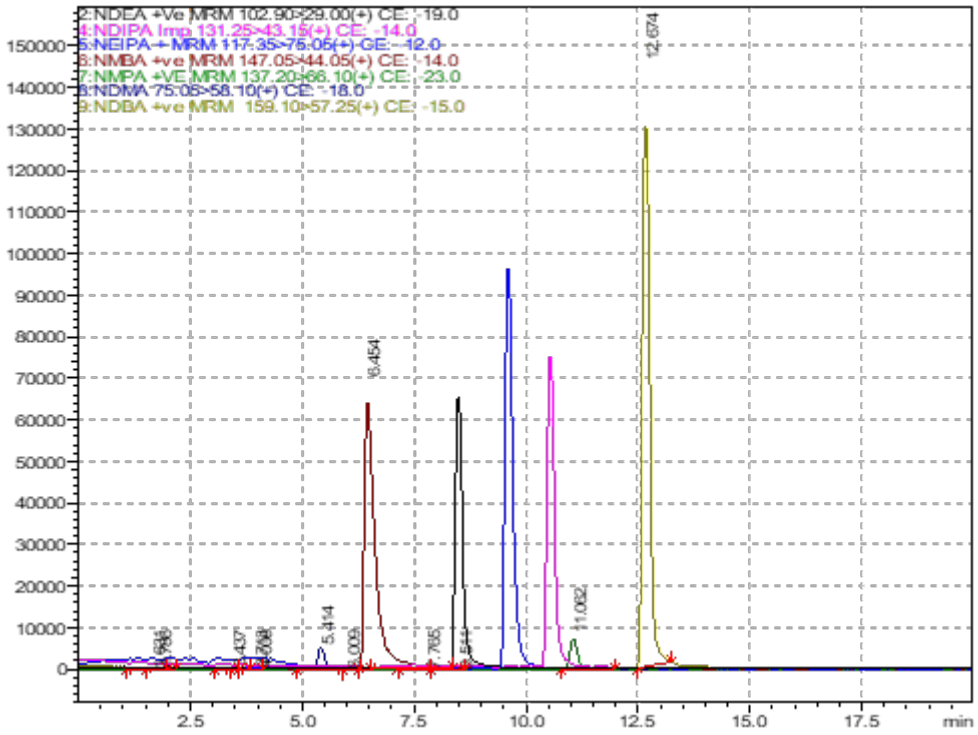

Figure 2: Overlain Total Ion Chromatogram of seven nitrosamine impurities.

Table 4: Retention Time (minutes).[$]

Injection No.	NDEA	NDIPA	NEIPA	NMPA	NDBA	NDMA	NMBA
1	8.48	10.54	9.6	11.06	12.67	5.41	6.45
2	8.47	10.51	9.62	11.12	12.49	5.49	6.49
3	8.48	10.45	9.71	11.36	12.67	5.36	6.41
4	8.46	10.57	9.68	11.48	12.31	5.38	6.5
5	8.45	10.56	9.64	11.35	12.52	5.4	6.39
6	8.48	10.55	9.64	11.1	12.79	5.55	6.38
AVG	8.47	10.53	9.65	11.25	12.58	5.43	6.44
STD	0.0126	0.0442	0.0402	0.1734	0.1701	0.0730	0.0512
RSD	0.149	0.420	0.417	1.542	1.353	1.345	0.796

[$] Elution time for each impurity during Method Precision.

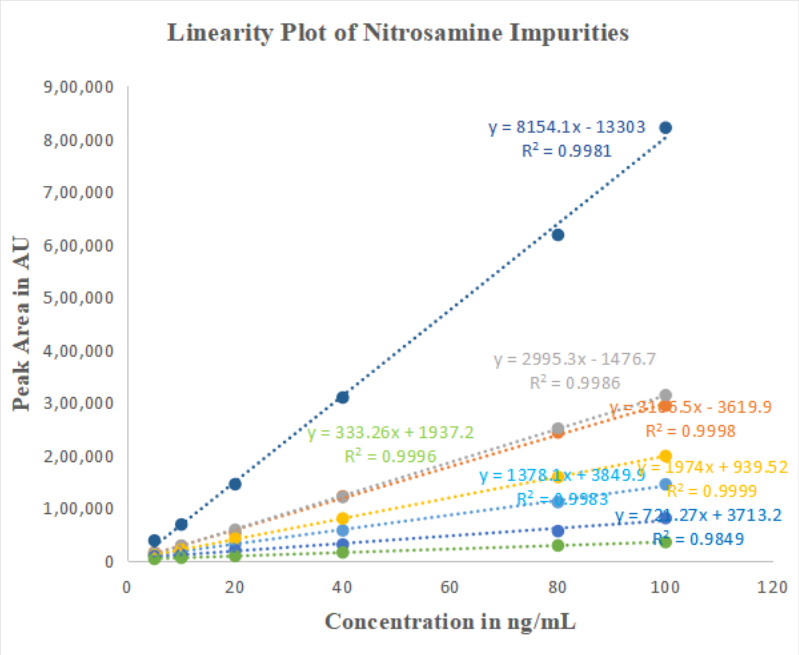

Figure 3: Picture of Trend line for series of concentrations of Nitrosamine Impurities.

Table 5: Retention Time (minutes).[@]

Injection No.	NDEA	NDIPA	NEIPA	NMPA	NDBA	NDMA	NMBA
1	8.47	10.49	9.68	11.32	12.7	5.49	6.39
2	8.46	10.51	9.65	11.4	12.69	5.41	6.51
3	8.45	10.56	9.67	11.29	12.57	5.62	6.35
4	8.51	10.58	9.7	11.17	12.62	5.53	6.72
5	8.39	10.47	9.59	11.36	12.41	5.76	6.48
6	8.37	10.48	9.63	11.45	12.8	5.39	6.66
AVG	8.49	10.57	9.61	11.33	12.63	5.53	6.52
STD	0.0523	0.0460	0.0394	0.0974	0.1337	0.1389	0.1463
RSD	0.616	0.436	0.411	0.860	1.059	2.511	2.245

[@] Elution time for each impurity during Intermediate Precision.

Table 6: Regression co-efficient values at each level of concentration.

Series*	S1	S2	S3	S4	S5	S6	S7
ng/mL	NDEA	NDIPA	NEIPA	NMBA	NMPA	NDMA	NDBA
5	10,903	15,443	14,390	9,822	8,158	3,222	38,101
10	19,429	28,024	28,359	20,776	11,261	5,314	68,442
20	31,309	52,632	58,373	41,618	19,700	8,746	1,44,940
40	57,200	1,22,025	1,20,695	79,815	30,483	15,606	3,09,289
80	1,10,961	2,43,069	2,50,345	1,58,592	55,923	28,622	6,17,654
100	1,44,708	2,93,746	3,13,588	1,98,396	80,679	35,095	8,21,042
Y-Intercept (+/-c)	3849.9	-1476.7	-3619.9	939.52	3849.9	1937.2	3713.2
Slope(m)	1378.1	2995.3	3166.5	1974	1378.1	333.26	721.27
r^2	0.9983	0.9986	0.9998	0.9999	0.9983	0.9996	0.9849

*S1-S7 Peak area represents each of the corresponding impurities.

Table 7: Obtained Signal-to-Noise Ratio.^

Std. Conc (ng/mL)	NDEA	NDIPA	NEIPA	NMBA	NMPA	NDMA	NDBA
	S/N Ratio						
5	4.35	3.24	4.43	6.82	1.9	1.95	9.72
10	6.66	5.55	18.47	12.23	5.04	2.64	18.28
20	12.55	9.74	40.25	38.64	8.68	6.33	34.59
40	28.24	22.29	56.46	85.79	18.04	9.42	84.8
80	47.7	44.57	209.79	180.1	44.08	16.18	243.39
100	68.09	52.3	214.22	141.64	54.44	18.47	308.8

^ generated by Lab solutions.

Table 8: Rows showing spiked concentration whereas columns split as Concentration recovered and %Recovered.

	Concentration and %Recovered													
Std. Conc (ppm)	NDEA		NDIPA		NEIPA		NMBA		NMPA		NDMA		NDBA	
5	4.80	95.9	5.19	103.9	5.11	102.3	4.89	97.9	5.06	101.1	4.78	95.7	5.24	104.8
10	11.01	110.1	9.53	95.3	9.66	96.6	10.32	103.2	9.53	95.3	10.78	107.8	9.17	91.7
20	19.66	98.3	18.02	90.1	19.42	97.1	20.65	103.3	21.70	108.5	20.60	103	19.08	95.4
40	38.53	96.3	41.96	104.9	39.70	99.2	39.59	99.0	37.25	93.1	40.24	100.6	40.38	100.9
80	77.69	97.1	83.71	104.6	81.88	102.3	78.63	98.3	73.93	92.4	77.52	96.9	80.34	100.4
100	102.28	102.3	101.19	101.2	102.46	102.5	98.36	98.4	109.62	109.6	96.05	96	106.69	106.7

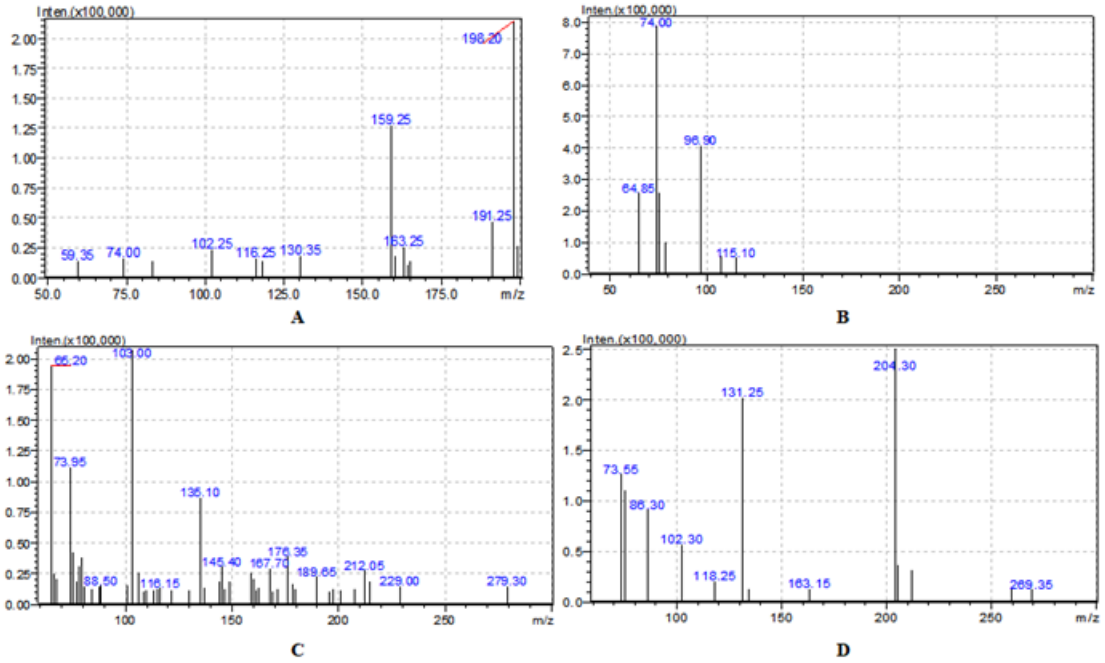

Figure 4: Mass Spectra depicting Parent ions(*m/z*) A: NDEA: Q1 Scan(C+) Ret. Time : [0.334->0.737]-[0.820->0.952] Scan# : [365->805]-[895->1039] B: NDMA: Q1 Scan(C+) Ret. Time : [0.256->0.839]-[0.866->0.989] Scan# : [59->191]-[197->225] C: NDBA:Q1 Scan(E+) Ret. Time : [0.702->1.212]-[1.386->1.968] Scan# : [937->1617]-[1849->2015]. D: NDIPA: Q1 Scan(C+) Ret. Time: [0.352->0.810]-[0.862->0.972] Scan# : [385->885]-[941->1189]

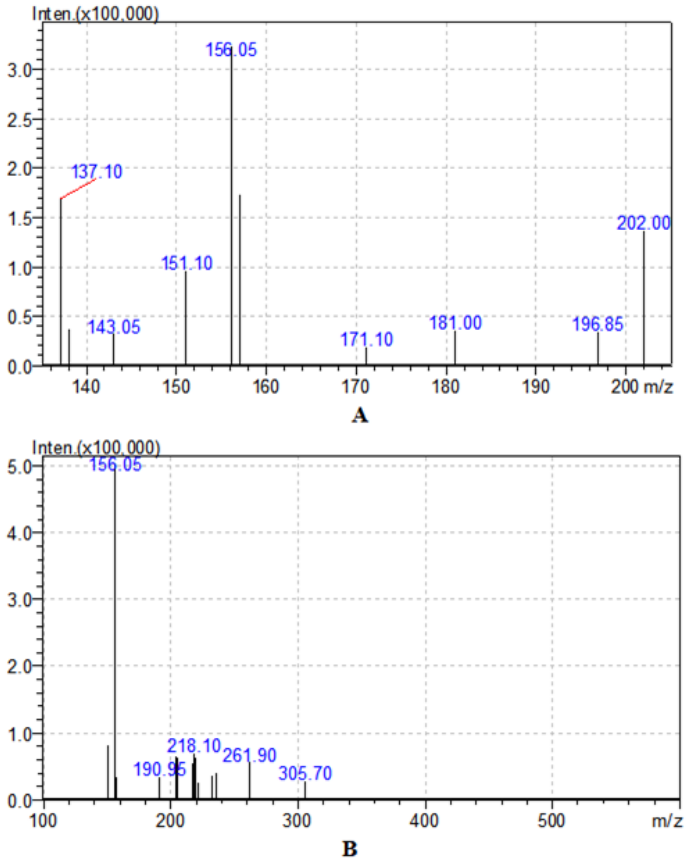

Figure 5: A: Favipiravir API in full scan spectrum mode: Q1 Scan(C-) Ret. Time: [6.728->7.132]-[8.972->11.665] Scan# : [1350->1431]. B: Favipiravir DP in full scan spectrum mode: Q1 Scan(E-) Ret. Time: [6.685->7.285]-[8.541->11.992] Scan# : [156->292].

Linearity and Range

The concentration values indicated in Table 6, with increasing standard concentrations used for calibration. The r^2 values for most analytes are very close to 1 as in Figure 3, indicating a strong linear relationship between concentration and instrument response. This suggests the calibration curves are reliable for these analytes. NDBA has a lower r^2 compared to others, suggesting a potentially less ideal fit for its calibration curve. The purpose of analyzing unknown concentration of Favipiravir samples using this calibration data can be efficiently served.

LOD and LOQ

All standards (5 to 100 ng/ mL) fall above the Limit of Detection (LOD) of 3 ppm, indicating that the analyte can be reliably detected in these samples inline with regulatory criteria. However, only samples with concentrations of 10 ng/ mL and above can be quantified with certainty, considering the Limit of Quantification (LOQ) of 10 ng/ mL. NDEA (12.3), NDPIA (12.7), NEIPA (13.7), NMBA (13.6) and NMPA (12.4) show (Table 7) concentrations above the LOQ (10 ng/mL) in all tested concentrations (5 to 100 ng/mL). This means the amount of these analytes can be reliably quantified. Typically determined using the signal-to-noise ratio in the chromatogram. For samples labeled NDMA (12.9) and NDBA (12.5), only concentrations of 40 ng/ mL and above can be confidently quantified based on the LOQ. Concentrations at 20 ng/ mL and below fall below the LOQ and cannot be definitively quantified. *m/z* values were depicted in Figures 4 and 5.

Accuracy

Table 8 shows the recovery percentages of seven analytes at various concentrations. Recovery indicates how much of the added analyte is retrieved. Most recoveries fall within an acceptable range, typically considered to be between 70% and 120%. Most recoveries are within an acceptable range, suggesting the method is effective. There seems to be a slight trend of decreasing recoveries with lower concentrations (5 and 10 ng/ mL) for some analytes (NDMA, NDBA, NMPA). This indicates challenges in accurately measuring very low amounts of these specific analytes. Recovery rates were slightly varied between different analytes. NDEA and NMBA showed consistently high recoveries across all concentrations, while NDMA and NDBA exhibited a wider range, particularly at lower concentrations.

CONCLUSION

This study addressed the critical need for a comprehensive method to detect potential nitrosamine impurities in Favipiravir API, a medication used against SARS-CoV-2. Existing methods focused on other substances and did not encompass the full range of these impurities. We developed a novel LC-MS/MS method using gradient elution with optimized parameters to achieve effective separation of seven nitrosamine impurities (NDEA, NDIPA, NEIPA, NMBA, NMPA, NDMA and NDBA) within a 20-min run. The method demonstrated excellent linearity (r^2=0.995-0.999) across a range of concentrations for each impurity, indicating a strong relationship between the amount of impurity present and the instrument's response. Furthermore, recovery studies yielded results of 83.7% and 107.2% at the lowest concentration level and 91.1% to 101.8% at various spiking levels, signifying good accuracy. This demonstrates the method's ability to reliably quantify these impurities at trace levels. In conclusion, this novel LC-MS/MS method offers a valuable tool for quality control in Favipiravir API production. It provides a comprehensive approach for detecting a broad spectrum of potential nitrosamine impurities, contributing to the safety and quality of this medication. The method has been validated to meet ICH Q2(R1) and USP<1469> standards, ensuring reliability and accuracy. The risk of nitrosamine development in Favipiravir API may be evaluated for their existence under stability settings and current research can be expanded to assess them in intermediate products during the manufacturing process.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

Min: Minute; **mL:** Millilitre; **g:** Gram; **mg:** Milligram; **µg:** Microgram; **ng:** Nanogram; **NMT:** Not more than; **RSD:** Relative standard deviation; **LOD:** Limit of Detection; **LOQ:** Limit of Quantification; **VF:** Volumetric flask; **LC-MS/MS:** Liquid chromatography hyphenated with mass spectrometry; **NDMA:** N-Nitroso dimethyl amine; **NDEA:** N-Nitroso diethyl amine; **NDIPA:** N-Nitrosodiisopropylamine; **NEIPA:** N-Nitrosoethylisopropylamine; **NMBA:** N-Nitroso-N-methy l-4-aminobutyric acid; **NMPA:** N-Nitrosomethylphenylamine; **NDBA:** N-Nitrosodi-n-butylamine; **ESI:** Electron Spray Ionization; **API:** Active Pharmaceutical Ingredient; **S/N:** Signal to Noise ratio; **USP:** United States Pharmacopoeia.

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