Original Article
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DEVELOPMENT OF GC-MS/MS METHOD FOR SIMULTANEOUS ESTIMATION OF FOUR NITROSOAMINES GENOTOXIC IMPURITIES IN VALSARTAN

VALSARTAN'DA DÖRT NITROSOAMIN GENOTOKSIK SAFSıZLıĞıN EŞZAMANLı TAHMINI IÇIN GC-MS/MS YÖNTEMININ GELIŞTIRILMESI

GC-MS/MS ANALYSIS OF NITROSOAMINES IMPURITIES IN VALSARTAN
VALSARTAN'DA NITROSOAMIN SAFSıZLıKLARıNıN GC-MS/MS ANALIZI

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ABSTRACT
Objectives: Recently, N-nitrosamines were unexpectedly detected in valsartan and other generic sartan products. Taking into this account, we developed sensitive & stable multiple reactions monitoring mode-based "GC-MS/MS" approach for the quantification of "four N-nitrosamines" in valsartan, especially, N-nitrosodiisopropylamine, N-nitroso ethyl isopropylamine, N-nitrosodiethylamine, and N-nitrosodimethylamine.
Materials and Method: Gas chromatography as well as mass spectrometry conditions, were optimized. With the parameter's specificity, sensitivity, linearity, precision, and accuracy. The approach was validated as per the ICH: "International Council for Harmonization" recommendations.
Results: The identification limits and limits of quantification of N-nitrosamines in valsartan varied between 0.02 to 0.03 ppm, and 0.06-0.09 ppm respectively. The obtained values were satisfactory with limits established by United States Food and Drug Administration for sensitivity requirements. The regression coefficients over 0.999 for four N-nitrosamines in the calibration curve demonstrated the strong linearity of the process. The retrievals of "N-

nitrosamines” in valsartan between 91.9 - 122.7 percent. For the intra-day as well as inter-day accuracy studies, the RSD (“relative “standard deviation”)” was less than 9.15 %.
Conclusion: The proposed approach had rapid analysis capability, high precision, accuracy, and good sensitivity, which give a reliable approach for N-nitrosamines quality control in valsartan.

Keywords: GC-MS/MS, Valsartan, N-nitrosamine genotoxic impurity.

Soyut

Hedef: Son zamanlarda, valsartan ve diğer jenerik sartan ürünlerinde beklenmedik bir şekilde N-nitrozaminler tespit edildi.Bu hesabı dikkate alarak, valsartandaki "dört N-nitrozamin" in niceliği için hassas ve kararlı çoklu reaksiyon izleme modu tabanlı "GC-MS/MS" yaklaşımı geliştirdik, özellikle N-nitrosodiisopropylamin, N-nitroso etil izopropylamin, N-nitrosodiethylamine ve N-nitrozodimethylamine.
Malzemeler ve Yöntem: Gaz kromatografisi ve kütle spektrometresi koşulları optimize edilmiştir. Parametreleri ile spektrometre, hassasiyet, doğrusallık, hassasiyet ve doğruluk.yöntem, Uluslararası Uyum Konseyi yönergelerine uygun olarak doğrulanmıştır.
Sonuçlar : Valsartandaki N-nitrozaminlerin tespit ve nicelik sınırları sırasıyla 0,02 ila 0,03 ppm ve 0,06-0,09 ppm arasında değişmektedir.Elde edilen değerler, Amerika Birleşik Devletleri Gıda ve İlaç İdaresi tarafından duyarlılık gereksinimleri için belirlenen limitlerle tatmin ediciydi.Kalibrasyon eğrisinde dört N-nitrozamin için 0.999'un üzerindeki regresyon katsayıları yöntemin iyi doğrusallığını gösterdi.Valsartan'daki N-nitrozaminlerin geri kazanımları % 91.9 ile % 122.7 arasında değişmektedir. Gün içi ve gün içi hassas çalışma için göreli standart sapma %9,15'ten azdı.
Sonuç: Önerilen yöntem, valsartandaki N-nitrozaminlerin kalite kontrolü için güvenilir bir yöntem sağlayan iyi hassasiyet, doğruluk, yüksek hassasiyet ve hızlı analiz yeteneğine sahipti.
Anahtar Kelimeler: Valsartan, N-nitrozamin genotoksik safsızlık, GC-MS/MS.

INTRODUCTION

In the production process of the APIs: “active pharmaceutical ingredients”, impurities are incorporated through various sources, like catalysts, solvents, reagents, intermediates, starting materials as well as by-products. When compared to other impurities, genotoxic impurities (GTIs) are of a special kind that could inspire mutations at the genetic level, which leads to chromosomal breakage and rearrangements, and even present in low concentration have increased the risk of cancer.[1] Taking into account the toxic effects of genotoxic impurities (GTIs) the international regulatory bodies had set an exposure limits threshold for GTIs, specifically, 1.5 µg/day for long-run therapy with greater thresholds of clinical shorter intervals. As these GTIs are present in very low concentrations, the pharmaceutical industries are facing an uphill task to develop robust analytical, sensitive, and high efficient methods for their determination.[2,3]
Valsartan belongs to the category of antihypertensive drug which selectively inhibits angiotensin receptor type II. It is used to treat mild to moderate essential hypertension. The angiotensin-II mediated unwanted effects are reduced to a significant extent by valsartan. Recalls for valsartan

were issued between mid-to-late 2018. The cause of the recalls was due to the detection of genotoxic impurities (GTIs) such as NDEA: "N-nitrosodiethylamine" or NDMA: "N-nitrosodimethylamine" in valsartan in unacceptable limits.[4] The nitrosamine impurities have been produced during the drug substance synthesis in which the sodium azide, which is applied in the production of the tetrazole moiety was eliminated using sodium nitrite and later under acidic circumstances would form nitrous acid, a powerful nitrosylating agent. The dimethylamine and diethylamine which might be present as impurities in dimethylformamide (DMF) may N-nitrosylated in the synthesis might result in the formation of NDMA and NDEA. Likewise, in certain production processes for valsartan, the reagent triethylamine can degrade to produce diethylamine, and latter N-nitrosylated to produce NDEA. N-nitroso compounds are included in the "cohort of concern" as they exhibit great carcinogenic potency.[5,6] The appropriate regular intake limits for NDEA, NDMA, and other impurities in some products were published by FDA. The limit of N-Nitrosoethylisopropyl amine (NEIA) and N-Nitrosodiisopropyl amine (NDIPA) is 96 ng/day.

Recently, for detecting N-nitrosamines in water, food, and personal care products, the analytical techniques widely used are GC[7-18], LC[19,20] and supercritical fluid chromatography-tandem mass spectrometry .[21] FDA has determined the interim appropriate regular intake levels for N-nitrosamines in valsartan (Table 1) and employed GC-MS/MS by using liquid injection as well as headspace,[22-23] Rapid Fire-MS/MS,[24] and HPLC-HRMS[25] for quantitation of the N-nitrosamines in valsartan.

In the analysis of water, food, personal care items the extraction, as well as purification measures, are critical and important. However, in the pharmaceutical industry, these were much not much applicable. Liu et al reported a GCMS/MS approach for the detection of four "N-nitrosamines" in valsartan,[26] but the method suffers from the drawback of long run time and less accuracy. So, we established through direct injection for quantification of four "N-nitrosamines", a simple, sensitive, precise, and rapid GCMS/MS approach for valsartan. The LODs and LOQs values were at acceptable limits as per the sensitivity requirements set by FDA. Proposed GCMS/MS approach validation was performed as per the ICH guidelines.

MATERIALS AND METHOD

Chemicals and materials

Valsartan drug was found as a gift sample by the local pharma industry. NDMA (purity ≥ 98.3%), NDEA (purity ≥ 100.0 %), NDIPA (purity ≥ 99.9%), and NEIA (purity ≥ 99.3%) standards were acquired by Sigma Aldrich. The solvents used methanol, acetonitrile, ethyl acetate, 1-methyl-2-pyrrolidinone all HPLC-grade were purchased from Merck Ltd India, Mumbai.

Instrumentation and optimized GC-MS/MS conditions

The Agilent "7890B" "gas chromatography"-tandem mass spectrometry equipped with an "Agilent 7693A" auto sampling device and 7697 a Headspace Sampler is examined using N-nitrosamines. The analytical column used was DM-WAX ("30 m x 0.25 mm, 0.5 µm"). This detection was conducted at a 7000C "triple quadrupole mass spectrometer" consist of EI: "electron ionization" ion source. The temperature programming in the GC oven was done by maintaining oven temperature of 70 °C for 4 minutes, which first elevated at 20 °C·min^{-1} to 240

°C and maintained for 3 minutes. The run interval was fixed for 10 minutes. The flow rate was 3.0 mL/min for helium as the carrier gas. The injection temperature, as well as injection interface, were maintained at 240 °C. The volume of injection in the split mode (1:2) was 1 µL. The MS was run at 70 eV in EI mode with a 150°C "quadrupole temperature". The ion source has adjusted to a temperature of 230 °C. It was 4 minutes to delay the solvent. The data recovery mode for the quantitative estimation of "four N-nitrosamine GTIs" was chosen as the MRM: "Multi Reaction Monitoring" mode. Table 2 provides a summary of the data for precursor ions, productions as well as enhanced CE: "collision energy" for four N-Nitrosamine GTIs.

Preparation of sample and standard solutions

The standard stock solutions of 1 mg/mL concentration of NDMA, NDEA, NEIA, and NDIPA were prepared through dissolving weighed reference standards in 1-methyl-2-pyrrolidinone, respectively, and stored in 4 °C. A sequence of standard working solutions of NDMA at the levels of 0.093, 0.155, 0.232, 0.309, 0.387, and 0.464 ppm (µg/g API) in 1-methyl-2-pyrrolidinone was found from a stock solution via the serial dilution process. The sequence of NDEA standard working solution was concentrated in 0.062, 0.154, 0.231, 0.308, 0.384, and 0.461 ppm(µg/g API), respectively. The working solution concentrations for NEIA were 0.090, 0.150, 0.224,0.299, 0.374 and 0.449 ppm (µg/g API), respectively. The concentration of NDIPA were 0.088, 0.146, 0.220, 0.293, 0.366 and 0.439 ppm(µg/g API). Here 1 ppm corresponds to 0.25 µg/mL of NDMA, NDEA, NEIA, and NDIPA respectively. At 250 mg/mL concentration Valsartan was prepared. A mixed standard solution of NDIPA, NEIA, NDEA, and NDMA was prepared from standard stock solution after subsequent dilution with 1-methyl-2-pyrrolidinone to obtain the concentration within the linearity range. The resulting mixture was sonicated for 30 min and kept in a centrifuge tube around 1 minute before being centrifuged for 10 minutes at 2500 rpm. The supernatant was passed to the chromatography injection vial through the 0.22 µm "nylon syringe" filter.

Method validation

The developed "GC-MS/MS" approach with MRM mode for the detection of four N-nitrosamines was validated for parameters, like solution stability, precision, accuracy, LOQ, LOD, linearity, sensitivity, specificity, and system suitability. For LODs "signal-to-noise" (S/N) ratio was 3, as well as LOQs, were S/N = 10. For the accuracy of the proposed method, the recovery studies were carried out for evaluation. The precision studies were evaluated by inter-day and intra-day RSDs of six specimens spiked across 3 continuous days at a single concentration.

RESULTS & DISCUSSION

Method development

Selection of solvent

Considering the trace level of N-nitrosamine genotoxic impurities NDMA, NDEA, NEIA, and NDIPA in valsartan and solubility parameter, 1-methyl-2-pyrrolidinone was selected as a solvent for the preparation of solutions for GC-MS/MS analysis.

Capillary Column Selection

Under the given temperature program three different capillary columns were tried to obtain the best chromatographic separation. The columns were: HP-35MS, HP-5MS, and DM-WAX in the order of increasing polarity. A 10 µg/mL normal blend was inserted in all three cases. The identification of the compounds was dependent on the spectra of EI utilizing the "National Institute of Standards and Technology" (NIST) library. Liu et al. Reported separation of four nitrosamines using DB-WAXetr capillary column without the inclusion of NDEA. We have used the DM-WAX column, which has sufficient polarity to isolate all nitrosamines with better peak shapes, resolution, less analysis time with applicable for the analysis of most polar and volatile compounds, such as NDMA and NDEA.

Mass Spectrometry

In the analysis of pharmaceuticals, the most crucial aspect is the trace detection method for GTIs. Considering the sensitivity criteria, the MRM mode is superior to SIM mode for the quantification of N-nitrosamines. Consequently, the MRM mode was used to quantify four N-nitrosamine GTI in valsartan as the MS approach. The mass spectra of valsartan and four N-nitrosamine GTIs with chromatograms are shown in Fig 3.

Method validation

The suggested approach for "four N-nitrosamine GTIs" was validated as per the International Council for Harmonization recommendations.

Specificity

For the specificity of the recommended approach, 1-methyl-2-pyrrolidinone, the valsartan matrices, and mixed standards of four N-nitrosamines were undergone by the "GC-MS/MS" examination. Figure 2 clearly indicates the retention times of four N-nitrosamines no interference peaks from the 1-methyl-2-pyrrolidinone, and the valsartan matrices were observed, indicating the specificity of the approach for the detection of four N-nitrosamines in valsartan.

Linearity and sensitivity

The data for linearity, LOQs, and LODs outcomes were summarized in Table 3. The standard curve ("y = Ax + B", in which A signifies the slope & B signifies the intercept) was obtained by plotting the chromatographic peak area (N-nitrosamines, y) to normal N-nitrosamines (x)concentrations. To validate the linearity of the approach six standard concentrations were used. The regression coefficients (R^2) of the standard curve for four N-nitrosamines were > 0.99 in the given concentration range, indicates better linearity and appropriate for quantitative examination. Therefore, based on LODs and LOQs, the sensitivity of the method was evaluated. In Table 3, the LODs and LOQs for NDEA, NEIA, NDIPA & NDMA in 1-methyl-2-pyrrolidinone were presented. These low values of LOQs & LODs for this "GC-MS/MS" approach were acceptable and suitable to detect N-nitrosamines in valsartan.

Accuracy

The accuracy of the method was estimated from the recovery results of four N-nitrosamines. To evaluate the output of the recommended approach, improvements of four N-nitrosamines were

determined after Valsartan samples spiked with 3 separate levels of four N-nitrosamines at 50 % (NDIPA-0.146 ppm, NDEA- 0.154 ppm ,NDMA- 0.155 ppm, NEIA-0.150 ppm), 100 % (NDIPA-0.293 ppm, NDEA- 0.308 ppm ,NDMA- 0.309 ppm, NEIA-0.299 ppm) and 150 % (NDIPA-0.439 ppm, NDEA- 0.461 ppm , NDMA- 0.464 ppm, NEIA- 0.449 ppm) of the limits, respectively. The recoveries for NDIPA, NDEA, NDMA, and NEIA in valsartan in the range of 87.68 to 122.75 %. Considering the ultra-trace essence of the study, the recovery of N-nitrosamines was found in the acceptable range of 70-130 %, indicating the accuracy of the proposed method for N-nitrosamines.

Precision

To study the method precision, the inter-day & intra-day accuracy were performed. The intra-day precision measurements were carried out by comparison of the "standard deviation" of the recovery proportions of the spiked specimens analyzed on the same day. For inter-day accuracy, spiked samples were tested for three distinct days. The intermediate precision was evaluated by results from the study on a different day with different analysts and with freshly prepared solutions. As reviewed in Table 5, this "GC-MS/MS" approach demonstrated acceptable % RSD values for the inter-day, intraday precision as well as intermediate accuracy was in between 1.45 to 6.38 %, 2.88-9.15 %, and 2.8-3.7 %.

Stabilities of four N-nitrosamines in 1-methyl-2-pyrrolidinone

To study the four N-nitrosamines solution stabilities in 1-methyl-2-pyrrolidinone, 0.3 ppm standard solutions were prepared and analyzed every 4 hours to a recently prepared standard. Each solution was placed at 25 °C in the dark. The recovery percentage of N-nitrosamines from these stock solutions were in between 97.51 to 105.04 percent, and the differential recoveries of N-nitrosamines at 0 hours & 24 hours at just 10 %, indicate the stability of stock solution at least 24 hours.

Applications in samples

This "GC-MS/MS" process was utilized in the determination of "four N-nitrosamine GTIs" in four batches of commercial valsartan-containing products & none of the four "N-nitrosamines" were observed in four batches of the commercially available formulation.

CONCLUSION

A simple and sensitive MRM mode-based GC-MS/MS approach was created for the estimation of four genotoxic impurities i.e NEIA, NDIPA, NDEA, and NDMA in Valsartan. The reported GC-MS/MS approach demonstrates satisfactory sensitivity & selectivity. The run time was under 10 minutes. The results of four N-nitrosamines in LOQs & LODs ranged 0.06-0.09 ppm and 0.02-0.03 ppm correspondingly, indicating the suitability of four N-nitrosamines in valsartan for sensitive quantification.

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Table 1: Interim limits for NDMA and NDEA in valsartan set by FDA

Drug	Maximum daily dose (mg/day)	Acceptable intake NDMA (ng/day)	Acceptable intake NDMA (ppm)	Acceptable intake NDEA (ng/day)	Acceptable intake NDEA (ppm)
Valsartan	320	96	0.3	26.5	0.083

Table 2 :Multiple reactions monitoring (MRM) transitions and optimized collision energy for four N-nitrosamine GTIs

Analyte	Precursor→Product(m/z)	Dwell Time (ms)	Collision Energy(eV)
N-Nitrosodimethylamine (NDMA)	74→44	200	5
N-Nitrosodiethylamine (NDEA)	102→85	200	5
N-Nitroso ethylisopropyl amine(NEIA)	116→99	200	5
N-Nitroso diisopropyl amine(NDIPA)	130→88	200	5

Table 3 : Calibration curves, LODs, and LOQs for four N-nitrosamines

Analyte	Linearity Range(ppm)	Regression equation	R^2	LOD(ppm)	LOQ(ppm)
NDMA	0.093-0.464	Y=60227X-444.56	0.9985	0.03	0.09
NDEA	0.062-0.461	Y=13714X-146.75	0.9984	0.02	0.06
NEIA	0.09-0.449	Y=28067X-432.89	0.9959	0.03	0.09
NDIPA	0.088-0.439	Y=13714X-146.75	0.9984	0.03	0.09

Table 4: Accuracy data of four nitrosoamines

Analyte	Valsartan Concentration (mg/mL)	Mean % Recovery at 50 % level ± SD	Mean % Recovery at 100 % level ± SD	Mean % Recovery at 150 % level± SD
NDMA	250	103.21 ± 0.23	101.15 ± 0.98	102.29 ± 1.89
NDEA		91.93 ± 1.75	87.68 ± 0.76	101.6 4± 0.49
NEIA		114.42 ± 0.31	112.21 ± 1.38	117.83 ± 1.66
NDIPA		111.41 ± 1..73	113.62 ± 0.99	122.75 ± 0.26

Table 5: Precision results of four nitrosoamines

Drug(API)	Analyte	Con.(ppm)* (µg/g API)	System Precision (% RSD)	Method Precision (% RSD)		Intermidiate Precision (% RSD)	
				Interday	Intraday	Analyst I	Analyst II
Valsartan (250 mg/mL)	NDMA	0.309	6.43	1.44	1.37	2.53	2.74
	NDEA	0.308	8.52	3.46	3.83	4.28	3.83
	NEIA	0.299	7.02	2.26	2.69	6.35	5.97
	NDIPA	0.293	9.21	2.79	2.93	6.41	7.27

*1 ppm corresponds to 0.25 µg/mL of NDMA, NDEA, NEIA, and NDIPA respectively.

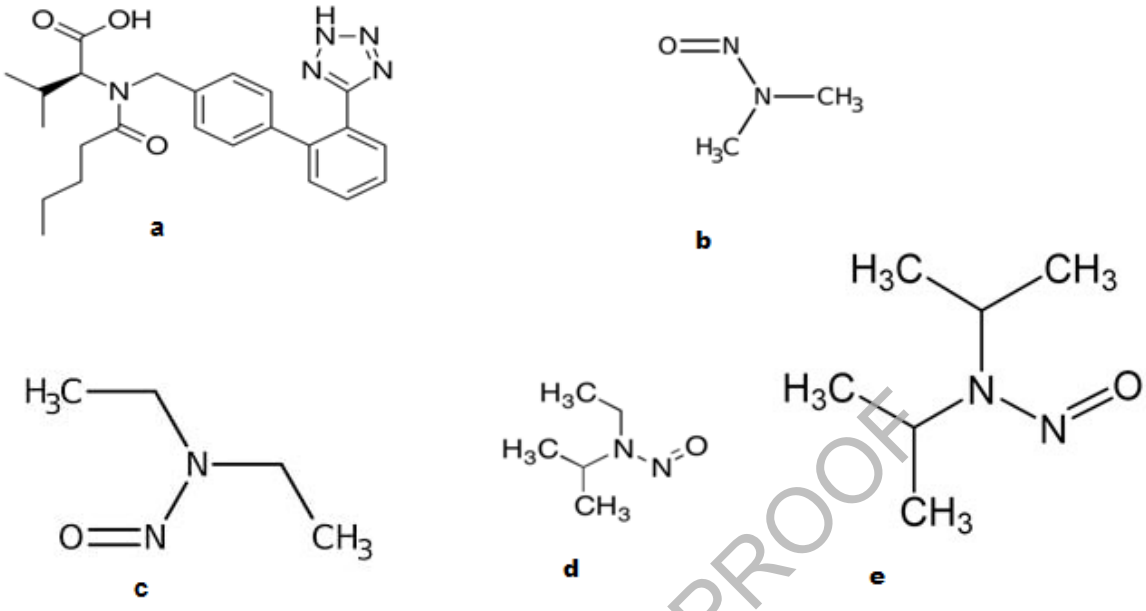


Fig 1 : a) Structure of valsartan b) N-Nitrosodimethylamine (NDMA) c) N-Nitrosodiethylamine (NDEA) d) N-Nitroso ethylisopropyl amine (NEIA) e) N-Nitroso diisopropyl amine (NDIPA)

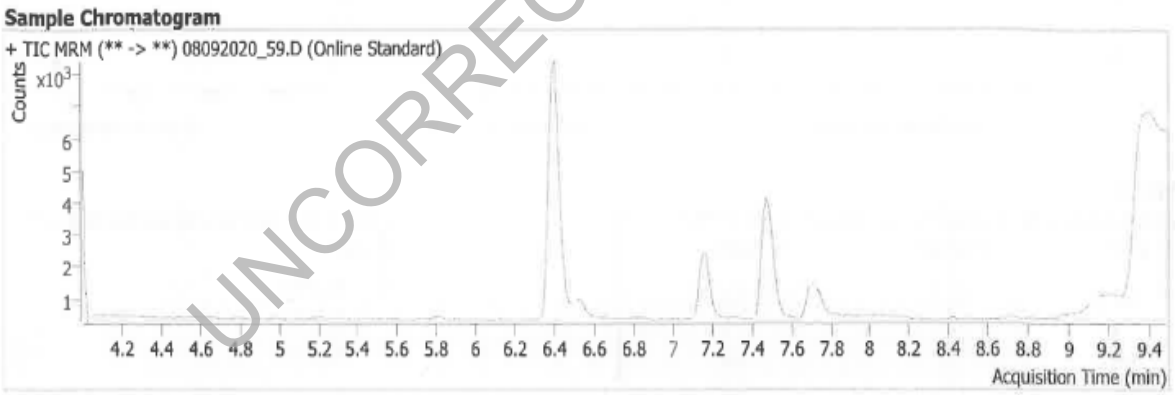
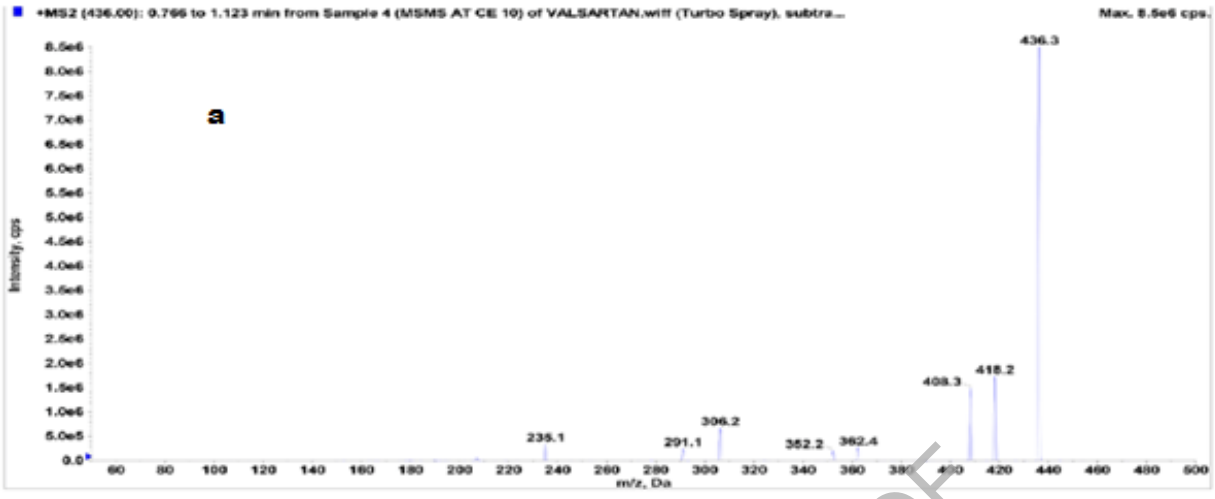
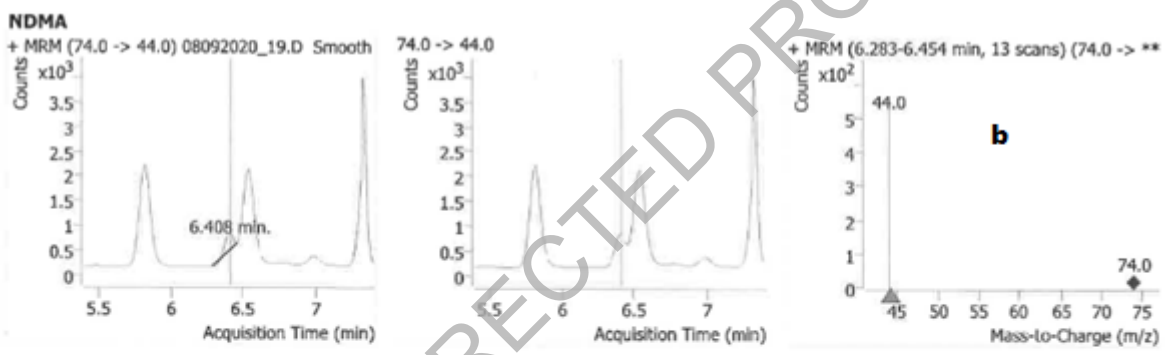
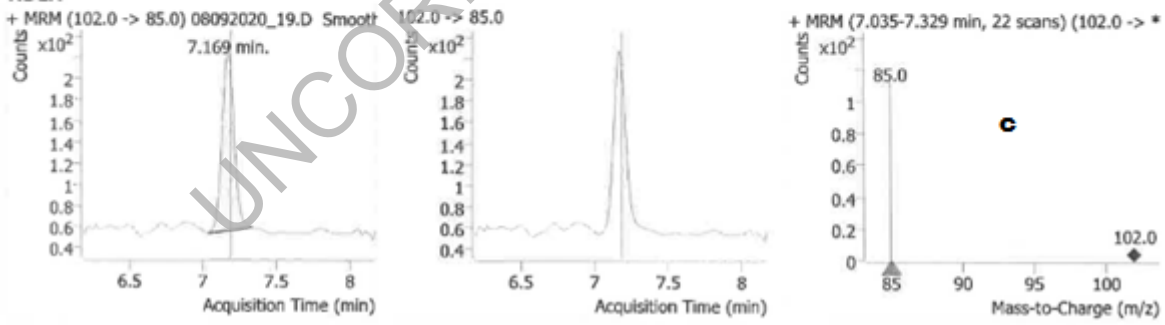


Fig 2 : GC chromatograms of mixed standard solution of N-nitrosamines







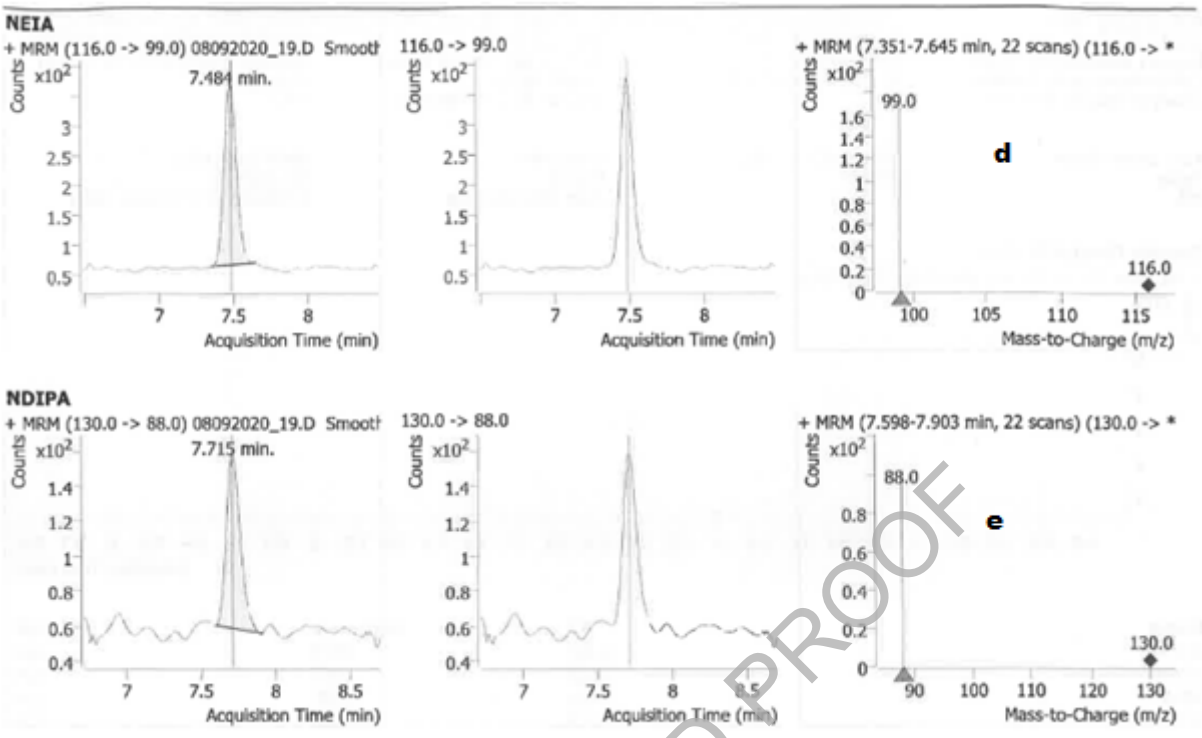


Fig 3 : a) Mass specrtra of valsartan b) N-Nitrosodimethylamine (NDMA) c) N-Nitrosodiethylamine (NDEA) d) N-Nitroso ethylisopropyl amine (NEIA) e) N-Nitroso diisopropyl amine (NDIPA)

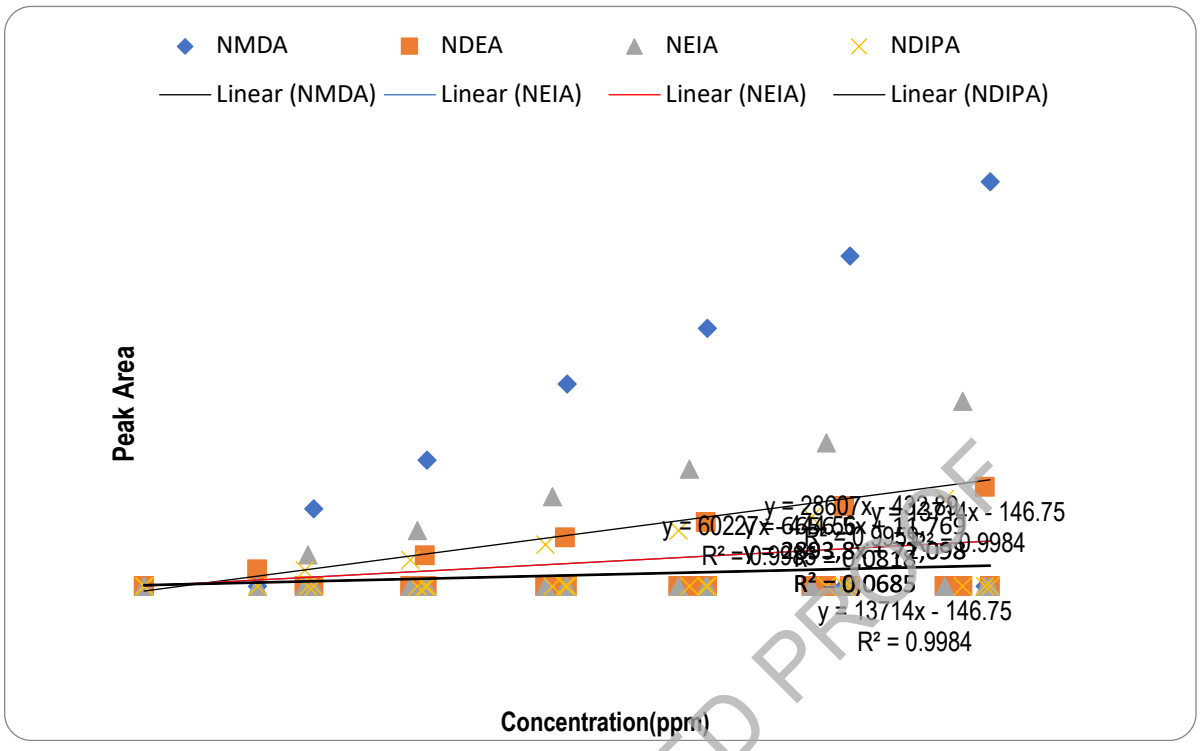


Fig 4: Calibration curve of four N-nitrosamines