Research Article

Development of Single Validation Method for Detection and Quantification of Six Nitrosamine Impurities in Valsartan by Using Ultra-high Performance Liquid Chromatography with Tandem Quadrupole Mass Spectrometry

Althaf Hussain Shaik [a, b], Ramakrishna Reddy Kotanka[c]*, Kiran Kumar Ruthala [b], Srinivasa Rao Gandholi [b], Kirankumar Nalla[d], Santosh R Kanade[d]

[a] Research Scholar-Department of Chemistry, REVA University, Bangalore, Karnataka, India.

[b] YMC India Pvt Ltd- R&D Lab, Hyderabad, University of Hyderabad, Telangana, India.

[c] Associate professor Department of Chemistry, REVA University, Bengaluru, Karnataka, India.

[d] Department of Plant Sciences, school of life sciences, University of Hyderabad, Hyderabad-500046, Telangana, India.

***Corresponding author's E-mail:** k.ramakrishnareddy@reva.edu.in

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ABSTRACT

In foods, nitrosamines have been an issue for many years, and this concern has been extended to pharmaceuticals also in recent times. In 2018 U.S. Food and Drug Administration. globally recalled some of the drug products due to contamination of Nitrosamine impurity in valsartan drugs. Hence detection of trace-level nitrosamines in active pharmaceutical ingredients (API) and drug products can be challenging and require sophisticated and robust tools and techniques to meet current regulatory standards. It is very difficult to quantify those trace-level impurities in a single method. Therefore, we developed a single method and validated for detection and quantification of six Nitrosamine impurities namely N-nitroso dimethylamine (NDMA) N-nitroso diethylamine (NDEA), N-ethyl-Nisopropylnitrosoamine (NEIPA), N- nitrosodibutylamine (NDBA), N-diisopropylnitrosoamine (NDIPA), and N-nitroso-n-methyl aniline (NMPhA) in valsartan drug by using Ultra-High Performance Liquid Chromatography with Triple Quadrupole Mass Spectrometry with Atmospheric pressure chemical ionization (APCI) source in +Ve (Positive) mode with Multiple reaction monitoring (MRM) Scan. In this method, we achieved the Limit of quantification (LOQ) and limit of detection (LOD) levels are 1.5ppb and 0.5 ppb concentrations respectively and in linearity performed from LOQ to 150%(9ppb) levels and obtained a correlation coefficient of all six Nitrosamine impurities are not less than 0.998. Accuracy (Recovery) at LOQ Level achieved between 80 to 120%. This method was validated in line with current regulatory requirements and guidelines.

Keywords: Nitrosamine's impurities, valsartan, Ultra-High Performance Liquid Chromatography, Triple Quadrupole Mass Spectrometry.

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INTRODUCTION

In foods, nitrosamines have been an issue for many years. This concern has recently extended to pharmaceuticals. Most notably, unwanted nitrosamine side products were detected in the Sartan class blood pressure medications (angiotensin-II-receptor antagonists) of many pharmaceutical companies, leading to a long string of product recalls and thus sales losses and potentially reputational damage. Nitrosamines in at least four categories of drugs are currently receiving scrutiny. Many professionals expect that nitrosamine impurities testing could extend to a lot more than APIs and finished drug products in the future [1-3]. Pharmaceutical impurities are unwanted chemicals that will form during the synthesis of active pharmaceutical ingredients (APIs) and or degradation and can also while storing conditions, contaminations, or excipient interactions. As per the regulations of ICH M7 guidance, these impurities need to be quantified at trace levels so that we can eliminate the risk of carcinogenic effect for human consumption. recently in July 2018 US-FDA (Food and Drug Administration) globally recalled and recommended to drug manufacturers should quantify Nitrosamine levels in their drugs and if those impurities have more than acceptable limits, should reduce or remove these impurities as per regulatory requirements. Nitrosamines are a high potential and increase the risk of cancer, these impurities in APIs and drug products create a significant risk to health and safety even in small quantities and thus are a major concern for drug makers. Those impurities may form multiple ways through degradation products generated during formulation or drug storage conditions or from environmental contaminants.

Recently two carcinogenic impurities have been identified in Sartan drugs, a class of medications used to treat high blood pressure and heart failure [5] prompting recalls of angiotensin receptor blockers (ARBs) valsartan and irbesartan which were contaminated with N-

Nitrosodimethylamine (NDMA) and N-Nitrosodiethylamine (NDEA) respectively [5]

Chemical structures for six nitrosamine impurities:

Since then, several other N-Nitrosamines have also been identified and are being investigated by regulators that are N-Nitrosodiispropylamine (NDIPA), N-Nitrosoethylisopropylamine (NEIPA), N-Nitrosodibutylamine (NDBA), and N-Nitroso-N-methyl-4-aminobutyric acid (NMBA), N-NitrosoMethylPhenylamine (NMPhA)[4] the chemical structures of Nitrosamine impurities were shown in (Figure-01).

Chemical structures for six nitrosamine impurities:

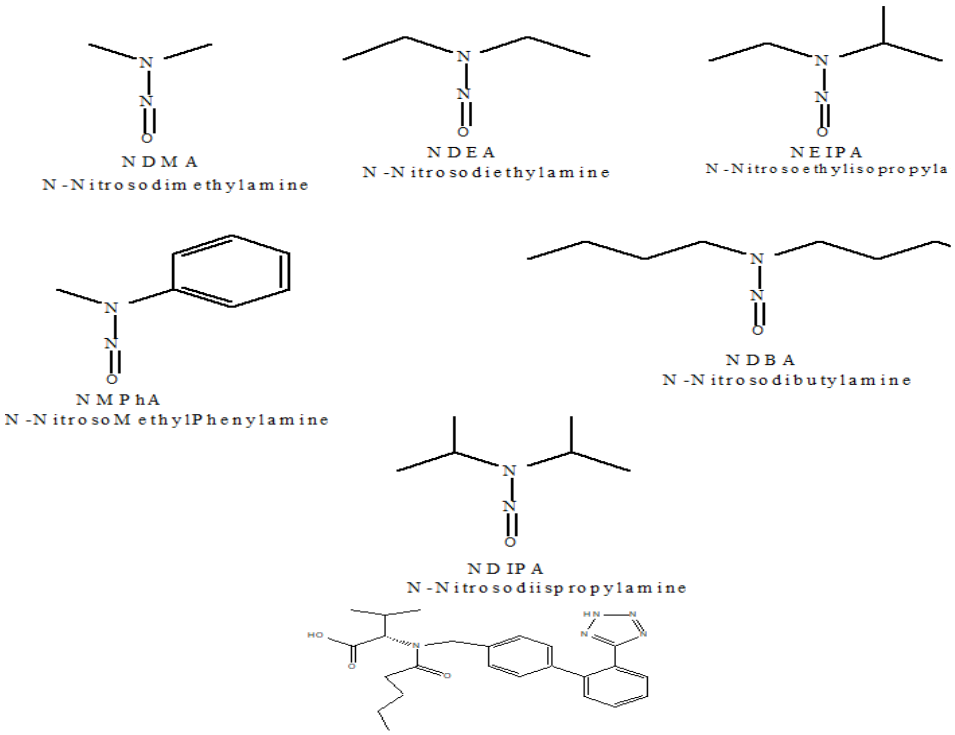

Figure 1: (2*S*)-3-methyl-2-[pentanoyl-[[4-[2-(2*H*-tetrazole-5-yl) phenyl] phenyl] methyl] amino] butanoic acid (Exact Mass: 435.23).

Nitrosamines have now also been identified in ranitidine medications and metformin. To quantify those impurities, there is need a for an analytical method, it should be quantified trace-level impurities in the drug. Therefore, to address this issue, we developed a single method that can detect and quantify six nitrosamine impurities in a single method at a trace level. It was validated in line with regulatory requirements. This method is reproducible and very sensitive.

Some diverse pathways for formation of nitrosamine impurities in pharmaceutical products:

1) Sodium Nitrate and other Nitrates react in the presence of secondary and tertiary amines[1] and form these impurities.
2) Contaminated Raw materials and intermediates from non-qualified vendors.
3) Recovered Solvents, some Catalysts, and Reagents are potential Sources of Contamination.

Limits of acceptable intake for nitrosamine impurities:

FDA recommends below acceptable intake (AI) limits for the six Nitrosamine impurities NDMA, NDEA, NMPA, NIPEA, NMPhA and NDIPA [1]. While determining the limits for Nitrosamine impurities should use the below Acceptable intake limits (Table 01).

Table 1: FDA-approved limits of acceptable intake of Nitrosamine Impurities in (ng/day)

Nitrosamine impurities	Acceptable intake (ng/day) [1]
NDMA	96
NDEA	26.5
NEIPA	26.5
NDIPA	26.5
NDBA	26.5
NMPhA	26.5

If a drug product contains more than one Nitrosamine impurities, The limit for total Nitrosamine impurities is 0.03 ppm or 26.5 ng/day acceptable intake is considered acceptable. hence validation has been performed on all impurities with 26.5ng/day based on the Maximum daily dosage (MDD) of the valsartan drug. MDD of valsartan drug is 320mg/day. the main aim of our research paper is to quantify six potential genotoxic nitrosamine impurities in valsartan drug substance at trace levels. During extensive literature search for analytical methods for the

determination of nitrosamine impurities in valsartan, there were several LC-MS/MS and LC-HRMS methods published by regulatory agencies for nitrosamines determination in Losartan, Irbesartan, Valsartan drugs with a limit of quantification at 0.05 ppm with respect to drug substance concentration [6-8].

MATERIALS AND METHODS

Reagents and chemicals

We have procured LCMS grade solvents and reagents which are good quality and highest purity (>99.8%). Water and methanol were purchased from Honeywell (Charlotte, NC, USA). Other necessary chemicals like formic acid procured from Fluka. Valsartan and six nitrosamines' impurities were procured from Saanvika Pharma limited, Hyderabad, India.

Instrumentation used

Agilent Triple quadrupole with 1290 infinity II UHPLC was used for performing all the analyses for the current study.

Mobile phase preparation

Preparation of mobile phase A: B was done by adding 10mM Ammonium formate in 1 liter Water: Methanol 100%. Diluent 85:15:0.1% v/v/v (Water: Methanol: Formic Acid), Needle wash Water: Methanol (25:75 v/v). performed mobile phase degassing and stored at ambient temperature for further use.

Chromatographic and mass spectrometry conditions:

The chromatographic conditions and mass spectrometry conditions employed for the present study were mentioned in the Table 2.

Table 2: Chromatographic conditions**,** Mass Spectrometry Conditions in APCI +Ve Polarity.

Column and Dimensions:	Poro-shell HPH-C_{18}(150X4.6mmid 2.7 µ).			
Flow Rate/min	0.4ml/min			
Column Temperature:	40 ºC.			
Run Time:	23minitues.			
Flow Mode	Gradient.			
Injection Volume:	20 µL.			
Time (Min)/Conc. of %B	0/1, 8/65, 10/75, 12/80, 13.5/80, 14/95, 19/95, 19.10/1, 23/1			
S.No.	**Compound Name**	**Precursor ion (m/z)**	**Product Ion(m/z)**	**CE(V)**
1	NDMA	**75.1**	**43.1**	**17**
2	NDEA	**103.1**	**47.1**	**16**
3	NEIPA	**117.1**	**47.1**	**16**
4	NMPhA	**137**	**66.1**	**18**
5	NDIPA	**131.1**	**43.1**	**7**
6	NDBA	**159.1**	**41.1**	**24**

Source parameters:

Gas Temperature (ºC)/300 ºC, Capillary Voltage(V)/3000V, Corona Current/4 µA, Dwell time 30ms.Vaporizer(ºC)/350ºC.

Diversion program & chemicals and standards used in the study

After an 18min run time, Flow Path should divert into waste. Six Nitrosamine impurities standards and valsartan drugs are Gifted from Saanvika Pharma, ammonium formate buffer from biosolve, formic acid from biosolve, methanol from JT Baker, we used Sartorius analytical balance for weighing (statistical mode), micropipettes are from Eppendorf research plus grade (100 to 1000µL), milli-Q water from JT Baker, Glassware and volumetric flasks used from Borosil Class-A.

Protocol for preparation of standards and samples for analysis

(1) Preparation of standard stock-01

Accurately weighed and transferred each 3mg of all six Nitrosamine impurities (NDMA, NDEA, NDIPA, NEIPA, NMPhA, NDBA) in 25ml Class-A volumetric flask and added diluent mixed well and made up to the mark with diluent.

(2) Preparation of standard stock-02

Taken 0.5mL(500µL) from standard stock-01 into 100mL class-A volumetric flask and made diluted with diluent up to mark and mixed well.

(3) Standard solution preparation-6ppb (0.08ppm w.r.t test concentration)

Accurately taken 1mL from Standard stock solution-02 into 100mL class-A volumetric flask and made diluted with diluent up to mark and mixed well. This solution is used for system suitability.

(4) Sample solution preparation:

Accurately weighed and transferred 365mg of valsartan drug in 15mL Centrifuge tube added 5mL diluent mixed well by using vortex mixture after extraction centrifuged the sample for 10min at 4000rpm. Filtered the supernatant using a 0.22-micron PVDF syringe filter and took this filtrate sample into HPLC vials for analysis.

Method validation parameters

Specificity:

In specificity, the parameter injected 6ppb standard solution and valsartan sample solution for a retention time of standards and sample.

Linearity solution preparations from LOQ to 150% level (1.5ppb to 9 ppb):

From Standard, stock solution-02 prepared different levels of linearity solutions are (1.5, 3.0, 4.2, 6.0, 7.8, 9.0ppb levels) and injected. Injected 150%level six injection and the remaining all levels are three injections. and six injections from the standard solution as system suitability and system precision parameter. Injected blank after 150% level for carryover test.

LOQ level standard solution preparation (1.5ppb/0.02ppm w.r.t test concentration):

Accurately taken 5ml of Standard solution (6ppb) into 20ml Class-A volumetric flask, added diluent and mixed well, made up to the mark with diluent and injected six injections.

LOD level standard solution preparation (0.5ppb/0.0066ppm w.r.t test concentration):

Accurately taken 3.3ml of LOQ Standard solution into 10ml Class-A volumetric flask added diluent and mixed well, made up to the mark with diluent.

Accuracy at LOQ level three individual preparations:

Accurately weighed and transfer 365mg of valsartan API in a 15mL Centrifuge tube added 5mL of LOQ Standard solution mixed well by using vortex mixture after extraction centrifuged the sample for 10min at 4000rpm. Filtered the supernatant using a 0.22-micron PVDF syringe filter and took this filtrate sample into HPLC vials for analysis.

RESULTS AND DISCUSSION

Chromatographic method development

In our lab-developed single validation method for the detection and quantification of six Nitrosamine impurities namely NDMA, NDEA, NEIPA, NDBA, NDIPA, and NMPhA in valsartan drug by using Ultra-High Performance Liquid Chromatography with Triple Quadrupole (tandem Quadrupole) Mass Spectrometry (UHPLC-MS/MS) with APCI source in +Ve (Positive) mode with MRM Scan, parameters and obtained results were discussed in the below sections.

Various mobile phase pH conditions and gradient conditions were evaluated to achieve good peak shapes and critical separation between valsartan and all the six nitrosamine impurities and finally 10mM Ammonium formate in water as mobile phase A given the better peak shapes and sensitivities. Few of the columns tested for separation between impurities and valsartan. Coelution observed between critical pair valsartan and NDBA when tried initially with Poroshell HPH 150 × 4.6 mm 2.7 µ column. Both the methanol and acetonitrile were evaluated for mobile phase B and concluded with 0.1% formic acid in Methanol due to better separation efficiency. Different flow rates were tried and concluded with 0.3 ml/min. Column temperature was concluded with 40˚C after evaluating different temperatures to achieve the separation. The retention times of impurities N-Nitroso dimethyl amine (NDMA), N-Nitroso-N-methyl-4-aminobutyric acid (NMBA), N-Nitroso diethyl amine (NDEA), N-Nitroso Ethyl Iso propylamine (NEIPA), N-Nitroso diisopropylamino (NDIPA) and N-Nitroso dibutyl amine (NDBA) were observed to be 4.75, 7.37, 8.85, 9.72, 10.45 and 12.25 min respectively and telmisartan eluted at 11.158 min. Injection volume was optimized to 20 µl. Representative chromatograms for standard and spike samples at 0.2 ng/ml (LOQ) with all six Nitrosamine impurities provided in (figure 02 & 03) and (table-03) Hence proven method can quantify from 1.5ppb level of impurities.

Specificity parameter:

In the specificity parameter, there is no interference of six Nitrosamine impurities was observed concerning blank and API. The retention time of NDMA-6.55min, NDEA-10.17min, NEIPA-11.57min, NMPhA-13.07min, NDIPA-12.76min, NDBA-15.82min, and Valsartan drug peak observed at 19.13min in UV absorbance. And the depicted trace levels of impurities in LOD areas were tabulated in (table 04) and as per the detected levels this method was proven as specific.

LOQ parameter and precision at LOQ:

In the LOQ parameter obtained percentage RSD of all six impurities of 6 injections precision is Not more than 15.0 and the results are tabulated below. And can quantify six impurities from 1.5ppb level in this method. this method was precise at LOQ level Concentration. The system was precise at the specification level. (Table 06) Hence proven method can quantify from 1.5ppb level of impurities. Hence proven method can quantify from 1.5ppb level of impurities.

Linearity and range (1.5 to 9ppb):

In the linearity parameter, from LOQ to 150% level the correlation coefficient and R^2 of six Nitrosamine impurities were obtained Not less than 0.999 and 0.997 respectively. and the range was performed at LOQ and 150% level of specification and met regulatory requirements. No carryover was observed after the 150% level (figure-04 & 05).

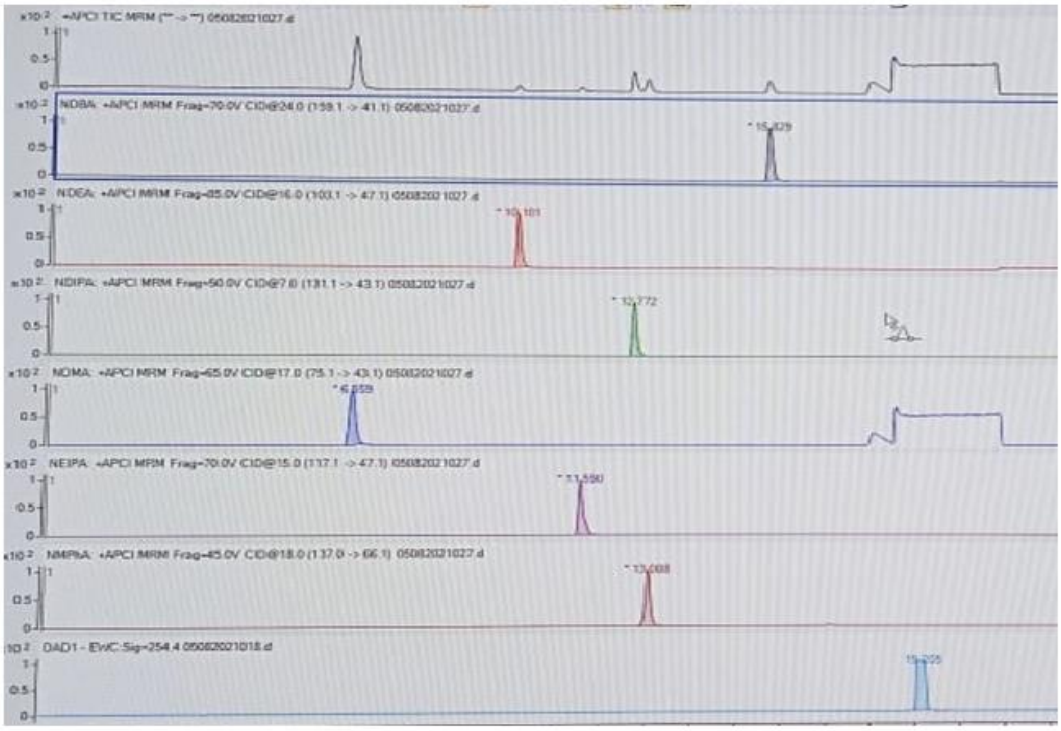

Figure 2: LOD Parameter: in LOD parameter all six Nitrosamine peaks was detected and areas and S/N ratio tabulated Below, and in this method can detect 0.5ppb level impurities

Table 3: LOD parameters

Standards	NDMA	NDEA	NEIPA	NDIPA	NMPhA	NDBA
LOD areas	16884	938	956	4681	2930	2790
S/N Ratio	20.98	11.90	36.38	30.89	26.37	21.31
Conc. of Solution(ppb)	0.5	0.5	0.5	0.5	0.5	0.5

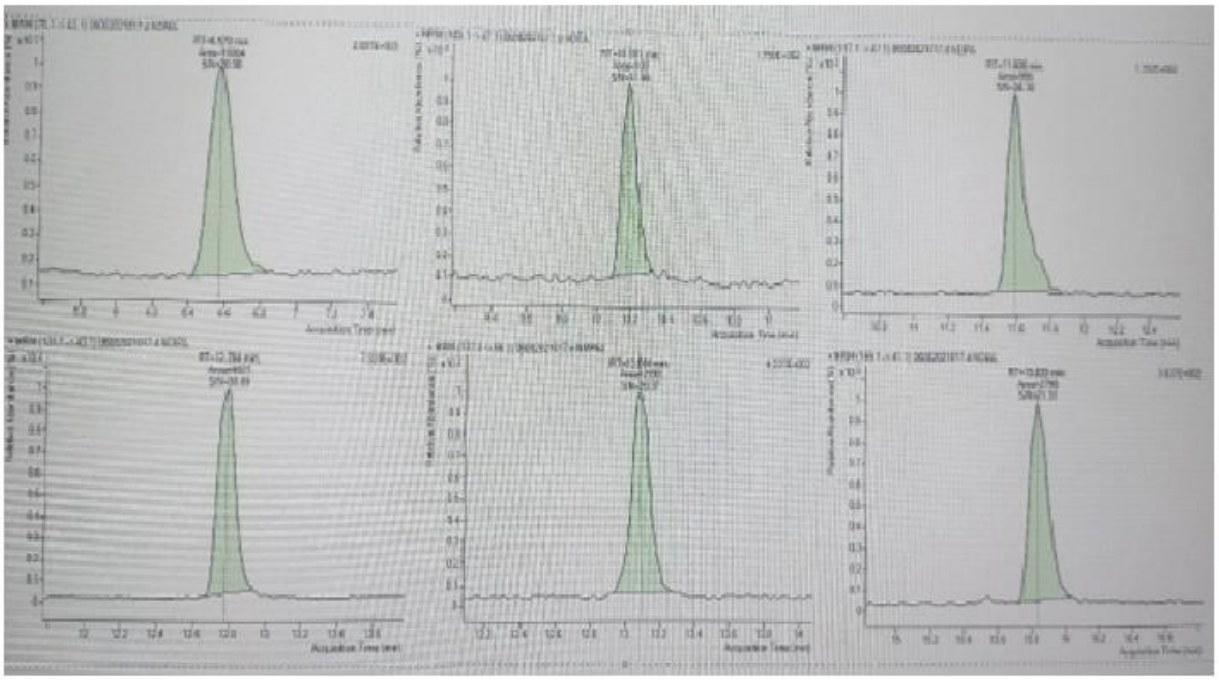

Figure 3: LOQ Parameter and Precision at LOQ**:** In LOQ parameter obtained percentage RSD of all six impurities of 6 injections precision Not more than 15.0 and results are tabulated below. And can quantify six impurities from 1.5ppb level in this method. this method was precise at LOQ level Concentration. System was precise at specification level. Hence proven method can quantify from 1.5ppb level of impurities.

Table 4: LOQ standards

LOQ Standards	NDMA	NDEA	NEIPA	NDIPA	NMPhA	NDBA
Injection-1/6	48831	2858	2727	13613	8825	7989
Injection-2/6	48848	2804	2824	13636	8905	8131
Injection-3/6	49513	2921	2744	13823	8950	7912
Injection-4/6	49686	2728	2816	14130	8872	7782
Injection-5/6	50397	2764	2865	14049	8928	8345
Injection-6/6	50078	2786	2870	13995	9049	7901
Average	49559	2810	2808	13874	8922	8010
RSD	1.28	2.47	2.14	1.57	0.86	2.50
S/N Ratio	54.30	39.23	83.18	68.56	71.69	34.68
Conc. of Solution(ppb)	1.5ppb	1.5ppb	1.5ppb	1.5ppb	1.5ppb	1.5ppb

Table 5: depicted trace levels of (0.5ppb) of impurities in LOD areas

Standards	NDMA	NDEA	NEIPA	NDIPA	NMPhA	NDBA
LOD areas	16884	976	963	4982	2926	2895
S/N Ratio	20.98	11.90	28.51	54.65	26.37	21.31
Conc. of Solution(ppb)	0.5	0.5	0.5	0.5	0.5	0.5

Table 6: Our single method can quantify from 1.5ppb level of impurities.

LOQ Standards	NDMA	NDEA	NEIPA	NDIPA	NMPhA	NDBA
Injection-1/6	49537	2974	2727	13966	8877	8064
Injection-2/6	48945	2919	2798	14053	8885	8318
Injection-3/6	49581	3003	2743	14196	8850	8047
Injection-4/6	49956	2782	2792	14457	8806	8037
Injection-5/6	50397	2877	2844	14330	8911	8367
Injection-6/6	50080	2917	2870	14365	8984	8148
Average	49749	2912	2796	14228	8886	8164
RSD	1.02	2.67	1.98	1.34	0.68	1.78
S/N Ratio	54.30	39.23	83.18	139.02	71.69	84.68
Conc. of Solution (ppb)	1.5ppb	1.5ppb	1.5ppb	1.5ppb	1.5ppb	1.5ppb

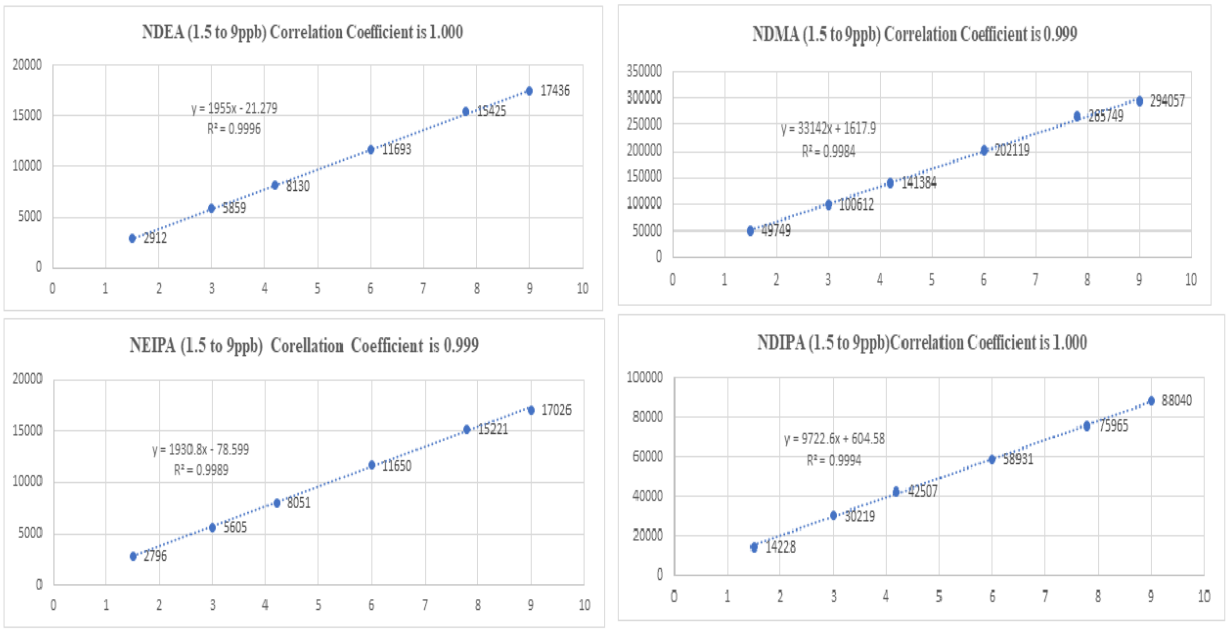

Figure 4: NDEA, NDMA NEIPA NDIPA impurities detection with obtained ppb levels were 1.5 to 9ppb with a correlation coefficient value.

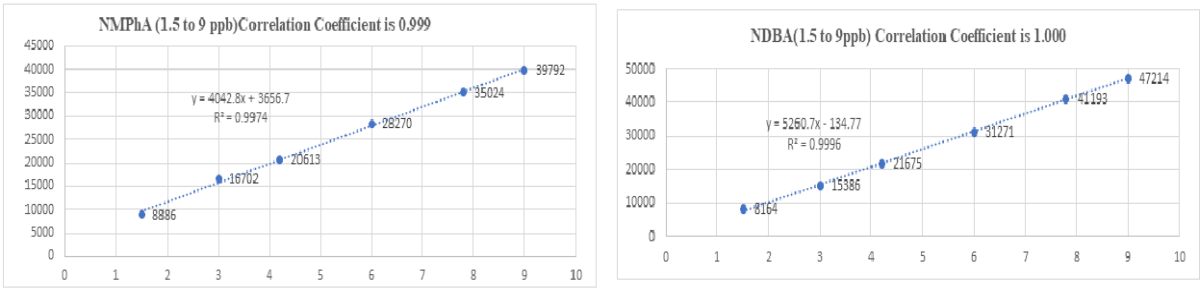

Figure 5: NMPhA and NDBA impurities detection obtained ppb levels were 1.5 to 9ppb with a correlation coefficient of 0.999 and 1.000

Table 7: Accurate and précised LOQ levels

Recovery	NDMA	NDEA	NEIPA	NDIPA	NMPhA	NDBA
Spiked solution-01	102.92	99.11	100.75	104.17	99.77	100.73
Spiked solution-02	102.77	102.78	104.69	101.60	97.50	100.72
Spiked solution-03	101.88	100.62	102.83	103.51	96.64	101.15

Accuracy (recovery at LOQ level):

In this method, we achieved recovery at the LOQ level three different preparations are in between 80 to 120% results are tabulated below. At 1.5 ppb/0.02 ppm w.r.t test level concentration recovery was obtained within regulatory requirements**.** (Table-07**)** Hence proven method was accurate and précised at LOQ level concentration.

Robustness:

To assess the robustness of our developed single method, we optimised different method conditions such as mobile phase flow rate and column temperatures were changed purposefully. And optimised changes for flow rate of the mobile phase was 0.3 mL/min and it got changed to 0.27 to 0.34 ml/min. and column temperature has been shifted to 36˚C and 44˚C (altered by 4.0˚C). these changes do not show any impact on chromatographic performance of all the six impurities due to the mentioned changes proving the robustness of the method.

Repeatability and solution stability:

our single validation method was further examined for repeatability at a limit of quantification by injecting all six replicate injections at 02.ng/ml (0.005 ppm) and mixture of six impurities and RSD% has been calculated. LOQ acceptance criteria is less than 20%, and the obtained RSD values for all six impurities were less than 4% and all are coming within the accepted criteria range. Valsartan solution stability and six impurities was examined by placing both spiked and un-spiked samples at 25˚C for 24 h alongside freshly prepared standard solutions. We did not observe any significant changes for all six impurities; hence it is confirming that the stability of impurities in sample solutions for at least 24 hours.

CONCLUSION

In summary, the single development and validation method by using Ultra High Performance Liquid Chromatography with Tandem Quadrupole Mass Spectrometry (UHPLC-MS/MS), this method was very sensitive for the analysis of six Nitrosamine impurities in a single method for the valsartan drug substances which is far sensitive than the currently available detection methods which have been published for nitrosamines by employing (UHPLC-MS/MS), in several drug substances, and it requires separate tests for all these six impurities, and there is no single method available to quantify nitrosamines in valsartan using LC-MS/MS till to date, only we could present in this work. (UHPLC-MS/MS) technique allowed us to quantify a maximum number of impurities compared to other detection techniques like LC & GC MS/MS, these methods have limitations to ionize impurities like NMBA impurity. We validated (ICHQ2(R1)) this method in line with regulatory requirements (ICH M7(R1)). We are successfully able to prove specificity, precision, LOD and LOQ, Accuracy at the LOQ level, Linearity and Range, carryover, and all validated parameters within regulatory acceptance criteria. Hence it is suitable to use the intended purpose of the valsartan drug. This method can use for other drugs (as per regulatory limits) but should prove specificity and accuracy for corresponding drugs.

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