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ORIGINAL ARTICLE

Cost Effective & Efficient Analytical Method Development for the Content Estimation of N-Nitrosodimethylamine, N-Nitrosodiethylamine, N-Nitrosoethylisopropylamine and N-Nitrosodiisopropylamine in Itraconazole by GCMS-HS

Baskar Venkatesan*, Neelu Jain
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 develop a method for content 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. This method used in SIM mode mass selective detection was developed 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 final method was developed after many trail and found suitable for the determination of NDMA, NDEA, NEIPA and NDIPA in ICR, respectively. The method was fully developed, complying Food and Drug Administration, ICH, and European Medicines Agency guidelines. Furthermore, verified precision, specific, LOQ precision, LOQ accuracy. The methods were successfully developed 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, NDEA, NEIPA, NDIPA, Itraconazole, Gas chromatography-mass spectrometry, Method Development.*

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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].

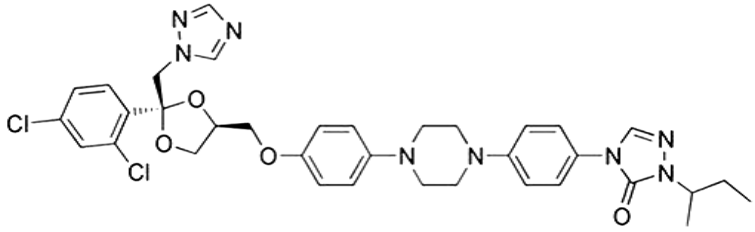


Figure 1: Chemical structure of itraconazole

Nitrosamines impurities:

Any molecule which containing the nitroso functional group contain nitrosamine impurities. 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. 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 [6-9].

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].

Table 1: Interim allowable daily intake limits

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

MATERIAL AND METHOD

Instruments/ chemicals & reagents /standards & samples: Instrument used in this Method Development Study are calibrated and used within the Calibration period.

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

MATERIAL AND METHODS

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 Ratio	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				
Detector gain mode	Relative the Tuning result				
Solvent cut time	2.50 min				
Start Time	3.00 min				
End Time	14.00 min				
Acquisition mode	MRM				
Event time	0.300sec				
Q3 Resolution	Unit				
Compound Name	N-Nitrosodimethylamine	Ch1-m/z	74.00>44.00	CE	5.00kV
Compound Name	N-Nitrosodiethylamine	Ch2-m/z	102.00>85.00	CE	5.00kV
Compound Name	N-Nitrosoethylisopropylamine	Ch3-m/z	116.00>99.00	CE	5.00kV
Compound Name	N-Nitrosodiisopropylamine	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

RESULT AND DISCUSSION

Development Trail-01:

Preparation of Standard/Diluent solutions:

Blank preparation: Transferred 1mL of N-methyl-2-Pyrrolidinone in to 20mL head space vial, crimped the vial immediately with cap and septa and placed into GCMS-HS system.

Preparation of NDMA standard stock solution (30ppm w. r. t test conc.): Weigh about 10.08mg of NDMA standard into 10mL volumetric flask, mix with 3mL of diluent and make up to the volume with diluent and mix well. Transfer 1.5mL 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 10.58mg of NDMA standard into 10mL volumetric flask, mix with 3mL of diluent and make up to the volume with diluent and mix well. Transfer 1.5mL 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 10.31mg of NDMA standard into 10mL volumetric flask, mix with 3mL of diluent and make up to the volume with diluent and mix well. Transfer 1.5mL 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 10.34mg of NDMA standard into 10mL volumetric flask, mix with 3mL of diluent and make up to the volume with diluent and mix well. Transfer 1.5mL 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, NIPEA & NDIPA w. r. t test conc.): Pipette 1.0mL of NDMA, NDEA, NIPEA& NDIPA standard stock solutions into 100mL volumetric flask dilute to volume with diluent and mix well. Transfer accurately 1.0mL of Standard solution into a 20mL HS vial and immediately crimp the vial immediately with cap and septa and placed into GCMS-HS system.
Observations and conclusion of trail-1: Peak shape of NDMA from standard solution-1 is not found satisfactory. Based on the data generated it is observed that NDMA peak shape is not found satisfactory and the peaks shapes of standard solution-2 and 3 is found satisfactory. Hence sequences were aborted and restart the analysis with fresh batch table.

Development Trail-02:

Preparation of LOQ Standard solution: Pipette 6.0mL of NDMA, NDEA, NIPEA & NDIPA standard stock solutions into 20mL volumetric flask dilute to volume with diluents and mix well.
Preparation of as such sample solution: Weighed and transferred 500.05 mg of Itraconazole sample in to 20mL head space vial, added 1mL of diluent and crimped the vial immediately with cap and septa and placed into GCMS-HS system. Prepare and inject sample in triplicate.
Preparation of LOQ spiked sample solution: Weighed and transferred 500.31 mg of Itraconazole sample in to 20mL head space vial, added 1mL of LOQ Standard Solution and crimped the vial immediately with cap and septa and placed into GCMS-HS system. Prepare and inject sample in triplicate.
Preparation of spiked sample solution: Weighed and transferred 500.76 mg of Itraconazole sample in to 20mL head space vial, added 1mL of Standard Solution and crimped the vial immediately with cap and septa and placed into GCMS-HS system. Prepare and inject sample in triplicate.
Observations and Conclusion of trail-2: Observation of development trail-2 is summarized in below mentioned tables:

Table-1: System Suitability results of Trail-2

S. No	NDMA AREA	NDEA AREA	NEIPA AREA	NDIPA AREA
1	12640	6400	10741	5883
2	12732	6678	11007	6187
3	12910	6808	11005	6257
4	12230	6684	10732	5841
5	13063	6831	10950	6419
6	12713	6982	11151	6528
AVG AREA	12715	6731	10931	6186
%RSD	2.0	2.7	1.4	4.1

Table-2: Specificity of Trail-2

S.No	Name of the Injection	Area			
		NDMA	NDEA	NEIPA	NDIPA
1	Blank	ND	ND	ND	ND
2	Standard solution-1	12640	6400	10741	4428
3	Standard solution-2	12732	6678	11007	4236
4	As such sample	ND	ND	ND	88
5	100% spiked solution	16365	10184	17039	10449

S.No	Name of the injection	Retention Time			
		NDMA	NDEA	NEIPA	NDIPA
1	Blank	ND	ND	ND	ND
2	Standard solution-1	6.541	7.334	7.659	7.895
3	Standard solution-2	6.538	7.334	7.657	7.895
4	As such sample	ND	ND	ND	ND
5	100% spiked solution	6.540	7.335	7.659	7.895

Table-3: Method Precision Results of Trail-2

S. No	Content			
	NDMA	NDEA	NEIPA	NDIPA
1	0.3173	0.3460	0.3203	0.3300
2	0.3064	0.3432	0.3248	0.3343
3	0.3208	0.3474	0.3337	0.3316

%Recovery at 100% spiked solutions:

S.No	NDMA	NDEA	NEIPA	NDIPA
1	104.9	109.1	104.9	109.2
2	101.3	108.2	106.4	110.7
3	106.1	109.6	109.3	109.8

Acceptance Criteria: Individual % recovery of NDMA, NDEA, NEIPA and NDIPA should be between 60.0 to 140. Individual % Recovery for NDMA, NDEA, NEIPA and NDIPA from 100% spiked samples is within the limits.

Table-4: LOQ Precision Results of Trail-2

S. No	Content			
	NDMA	NDEA	NEIPA	NDIPA
1	0.1332	0.1492	0.1144	0.1295
2	0.1213	0.1365	0.1155	0.1250
3	0.1251	0.1368	0.1138	0.1227

%Recovery at LOQ spiked solutions:

S.No	NDMA	NDEA	NEIPA	NDIPA
1	146.8	156.8	125.1	142.9
2	133.7	143.5	126.3	137.9
3	137.9	143.8	124.3	135.4

Acceptance Criteria: Individual % recovery of NDMA, NDEA, NEIPA and NDIPA should be between 60.0 to 140. Individual % Recovery for NDMA, NDEA, NEIPA and NDIPA from LOQ spiked samples is not within the limits.

Development Trail-03:

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 Ratio	2:1
Flow Control Mode	Linear velocity
Run Time	16.00 minutes
Column flow	3.00 mL/min

Purge flow	3.00 mL/min				
Ion source temp	230°C				
Interface temp	240°C				
Detector gain mode	Relative the Tuning result				
Solvent cut time	2.50 min				
Start Time	3.00 min				
End Time	14.00 min				
Acquisition mode	MRM				
Event time	0.300sec				
Q3 Resolution	Unit				
Compound Name	N-Nitrosodimethylamine	Ch1-m/z	74.00>44.00	CE	5.00kV
Compound Name	N-Nitrosodiethylamine	Ch2-m/z	102.00>85.00	CE	5.00kV
Compound Name	N-Nitrosoethylisopropylamine	Ch3-m/z	116.00>99.00	CE	5.00kV
Compound Name	N-Nitrosodiisopropylamine	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

Blank preparation: Transferred 1mL of N-methyl-2-Pyrrolidinone in to 20mL head space vial, crimped the vial immediately with cap and septa and placed into GCMS-HS system.
Preparation of NDMA, NDEA, NEIPA and NDIPA intermediate stock solution: Pipetted 0.7mL of stock solutions into 100mL volumetric flask, made up to volume with diluent and mixed well.
Preparation of NDMA, NDEA, NEIPA and NDIPA standard solution: Pipetted 1.0mL of stock solutions into 100mL volumetric flask, made up to volume with diluent and mixed well. Transferred 1.0mL of above standard solution into 20mL HS vial, crimped the vial and placed into GCMS-HS system.
Preparation of LOQ Standard solution: Pipette 3.0mL of NDMA, NDEA, NIPEA & NDIPA standard stock solutions into 10mL volumetric flask dilute to volume with diluent and mix well.
Preparation of as such sample solution: Weighed and transferred 200.27 mg of Itraconazole sample in to 20mL head space vial, added 1mL of diluent and crimped the vial immediately with cap and septa and placed into GCMS-HS system.
Preparation of LOQ spiked sample solution: Weighed and transferred 200.19 mg of Itraconazole sample in to 20mL head space vial, added 1mL of LOQ Standard Solution and crimped the vial immediately with cap and septa and placed into GCMS-HS system.
Preparation of 100% spiked sample solution: Weighed and transferred 200.34 mg of Itraconazole sample in to 20mL head space vial, added 1mL of 100% Standard Solution and crimped the vial immediately with cap and septa and placed into GCMS-HS system.
Observations and Conclusion Trail-3: Based on the results obtained from above experiment it is conclude that responses of analytes is low, but the % individual recovery of impurities at 100% spike level and LOQ level is within the criteria. Next experiment shall perform by injecting spike samples at 100% and LOQ level to finalize method.

Table -5: %Recovery at LOQ spiked solutions in Trail-3

S.No	NDMA	NDEA	NEIPA	NDIPA
1	77.9	69.2	62.5	64.2

%Recovery at 100% spiked solutions:

S.No	NDMA	NDEA	NEIPA	NDIPA
1	105.1	101.4	105.4	111.2

Development Trail-04: Standard details, solvent details and chromatographic condition same as used in development trail-3.
Preparation of standard vial: Pipetted 2.0mL of above standard solution into 20mL HS vial, crimped the vial and place into GCMS-HS system.
Preparation of LOQ Standard solution: Refer experiment number: PAN-RS-MD-004, page No: 04 of 05.
Preparation of as such sample solution: Weighed and transferred 400.60 mg of Itraconazole sample in to 20mL head space vial, added 2mL of diluent and crimped the vial immediately with cap and septa and placed into GCMS-HS system.
Preparation of LOQ spiked sample solution: Weighed and transferred 400.58 mg of Itraconazole sample in to 20mL head space vial, added 2mL of LOQ solution and crimped the vial immediately with cap and septa and placed into GCMS-HS system.
Preparation of spiked sample solution: Weighed and transferred 400.96 mg of Itraconazole sample in to 20mL head space vial, added 2mL of standard solution and crimped the vial immediately with cap and septa and placed into GCMS-HS system.
Observations and Conclusion of Trail-4: No blank interference is observed at Retention time of NDMA, NDEA, NEIPA and NDEA. Results obtained from development trail-4 are summarized in below mentioned tables:

Table-6: System Suitability results of Trail-4

S. No	Area			
	NDMA	NDEA	NEIPA	NDIPA
1	10284	5378	8431	4664
2	10365	5385	8589	4769
3	10383	5428	8556	5013
AVG AREA	10344	5397	8525	4815
%RSD	0.5	0.5	1.0	3.7

Table-7: Method Precision Results of Trail-4

S. No	Content			
	NDMA	NDEA	NEIPA	NDIPA
1	0.3347	0.3407	0.3325	0.3012
2	0.3345	0.3628	0.3453	0.3220

%Recovery at 100% spiked solutions:

S.No	NDMA	NDEA	NEIPA	NDIPA
1	94.9	92.1	95.1	86.0
2	94.8	98.1	98.8	92.0

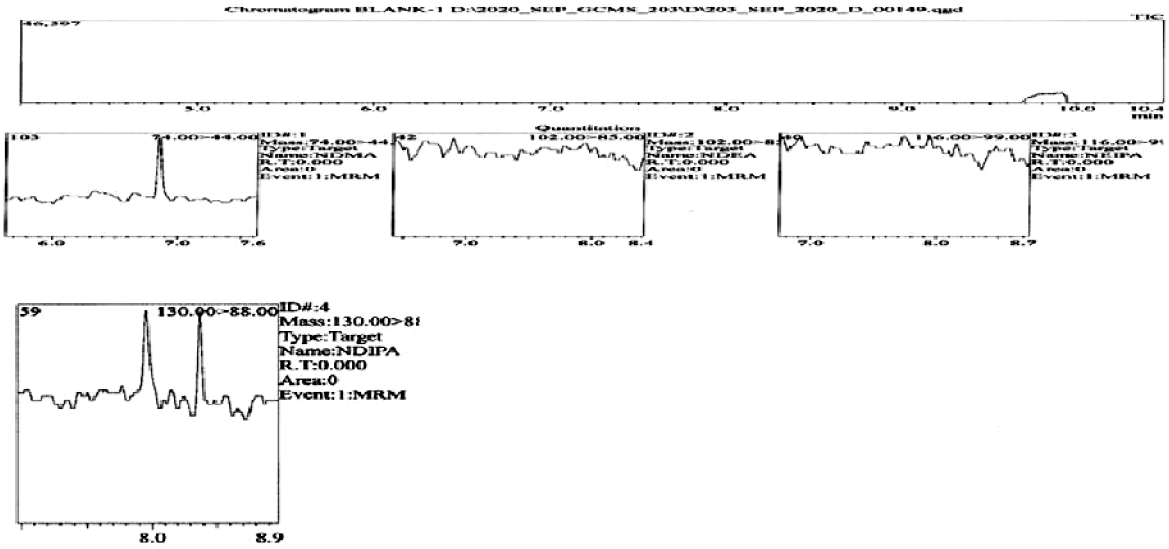
Table-8: LOQ Precision Results of Trail-4

S. No	Content			
	NDMA	NDEA	NEIPA	NDIPA
1	0.0936	0.0908	0.0840	0.0721
2	0.0997	0.0875	0.0875	0.0653

%Recovery at LOQ spiked solutions:

S.No	NDMA	NDEA	NEIPA	NDIPA
1	88.5	81.8	80.1	68.6
2	94.2	78.9	83.5	62.2

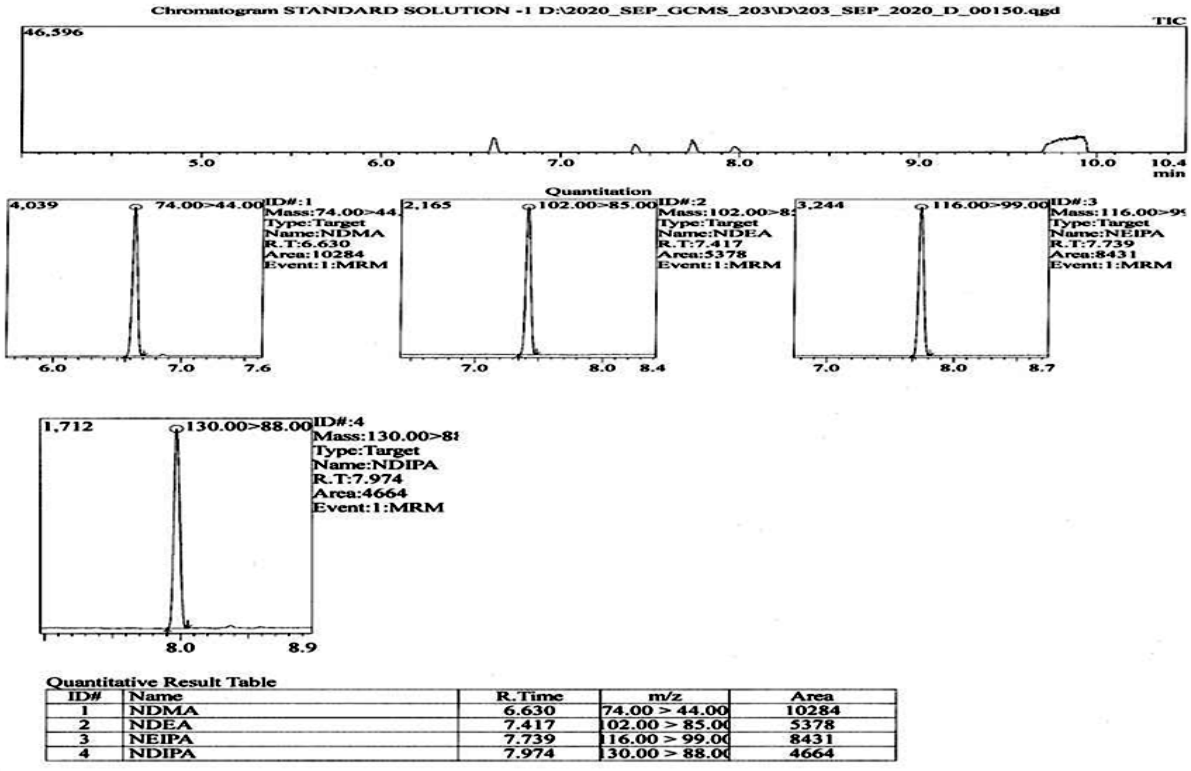
Acceptance Criteria: Individual % recovery of NDMA, NDEA, NEIPA and NDIPA should be between 60.0% to 140.0% and Individual % Recovery for NDMA, NDEA, NEIPA and NDIPA from 100% spiked samples and LOQ spiked samples is within the limits.



Quantitative Result Table

ID#	Name	R.Time	m/z	Area
1	NDMA	-	74.00 > 44.00	----
2	NDEA	-	102.00 > 85.00	----
3	NEIPA	-	116.00 > 99.00	----
4	NDIPA	-	130.00 > 88.00	----

Figure 2: Typical Blank Chromatogram



Quantitative Result Table

ID#	Name	R.Time	m/z	Area
1	NDMA	6.630	74.00 > 44.00	10284
2	NDEA	7.417	102.00 > 85.00	5378
3	NEIPA	7.739	116.00 > 99.00	8431
4	NDIPA	7.974	130.00 > 88.00	4664

Figure 3: Typical Standard Chromatogram

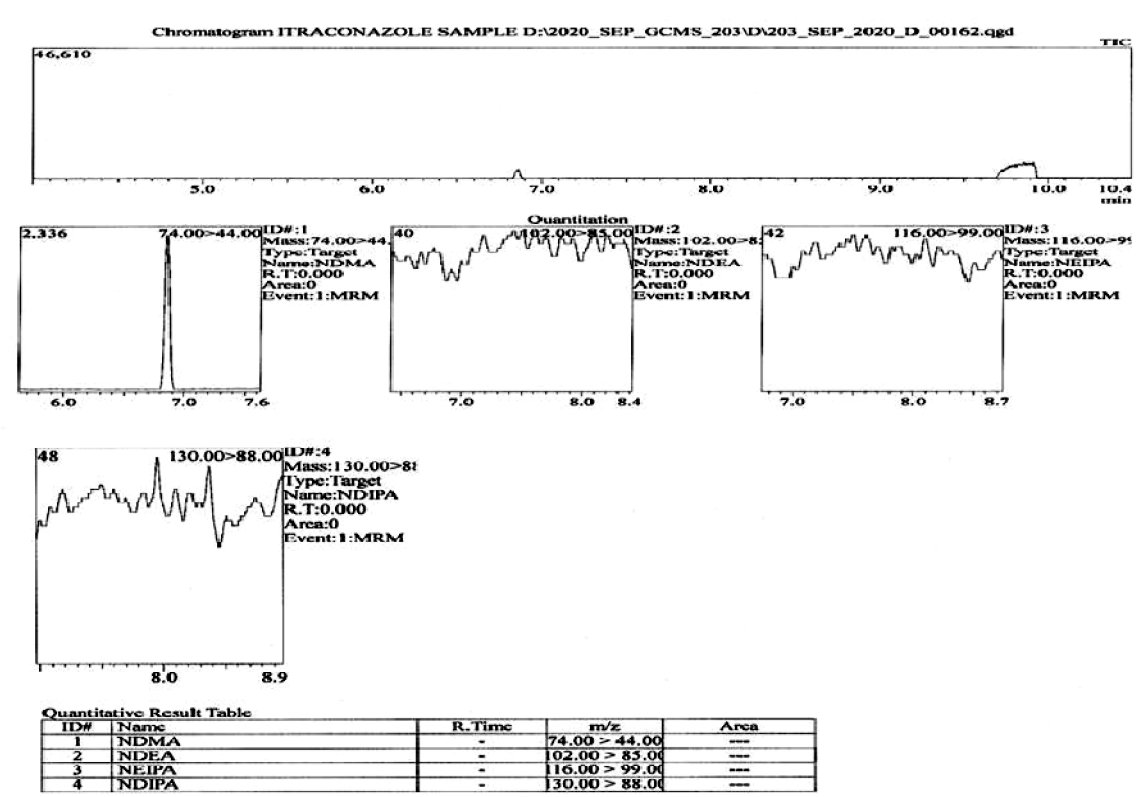


Figure 4: Typical Control Sample Chromatogram

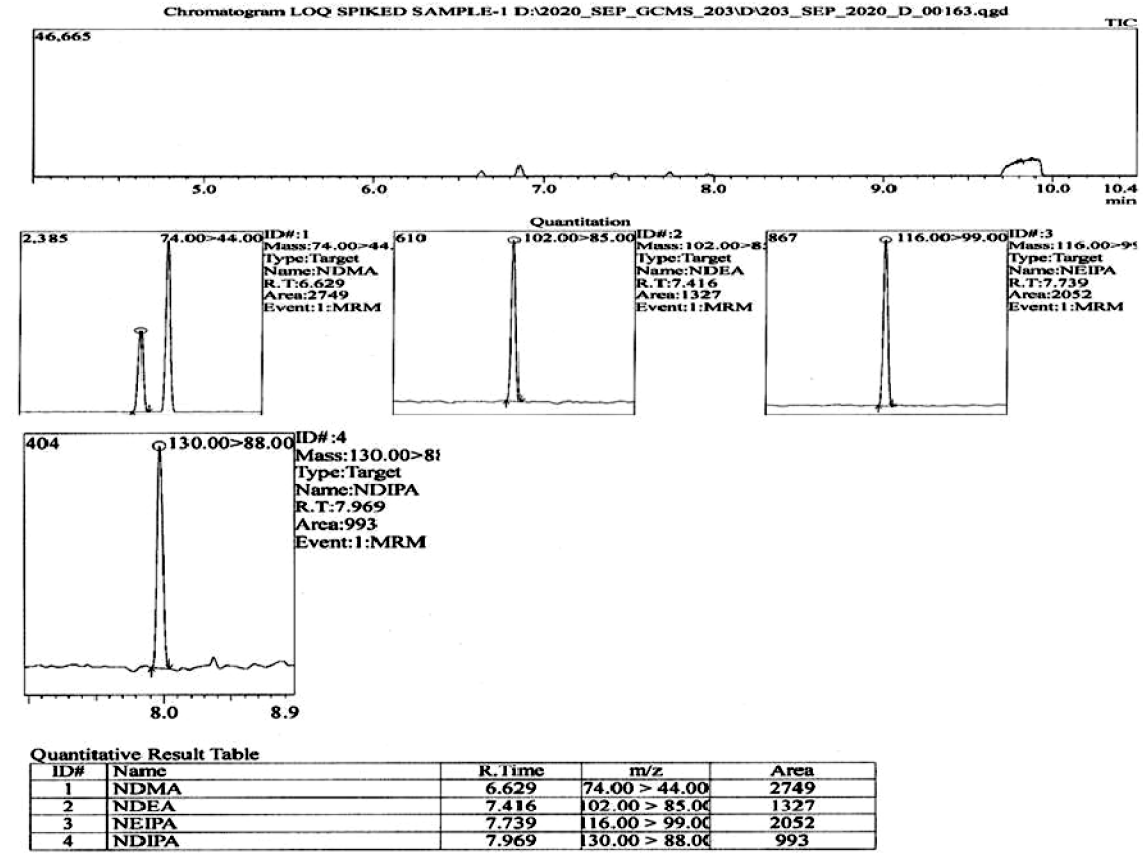


Figure 5: Typical Control Sample Chromatogram

CONCLUSION

A simple high throughput GC-MS method has been developed for the determination of Nitrosamine impurities in ICR API. After many development trails trail number 4 has been found suitable for the determination of nitrosamine impurities in Itraconazole. This method is specific, sensitive, and reproducible and has been successfully to monitor and control impurity level. The residue NDMA, NDEA NEIPA and NDIPA was determined in ppm levels also. The method well suits for the intended purpose.

ACNOWLEDGMENT

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CONFLICT OF INTEREST

Author has no conflict of interest while preparing the manuscript.

ABBREVIATIONS:

GC	:	Gas Chromatography
MS	:	Mass Spectrometer
HS	:	Head Space
RSD	:	Relative Standard Deviation
S No.	:	Serial Number
%	:	Percentage
LOQ	:	Limit of Quantification
API	:	Active Pharmaceutical Ingredient
NDMA	:	N-Nitrosodimethylamine
NDEA	:	N-Nitrosodiethylamine
NEIPA	:	N-Nitrosoethylisopropylamine
NDIPA	:	N-Nitrosodiisopropylamine
MP	:	Method Precision
ICR	:	Itraconazole

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