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ORIGINAL ARTICLE **OPEN ACCESS**

Cost Effective & Efficient Analytical Method Validation for the Content Estimation of N-Nitrosodimethylamine (NDMA) & N Nitrosodiethylamine (NDEA), N-Nitrosoethylisopropylamine (NEIPA) & N-Nitrosodiisopropylamine (NDIPA) In Itraconazole GCMS-HS

Baskar Venkatesan*, Pushpendra Sharma
Department of Science, Sri Satya Sai University of Technology and Medical Sciences
Sehore- (MP) - 466001
***Corresponding Author**'s E mail: Baskar_69@rediffmail.com

ABSTRACT
The Objective of this paper is to validate an accurate, precise, and linear gas chromatographic-mass spectrometry selective ion monitoring (SIM) method for quantitative estimation of N-nitrosodimethylamine (NDMA), N-nitrosodiethylamine (NDEA), N-Nitrosoethylisopropylamine (NEIPA) & N-Nitrosodiisopropylamine (NDIPA) as an impurity in Itraconazole (ICR) active pharmaceutical ingredient (API) at ppm level and validated as per International Council of Harmonization (ICH) guidelines. This method used in SIM mode mass selective detection was validated for the trace level analysis of an impurity. Chromatographic separation of NDMA, NDEA, NEIPA, NDIPA was achieved in, DB-WAX, 30.0 m X 0.25 mm, 0.5 µm Capillary column or Equivalent ZB-5 ms 30 m × 0.25 mm × 0.25 µm column, using helium carrier gas with 3.0 ml/min. The method was linear for NDMA (0.1161-0.1041 ppm), NDEA (0.1019-0.1004 ppm), NEIPA (0.1118-0.1066 ppm) and NDIPA (0.1045-0.0958 ppm) in ICR, respectively. The coefficient of correlation (r2) for these impurities was better than 0.999. The method was fully validated, complying Food and Drug Administration, ICH, and European Medicines Agency guidelines. Furthermore, verified precision, accuracy, LOQ precision, LOQ accuracy and robustness. The methods were successfully validated to determination and quantification of above mentioned genotoxic impurities in Itraconazole API. Hence, the method holds good for the routine trace analysis of these impurities in Itraconazole and various pharmaceutical industries as well as academics.
Keywords: *NMDA, NMEA, Itraconazole, Gas chromatography-mass spectrometry, Method validation.*

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INTRODUCTION

Itraconazole is an orally active triazole antifungal drug which has demonstrated a broad spectrum of activity and a favourable pharmacokinetic profile. It is a potent inhibitor of most human fungal pathogens including Aspergillus species. In non-comparative clinical trials itraconazole was shown to be extremely effective in a wide range of superficial and more serious 'deep' fungal infections when administered once or twice daily [1]. ICR, (+-)-ics-4[4-[4-[4-[[2-(2,4- dichlorophenyl)-2-(1H-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-yl] methoxy] phenyl]-1-piperazinyl] phenyl]-2,4-dihydro-2(1-methylpropyl)- 3H-1,2,4-triazol-3-one, is (Figure 1) a classical member of the triazole class and is an important drug in our arsenal to treat fungal infections because it exhibits broad-spectrum antifungal activity [2].

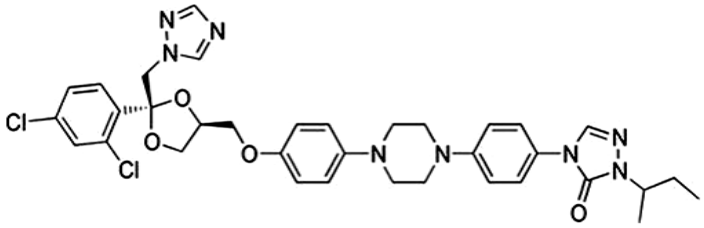


Figure 1: Chemical structure of itraconazole

Nitrosamines impurities:

Any molecule containing the nitroso functional group called nitrosamines. These molecules are of concern because nitrosamine, are classified as probable carcinogens by International Agency for Research on Cancer [IARC]. Nitrosamines are common in water and foods, including cured and grilled meats, dairy products and vegetables. Everyone is exposed to some level of nitrosamines. Although they are also present in some foods and drinking water supplies, their presence in medicines is nonetheless considered unacceptable.

Medicine Regulatory Authorities first became aware of the presence of the nitrosamine impurity, Nitrosodimethylamine (NDMA), in products containing valsartan in July 2018. Valsartan is an Angiotensin II Receptor Blocker (ARB) and belongs to a family of analogue compounds commonly referred to as the sartans. Further nitrosamine impurities were subsequently detected in other medicines belonging to the sartan family, including: N-nitrosodiethylamine (NDEA), N-nitrosodiisopropylamine (NDIPA), N-nitrosoethyliso- propylamine (NEIPA) and N -nitroso-N-methyl-4-aminobutyric acid (NMBA). Subsequently, in Sept 2019a nitrosamine impurity has been detected in batches of ranitidine, a medicine used to treat heartburn and stomach ulcers. On 6 December 2019, EMA confirmed that trace amounts of NDMA had been found in a small number of metformin-containing medicines outside the EU. There were no data indicating that EU medicines were affected [3-5].

Toxicity of NDMA and NDEA

NDMA and NDEA belong to group of highly potent mutagenic carcinogens. Despite the potency of these impurities, there is still a very low risk that nitrosamine impurities at the levels found could cause cancer in humans. Only limited impurity-specific toxicity data is available for NDMA and NDEA. Due to their structural similarity, NDIPA, NEIPA, and NMBA are considered by international regulators to exhibit a toxicological profile like NDMA and NDEA.

Table 1: Interim allowable daily intake limits

Impurity name Abbreviation	Chemical name	Allowable Daily Intake
NDMA	N-nitrosodimethylamine	96.0 ng/day
NDEA	N-nitrosodiethylamine	26.5 ng/day
NMBA	N-nitroso-N-methyl-4-aminobutyric acid	96.0 ng/day
DIPNA	N-nitrosodiisopropylamine	26.5 ng/day
EIPNA	N-nitrosoethylisopropyleamine	26.5 ng/day

Numerous analytical methods for the determination of pharmaceuticals and their metabolites in aqueous solutions have been described in the literature. Liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-MS (GC-MS) are the most widely used techniques [6, 7]. An MS is typically utilized in one of two-ways: Full scan or selected ion monitoring (SIM). The typical GC-MS instrument is capable of performing both functions either individually or concomitantly depending on the setup of the particular instrument. The primary goal of instrument analysis is to quantify an amount of substance. This is done by comparing the relative concentrations among the atomic masses in the generated spectrum in SIM certain ion fragments are entered into the instrument method, and only those mass fragments are detected by the MS. The advantages of SIM are that the detection limit is lower since the instrument is only looking at a small number of fragments (e.g., three fragments) during each scan [8]. SIM mode the MS is "targeting a limited mass range," the number of scans across the peak has increased resulting in better peak shape. This is an easy solution for getting better quantitation for early eluting peaks. Inspect the ions obtained for the peak in full scan mode and use at least one of the ions in SIM to obtain a better scan rate [9]. It has been demonstrated that GC-MS method offers several advantages over high-performance LC (HPLC) method including better sensitivity, specificity, and higher throughput. This paper presents a highly specific and sensitive GC-MS method for the estimation of NMDA and NMEA in ICR active pharmaceutical ingredient (API) as per International Council of Harmonization guidelines. This approach eliminated the time-consuming liquid-liquid extraction used in HPLC-ultraviolet method, increased the detection limit, and greatly reduced sample processing and instrument acquisition time. Thus, the paper reports an economical, simple, and accurate GC-MS method for estimation of NMDA and NMEA in ICR [10-11].

MATERIAL AND METHODS

Instruments/ chemicals & reagents /standards & samples:

S. No	Instrument/Materials	Make/Model/Lot No	Grade/Purity
1	GCMS	Shimadzu GCMS-TQ8040	NA
2	Analytical balance	RADWAG & XA 82/220.R2/LC&GC	NA
3	Column (DB-WAX)	(Dimension) 30m X 0.32mm, 0.5µm	NA
4	Methanol	SH8SA81209	HPLC
5	N-Methyl-2-Pyrollidinone	Spectrochem	GC
6	N-Nitrosodimethylamine	MNEA/001/08/2018	98.1
7	Nitraso Diethyl Amine	H5GMI	100
8	N-Nitrosoethylisopropylamine	L47-005	96.80%
9	N-Nitrosodiisopropylamine	L36-081(1)	96.70%
10	Itraconazole	RTFPAA024/II/19-20	NA

Methodology:

Chromatographic Conditions:

Instrument	GCMS-HS-TQ8040				
Column	DB-WAX, 30.0 m X 0.25 mm, 0.5 µm Capillary column or Equivalent				
Detector	MS				
Carrier gas	Helium				
Column Oven Program	Initial: 70°C Hold time for 4.0 minutes				
	Ramp rate: 20°C/minute at 240°C hold for 3.5 minutes				
Injection Mode	Split				
Split	1:2				
Flow Control Mode	Linear velocity				
Run Time	16.00 minutes				
Column flow	3.00 mL/min				
Purge flow	3.00 mL/min				
Ion source temperature	230°C				
Interface temperature	240°C				
Event Time	0.200sec				
Start Time	4.00min				
End Time	12.00min				
Solvent cut time	4.00min				
Detector gain mode	Relative the Tuning result				
Acquisition mode	MRM				
Q1 Resolution	Unit				
Q3 Resolution	Unit				
Compound Name	NDMA	Ch1-m/z	74.00>44.00	CE	5.00kV
Compound Name	NDEA	Ch2-m/z	102.00>85.00	CE	5.00kV
Compound Name	NEIPA	Ch3-m/z	116.00>99.00	CE	5.00kV
Compound Name	NDIPA	Ch4-m/z	130.00>88.00	CE	5.00kV

Head Space Parameters:

Oven Temperature	120°C
Sample Line Temperature	125°C
Transfer Line Temperature	130°C
Shaking Level	5
Pressurizing Gas Pressure	10 psi
Equilibrating Time	15.0 min
Pressurizing Time	0.2 min
Pressure Equilibration Time	0.2 min
Load Time	0.1 min
Load Equilibration Time	0.05 min
Injection Time	1.00 min
Needle flush time	5.0 min
GC Cycle Time	23.0 min

Preparation of blank solutions and standard solutions:
Preparation of Diluent: Use N-Methyl-2-Pyrrlodinone as diluent.
Preparation of blank: Pipette 2mL of diluent and transfer into 20mL HS vial crimp the vial immediately with cap and septa and place into GCMS-HS system.
Preparation of NDMA standard stock solution (30ppm w.r.t test conc.): Weigh about 10mg of NDMA standard into 10mL volumetric flask, mix with 3mL of diluent and make up to the volume with diluent and mix well. Transfer 0.7mL of above solution into 100mL volumetric flask and dilute to volume with diluent and mix well.
Preparation of NDEA standard stock solution (30ppm w.r.t test conc.): Weigh about 10mg of NDEA standard into 10mL volumetric flask, mix with 3mL of diluent and make up to the volume with diluent and mix well. Transfer 0.7mL of above solution into 100mL volumetric flask and dilute to volume with diluent and mix well.
Preparation of NEIPA standard stock solution (30ppm w.r.t test conc.): Weigh about 10mg of NEIPA standard into 10mL volumetric flask, mix with 3mL of diluent and make up to the volume with diluent and mix well. Transfer 0.7mL of above solution into 100mL volumetric flask and dilute to volume with diluent and mix well.
Preparation of NDIPA standard stock solution (30ppm w.r.t test conc.): Weigh about 10mg of NDIPA standard into 10mL volumetric flask, mix with 3mL of diluent and make up to the volume with diluent and mix well. Transfer 0.7mL of above solution into 100mL volumetric flask and dilute to volume with diluent and mix well.
Preparation of Standard solution: (0.3ppm of each NDMA, NDEA, NEIPA, NDIPA w.r.t test conc.). Pipette 1.0mL of NDMA, NDEA, NEIPA & NDIPA standard stock solutions into 100mL volumetric flask dilute to volume with diluent and mix well. Transfer accurately 2.0mL of Standard solution into a 20mL HS vial and immediately crimp the vial.
Preparation of NDMA, NDEA, NEIPA, NDIPA LOQ solution: (0.09ppm of each NDMA, NDEA, NEIPA, NDIPA w.r.t test conc.): Transfer 30mL of standard solution into 100mL volumetric flask and dilute to volume with diluent and mix well.
Preparation of NDMA, NDEA, NEIPA, NDIPA LOD solution: (0.03ppm of each NDMA, NDEA, NEIPA, NDIPA w.r.t test conc.): Transfer 10mL of standard solution into 100mL volumetric flask and dilute to volume with diluent and mix well.
Preparation of sample solution: (Prepare in duplicate): Weigh accurately 400 mg of Itraconazole sample in to 20mL head space vial, add 2mL of diluent crimp the vial immediately with cap and septa and place into GCMS-HS system.
Injection sequence:

S. No	Description	Number of injections
1.	Blank	2
2.	Standard solution	6
3.	Blank	1
4.	Sample solution preparation-1	1
5.	Sample solution preparation-2	1
6.	Blank	1
7.	Bracketing Standard	1

Additional blank injections can be injected to avoid the carryover and to obtain stable baseline.
Procedure: Inject blank solution (two) and standard solution (six injections) into the GCMS system and check the system suitability parameters. Then inject sample solution record the chromatograms. Measure the area response of NDMA, NDEA, NEIPA and NDIPA peak. Disregard the peaks due to blank. The retention time of NDMA about 6.7 minutes and NDEA peak is about 7.5 minutes, NEIPA about 7.8 minutes and NDIPA about 8.0 minutes.
System Suitability Requirements: % RSD calculated for the peak areas of NDMA, NDEA, NEIPA & NDIPA peak areas obtained from six injections of standard solution should be not more than 15.0.
The cumulative %RSD calculated for the peak areas of NDMA, NDEA, NEIPA & NDIPA from initial six injections and online standard solution should not be more than 15.0.
Calculation: Content of NDMA, NDEA, NEIPA & NDIPA in Itraconazole calculated by µg/g

$$\frac{AT-AB}{AS-AB} \times \frac{CS}{CT} \times \frac{P}{100} \times 1000000$$

Calculate the content of NDMA, NDEA, NEIPA & NDIPA in preparation-1 and preparation-2 of Itraconazole using above formula and report the average value.

Specification Limits: 0.3 ppm of each NDMA, NDEA, NEIPA and NDIPA w.r.t. test conc. As per the sponsor.
Where,
AT = Peak area of NDMA, NDEA, NEIPA & NDIPA obtained in test solution.
AB = Area response of peak in the chromatogram of the respective blank
AS = Average area of NDMA, NDEA, NEIPA & NDIPA in standard solution
CS = Conc. of NDMA, NDEA, NEIPA & NDIPA in standard solution (mg/mL)
CT = Test concentration (mg/mL)
P = Purity/ Assay of NDMA, NDEA, NEIPA & NDIPA Standard (%)

RESULT AND DISCUSSION:

System Suitability

The system suitability solutions were prepared by using NDMA, NDEA, NEIPA and NDIPA standard as per analytical test procedure and injected into the GC-MS HS system. The % RSD of N-Nitrosodimethylamine (NDMA) & N-Nitrosodiethylamine (NDEA), N-Nitrosoethylisopropylamine (NEIPA) & N-Nitrosodiisopropylamine (NDIPA) peak areas from six replicate injection of standard solution should be not more than 15.0. The system suitability parameters were evaluated and found well within the limits. The results are summarized in Table No-2.

Table No-2: System Suitability Results for NDMA, NDEA, NEIPA and NDIPA

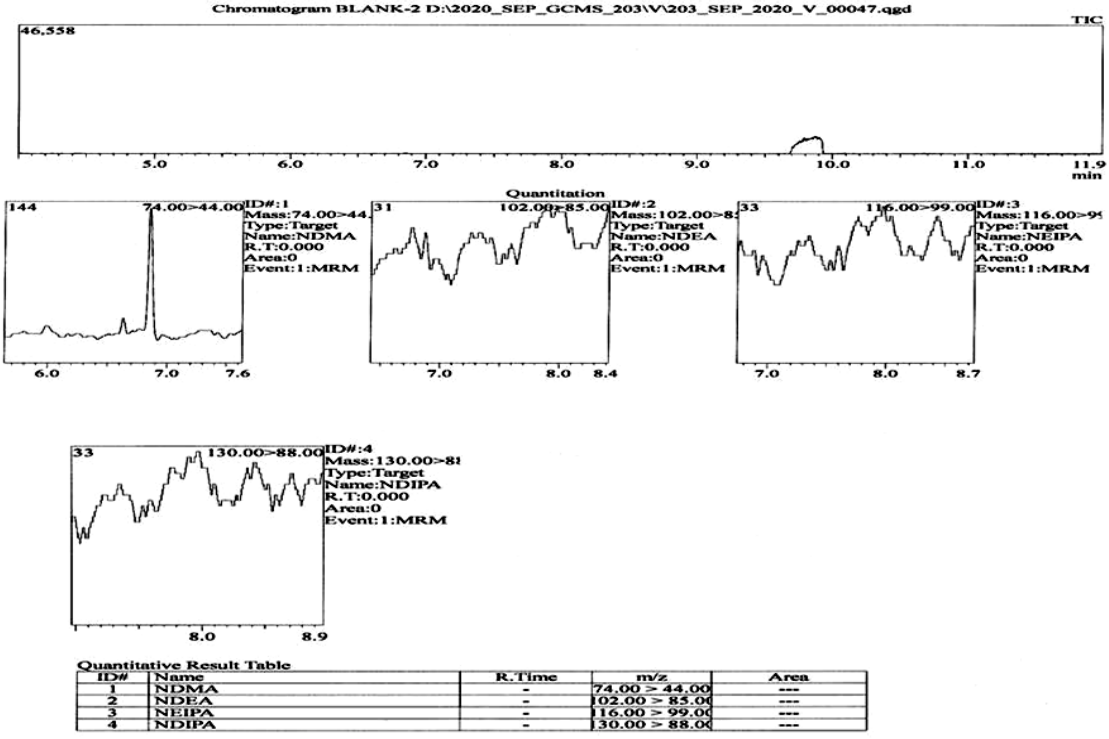
Preparation	Peak Area			
	NDMA	NDEA	NEIPA	NDIPA
Standard Solution-1	11581	5744	8971	4512
Standard Solution-2	11852	5977	8647	4759
Standard Solution-3	11964	5856	9330	5070
Standard Solution-4	11544	6350	8940	4877
Standard Solution-5	11897	5835	8843	4697
Standard Solution-6	11911	5790	8952	4966
Average	11792	5925	8947	4814
Standard Deviation	181	222	223	200
%RSD	1.5	3.8	2.5	4.2

Specificity (Blank Interference): Established the interference in blank. Specificity was conducted by preparing blank, individual standard solution at specification level, as such sample and spike preparation at specification level as per test procedure injected into the GC-MS HS system. The chromatogram of the blank should not show any peak at the retention time of NDMA, NDEA, NEIPA and NDIPA. If any Interference found at the retention time of NDMA, NDEA, NEIPA and NDIPA it should not be more than 5.0% of standard solution. No interference was found at the retention time of NDMA, NDEA, NEIPA and NDIPA in the blank. The results are summarized in table No-3 and figure no 2 to 5.

Table No-3: Blank interference results for NDMA, NDEA, NEIPA and NDIPA

Solution	Name	Retention Time (min)	Peak Area	Interference found at the retention time of NDMA, NDEA, NEIPA and NDIPA (Yes/No), in %
Standard	NDMA	6.624	11369	NA
	NDEA	7.411	7179	NA
	NEIPA	7.734	12683	NA
	NDIPA	7.968	6879	NA
Blank	NDMA	6.624	NA	No
	NDEA	7.411	NA	No
	NEIPA	7.734	NA	No
	NDIPA	7.968	NA	No

Figure 2: Blank Chromatogram



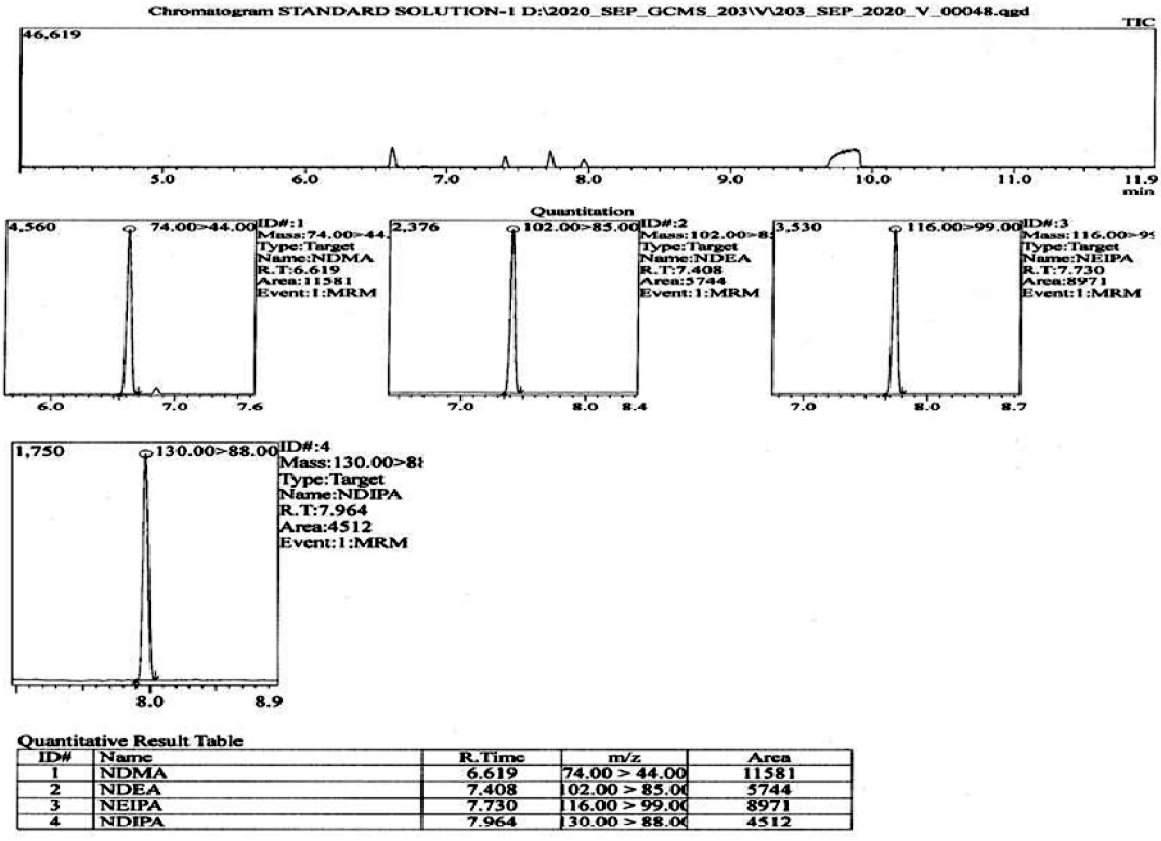


Figure 3: Standard Chromatogram

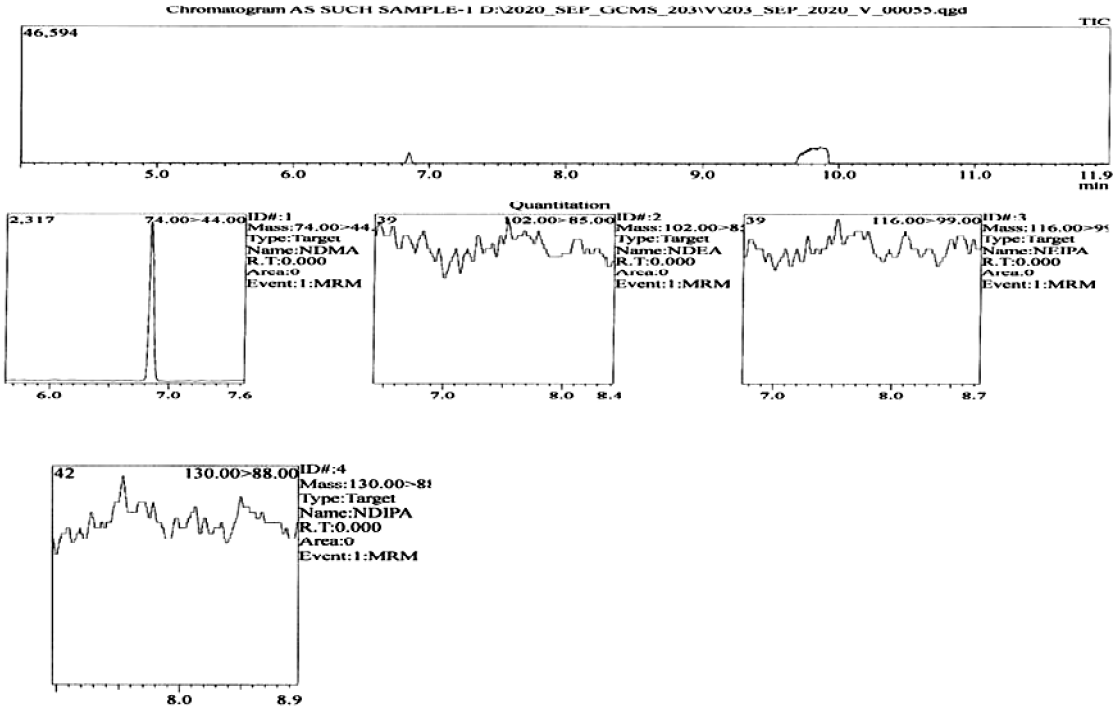


Figure 4: Control sample Chromatogram

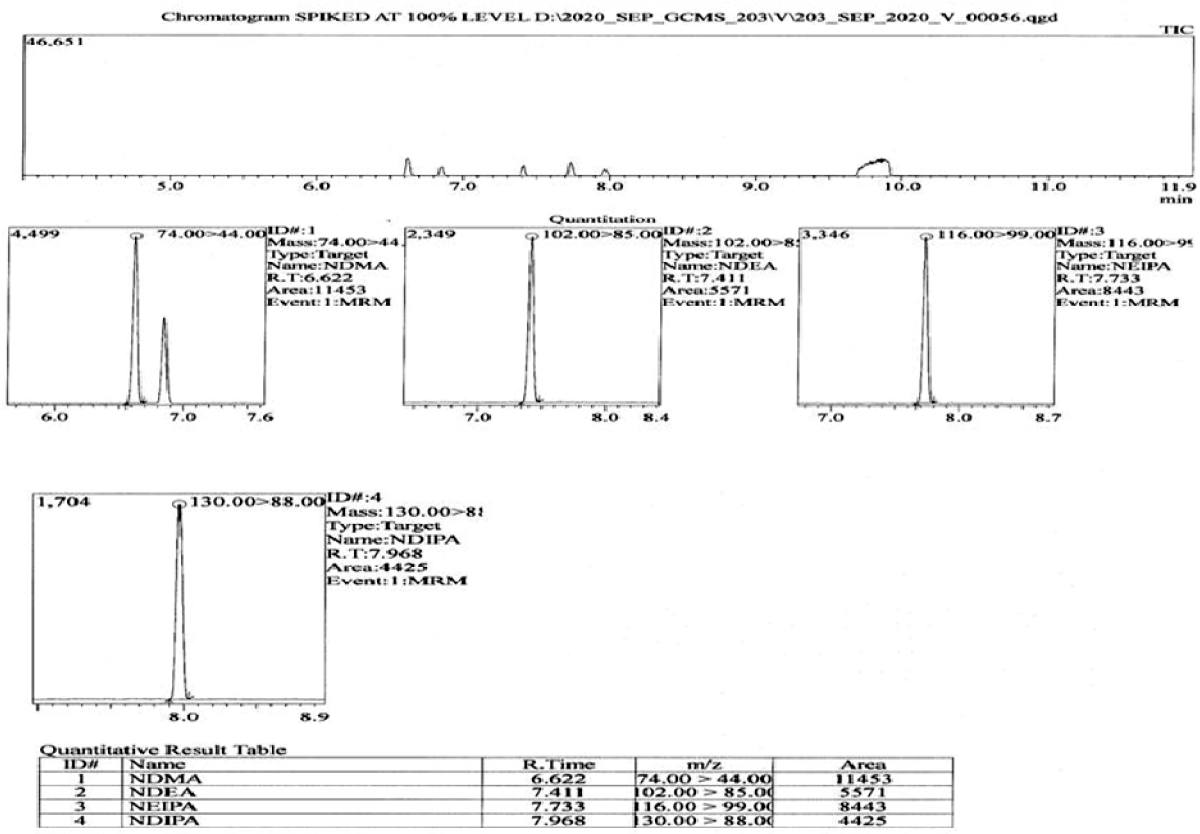


Figure 5: Control sample Chromatogram

LOD & LOQ Confirmation: Established and Confirmed Limit of Detection and Limit of Quantification for NDMA, NDEA, NEIPA and NDIPA. The system suitability parameters were evaluated and found to be well within the limits. S/N Ratio for NDMA, NDEA, NEIPA and NDIPA from LOD solution 17.43, 15.13, 35.28 and 10.68. S/N Ratio for NDMA, NDEA, NEIPA and NDIPA from LOQ solution 62.58, 66.30, 52.00 and 62.82. Results are described below.

Table No-4(a): LOD results for NDMA, NDEA, NEIPA and NDIPA

Name of analyte	S/N Ratio
NDMA	17.43
NDEA	15.13
NEIPA	35.28
NDIPA	10.68

Table No-4(b): LOQ results for NDMA, NDEA, NEIPA and NDIPA

Name of analyte	S/N Ratio
NDMA	62.58
NDEA	66.30
NEIPA	52.00
NDIPA	62.82

LOQ Precision: The Precision at LOQ Level was evaluated by preparing six spiked samples having NDMA, NDEA, NEIPA and NDIPA at about LOQ Level. % RSD calculated for the content of NDMA, NDEA, NEIPA and NDIPA obtained from six injections of LOQ standard solution should be not more than 20.0. The % Relative standard deviation value for the content of NDMA and NDEA was found to be well within the limits. The results are summarized in Table No-5.

Table No-5: LOQ Precision results for NDMA, NDEA, NEIPA and NDIPA

LOQ Preparation	Contents in ppm			
	NDMA	NDEA	NEIPA	NDIPA
Preparation-1	0.1161	0.1019	0.1118	0.1045
Preparation-2	0.1206	0.1143	0.1147	0.1082
Preparation-3	0.1217	0.1140	0.1161	0.0931
Preparation-4	0.1196	0.1170	0.1281	0.1114
Preparation-5	0.1157	0.1100	0.1191	0.1031
Preparation-6	0.1041	0.1004	0.1066	0.0958
Average	0.1163	0.1096	0.1161	0.1027
Standard Deviation	0.00645	0.00693	0.00726	0.00706
%RSD	5.5	6.3	6.3	6.9

LOQ Accuracy: Prepared sample solutions by spiking NDMA & NDEA standard solution at about LOQ Level. The % recoveries for all the samples at LOQ Level were found to be well within the limits. The results are summarized in Table No-6.

Table No-6: Batch Analysis Results for NDMA, NDEA, NEIPA and NDIPA:

Sample name	Batch Number	Average Content			
		NDMA	NDEA	NEIPA	NDIPA
Itraconazole	RTFPAA024/II/19-20	ND	ND	ND	ND
Itraconazole	RTFPAA025/II/19-20	ND	ND	ND	ND
Itraconazole	RTFPAA026/II/19-20	ND	ND	ND	ND
Itraconazole	RTFPAA001/IV/18-19/III	ND	ND	ND	ND
Itraconazole	RTFPAA002/IV/18-19/III	ND	ND	ND	ND
Itraconazole	RTFPAA003/IV/18-19/III	ND	ND	ND	ND

Method Precision (Repeatability): Method Precision of test method is determined by preparing six spiked samples for NDMA, NDEA, NEIPA and NDIPA at Specification level as per the test procedure. The %

Relative standard deviations for content of NDMA, NDEA, NEIPA and NDIPA in six spiked sample preparations were found 2.8, 3.0, 4.0 and 3.0 respectively (The % RSD for content of NDMA, NDEA, NEIPA and NDIPA from 6 replicate injections of spiked samples should be NMT 15.0%). System suitability parameters were found to be well within the limits and the results are summarized in Table No-7.

Table No-7: Method Precision results for NDMA, NDEA, NEIPA and NDIPA

Preparation	Content in ppm			
	NDMA	NDEA	NEIPA	NDIPA
Spiked at 100% Level Preparation-1	0.3744	0.3916	0.4038	0.3681
Spiked at 100% Level Preparation-2	0.3751	0.3704	0.3677	0.3674
Spiked at 100% Level Preparation-3	0.3679	0.3689	0.3694	0.3657
Spiked at 100% Level Preparation-4	0.3738	0.3879	0.3738	0.3666
Spiked at 100% Level Preparation-5	0.3494	0.3690	0.3616	0.3399
Spiked at 100% Level Preparation-6	0.3595	0.3647	0.3702	0.3595
Average	0.3667	0.3754	0.3744	0.3612
Standard Deviation	0.01032	0.01133	0.01494	0.01088
%RSD	2.8	3.0	4.0	3.0

Robustness: This study was performed by making small but deliberate variations in the method parameters. The effect of variations in flow rate of carrier gas and column oven temperature was studied. Under all the variations, system suitability requirement is found to be within the acceptance criteria and hence the proposed method is robust. The RSD of area counts for NMDA NDEA, NEIPA and NDIPA peak obtained from six replicate injections of standard solution should be not more than 15.0%. The data of robustness were shown in Table 8.

Table 8: Result of robustness

Parameter	Control	Increased	Decreased
Column Flow rate	3.0 mL/min	3.3 mL/min	2.7 mL/min
Result (% RSD)	5.28	5.58	5.69
Temperature	240°C	245°C	255°C
Result (% RSD)	4.71	4.39	4.83

CONCLUSION

A simple high throughput GC-MS method has been validated for the determination of Nitrosamine impurities in ICR API. This method is specific, sensitive, and reproducible and has been successfully to monitor and control impurity level. The residue NMDA and NMEA was determined in ppm levels also. The method well suits for the intended purpose.

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ABBREVIATIONS:

GC	:	Gas Chromatography
MS	:	Mass Spectrometer
HS	:	Head Space
RSD	:	Relative Standard Deviation
S No.	:	Serial Number
%	:	Percentage
QA	:	Quality Assurance
ATP	:	Analytical Test Procedure
NDMA	:	N-Nitrosodimethylamine
NDEA	:	N-Nitrosodiethylamine
NEIPA	:	N-Nitrosoethylisopropylamine
NDIPA	:	N-Nitrosodiisopropylamine
MP	:	Method Precision

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