

# Risk assessment of amine-based anti-hypertensive drugs for the possible presence of N-nitroso dimethyl amine by a verified GC-MS method

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**Abstract:** Recently recalled drugs by FDA were analyzed in common features and most of them had nitrosamine moiety. found to contain amine group in their structure. Their long term use make them susceptible for different diseases especially cancer. Due to the presence of N-nitrosamine moieties in these drugs, possible carcinogenicity can be induced. To screen out this assumption a study was designed for commonly used two brands of anti-hypertensive drugs containing above features. Most commonly used brands of these drugs from market were collected and analyzed for one of the potential carcinogenic moiety N-nitroso dimethyl amine by FDA laid down procedure on GC-MS-HS. After verification of the method results of the study have shown that the selected brands of the two drugs do not contain NDMA which was further analyzed and verified by spiking the samples with trace amount of NDMA standard. Results of the three brands of sample have shown that the amount after spiking was within the limit by FDA and safe to be used for respective patients.

**Keywords:** Anti-hypertensive, Cancer, GCMS-HS, NDMA, Nitrosamines

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## INTRODUCTION

Drugs used for the treatment of different ailments are associated with impurities and degradation products that are part of manufacturing process of active pharmaceutical ingredient (API). Various impurities in drugs have been identified and reported during and after manufacturing process (Guideline, 2006) which are considered to have serious safety concern. Nitrosamines are diverse group of organic compounds found widely dispersed in our environment including food, soil, water, drugs, cosmetics and are reported to be produced endogenously in human (Vermeer and Van Maanen; 2001). These compounds were reported to be carcinogenic in experimental animals (Souliotis *et al.*, 2002) and potential carcinogen for humans (class A2 carcinogens). Nitrosamines, a group of impurities found in different dosage forms when reach above Acceptable Daily Intake (ADI) limit prove itself to be carcinogenic. Different nitrosamines have been identified and reported to be present in drugs including NDMA, NDEA, NMPA, NDIPA, NIPEA, NDBA and NMBA (fig. 1). The simplest compound of this family, N-nitroso dimethylamine (NDMA), has been reported to be hepatotoxic and genotoxic in animals (White, 2020). Recovered solvents, catalysts and reagents may contain nitrosamines and can be the source of contamination of the product. A large number of people are being treated with multiple drugs which are being administered for longer duration, these long term multi drug therapy is

being associated with risk of developing complications including cancer.

Acceptable daily intake limit for N-nitroso dimethylamine (NDMA) defined by FDA is 96 ng per day, above this limit the risk of carcinogenicity in human's increases (Johnson *et al.*; 2021). NDMA was the first nitrosamine moiety reported in various batches of valsartan (Pottgård A *et al.*, 2018; Shephard and Nawarskas; 2020) which was later on recalled by FDA in June 2019 (Parr *et al.*, 2019). After that, in April 2020, nitrosamine formation in formulations of ranitidine, after that metformin (fig. 2) (Braunstein LZ, 2021) and recently quinapril, varenicline have also been recalled due to presence of high levels of nitrosamines above the acceptable limit.

Hypertension is one of prevailing disease in Pakistan requiring long term medication to reduce the risks of cardiovascular events (Battistoni and Volpe 2020). Wilkins *et al.* reported that now a days cardiovascular and neoplastic diseases are the main cause of mortality and morbidity in developed countries (Sheweita *et al.*, 2014). According to WHO, it is the 10<sup>th</sup> leading disease to cause death. Among the seventeen million deaths due to CVS failure, about seven million deaths are due to high blood pressure (Iqbal *et al.*, 2023). Coexistence of both diseases in an individual may worsen its general condition and therapeutic management (Wilkins *et al.*, 2017). Various drugs used in hypertension having secondary or tertiary amines may participate in cancer development in their users as exemplified by Valsartan and ACE inhibitors. Recent recalls of anti-hypertensive drugs by FDA

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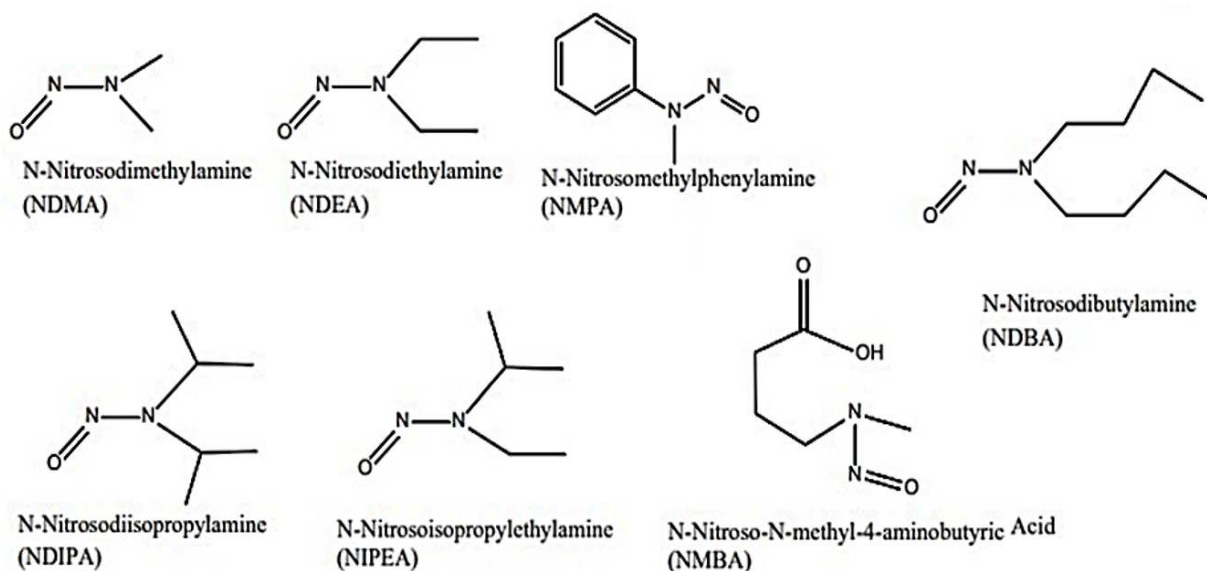


Fig. 1: Structure of seven different nitrosamine compounds reported in drugs declared by FDA.

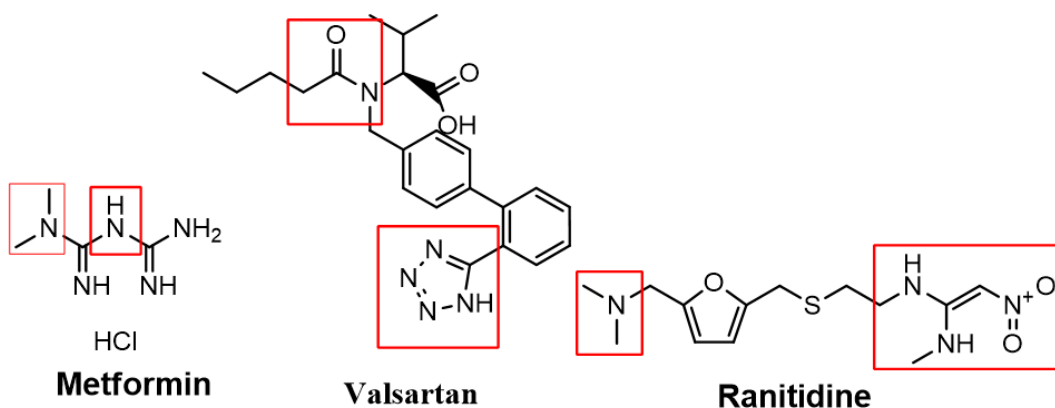


Fig. 2: Chemical structures of recalled drugs by FDA containing amines and having possible carcinogenic nitrosamines moiety.

(Charoo et al., 2019; Bharate SS, 2021) has raised concern about the safety of drugs used in long term treatment of hypertension. Lisinopril and Diltiazem are most commonly prescribed drug for the management of hypertension (fig. 3). Chemical structure of both drugs contains secondary and tertiary amines which are susceptible to produce carcinogenicity by producing nitrosamines so have potential to form one of the most common impurities NDMA at different stages. For that purpose, these drugs were screened for NDMA presence by using FDA validated analytical method on GCMS-HS so that their safety profile can be established against possible carcinogenicity in hypertensive patients. Presence of amines in anti-hypertensive drugs, lisinopril and diltiazem, make them susceptible to contain carcinogenic moieties nitrosamine that can worsen the treatment outcome of hypertensive patients. Keeping this assumption in view, this study was designed to evaluate different brands of these drugs to estimate these carcinogenic impurities using a standard analytical method of GCMS published by FDA.

## MATERIALS AND METHOD

NDMA reference standard Macklin (N871375) China, Samples of different brands of anti-hypertensive drugs lisinopril and diltiazem were purchased randomly from local market of different vicinities of city. Analytical grade methanol and acetonitrile (Merck). Dimethyl sulfoxide (DMSO)(Thermo-scientific). Headspace, GC 7890B, MSD 5977A, DB-Wax analytical column, GC-MS-HS and all accessories used in this study were of Agilent technology.

### Experiment undertaken

FDA published protocol of GCMS with head space was opted to screen lisinopril and diltiazem and verified by ICH guidelines for the detection and quantification of NDMA in selected brands of drugs (FDA, 2019). All instrumental parameters, Preparation of standard and sample, methodology and other conditions were kept same as described in approved methodology (Chan CC and Saraswat P, 2010).

**Table 1:** Preparation scheme for different concentrations of working standard solutions.

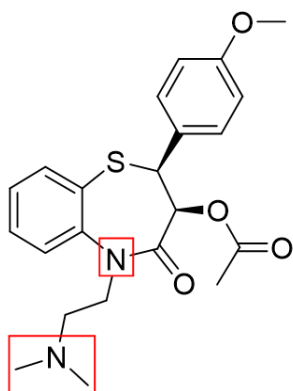
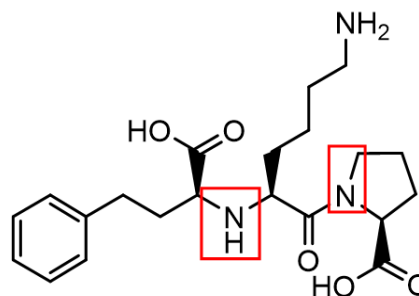
Concentration of stock solution (µg/mL)	Aliquot volume used (mL)	Total volume (mL)	Concentration of NDMA (µg/mL)
50	4.0	10.0	20.0
50	2.0	10.0	10.0
50	1.0	10.0	5.0
50	1.0	50.0	1.0
50	1.0	100.0	0.50
50	1.0	200.0	0.25
50	1.0	500.0	0.10

**Table 2:** Concentration of standard solution spiked for the accuracy determination.

Initial concentration of Std. (µg/mL)	Amount of standard spiked (µg)	Final concentration after spiking (µg/mL)
0.25	-	0.25
0.25	0.25	0.50
0.25	0.25	0.75
0.25	0.25	1.00

**Table 3:** Data used for determination of accuracy of analytical method.

S No.	Concentration (µg/mL)	Average response (n=6)	Percent Recovery
1	0.25	5349.50	107.100
2	0.50	9608.17	100.379
3	1.00	17320.17	92.591

**Diltiazem****Lisinopril****Fig. 3:** Chemical structure of anti-hypertensive drugs diltiazem and lisinopril.**Preparation of standard and working solutions**

Stock solution of NDMA (Standard) was prepared by weighing 10mg of NDMA reference standard and diluting it to 200mL with methanol. Different concentrations of NDMA solution were prepared from stock solution by following the dilution protocol with DMSO as given in table 1 (Araujo P 2009). Working standard solution of 0.1µg/mL was prepared by pipetting 1mL of NDMA standard stock solution and making up the volume with DMSO to 500mL. Similar protocol was followed for preparing working standard solution of 0.25µg/mL, 0.5µg/mL, 1.0µg/mL and 5.0µg/mL concentrations and diluting to 200mL, 100mL, 50mL and 10mL respectively. Working standard solution of 10.0µg/mL and 50.0µg/mL concentration were prepared by pipetting 2mL and 4mL of stock solution in 10mL and diluting up to the mark

with DMSO. An aliquot of 1mL of each working standard solution was pipetted in the 20mL head space vial and 5mL of dimethyl sulfoxide was added to it, crimped immediately and placed in head space carousel for analysis.

**Chromatographic conditions****GC-MS parameters**

To operate the GCMS, new method was created in Agilent Mass Hunter software and all the operating parameters were set as given in the method. Helium was used as carrier gas (mobile phase) on DB-Wax stationary phase with a flow rate of 3mL/min. Temperature of the inlet was set to 220° with 5:1 inlet split ratio. GC oven was programmed to maintain the temperature at 70degree for 4min, then ramped it gradually to 240° at the rate of

20° /min and held for 3.5min. GC run time was set to 16 minutes and GC cycle time was 23minutes.

#### **Head space parameters**

Head space was also controlled remotely via Mass Hunter software. Temperature of head space oven, loop and transfer line were set to 120, 125 and 130° respectively and monitored throughout the analysis. As per method 20mL vial size was selected with vial equilibrium time of 15min and injection time of 1.0 minute. The head space injection loop used for this study was of 1mL in size.

#### **MS parameters**

Before start of analysis, MS was auto tuned to ensure the proper working of mass spectrometer and to verify the absence of moisture, oxygen or any other impurity in carrier gas or any leakage in carrier gas transfer lines. In Mass Hunter, MS source temperature was set at 230° and Quad temperature was set at 150°. The MS was operated in SIM mode and 74.0 m/z was selected as SIM ion. Solvent delay of 4.0 min was set to avoid the saturation of detector with the dwell time of 200 minutes.

#### **Verification of analytical method**

##### **Accuracy**

For the estimation of accuracy, 0.25µg/ml standard solution was used as initial value and given protocol was followed (table 2).

##### **Linearity**

Different concentrations 0.25µg/mL, 0.50µg/mL, 1.0µg/mL, 5.0µg/mL and 10.0µg/mL of standard solution were prepared and injected one by one in triplicates into GC column via head space. The response of each standard solution obtained were plotted against their respective concentration to draw linearity curve.

##### **Repeatability**

For the estimation of repeatability, 6 replicates of 1.0µg/mL standard solution were prepared in head space vials and injected into GC-MS to obtain corresponding area of each replicate. Statistical tool was applied to calculate percent relative standard deviation (%RSD). To meet the repeatability criteria, the %RSD of all replicates should be within 2%.

#### **Limit of detection (LOD), Limit of quantification (LOQ)**

LOD and LOQ was determined by drawing calibration curve between concentration and response and finding the value of Y-intercept using Pearson coefficient of Correlation. The residual value was calculated using SPSS software.

$$\text{LOD} = 3.3\sigma / S$$

$$\text{LOQ} = 10\sigma / S$$

#### **System suitability**

For the evaluation of suitability of system, 6 replicates of 0.50µg/mL standard solution were analyzed and from the

response obtained, system suitability parameters were evaluated as per USP-23. NDMA standard of 0.25µg/mL concentration was analyzed for evaluation of signal to noise ratio which should be  $\geq 10$ .

#### **Standard calibration curve**

Two different calibration curves were drawn by injecting standard concentration mentioned in table 3 and plotting their respective peak responses. One calibration curve (curve-01) was drawn with standard solution of 0.10µg/mL to 20.0µg/mL in duplicate. The other (curve-02) was drawn with standard solution of 0.10µg/mL to 50µg/mL in duplicate.

#### **Preparation of sample**

Sample of different brands of lisinopril tablets was prepared by crushing 10 tablets of each brand separately and transferring to 20mL headspace vial with addition of 5ml of dimethyl sulfoxide (DMSO). Vials were immediately crimped with cap sealer and left undisturbed for 10 minutes. After it vials were vortexed for 10 minutes, then shaken on wrist action shaker for 30 minutes or until the content was completely dispersed. Same procedure was repeated for diltiazem tablets of each of selected brand.

#### **Spiked samples**

Spiked samples of each of selected brand were prepared similar to samples prepared in table 3. Before the addition of DMSO, 1ml of known concentration (0.5µg/ml) of NDMA standard solutions was added to head space vial and treated same as given above to estimate totally recovered drug.

*Ethical approval:* Not applicable

## **STATISTICAL ANALYSIS**

All the above data was analyzed by using instrument software Mass Hunter while statistical tools of Microsoft-Excel 2022 were used for calculation of validation parameters of analytical samples and spiked samples.

## **RESULTS**

#### **Calibration Curve: 1**

Response of the different concentrations of standard solution of NDMA (0.10µg/mL, 0.25µg/mL, 0.50µg/mL, 1.0µg/mL, 5.0µg/mL, 10.0µg/mL and 20.0µg/mL was noted in the form of calibration curve 1 as described in (fig. 6) while for calibration curve 2 same concentrations were used except one more 50.0µg/mL (fig. 7)

#### **Accuracy**

##### **Linearity**

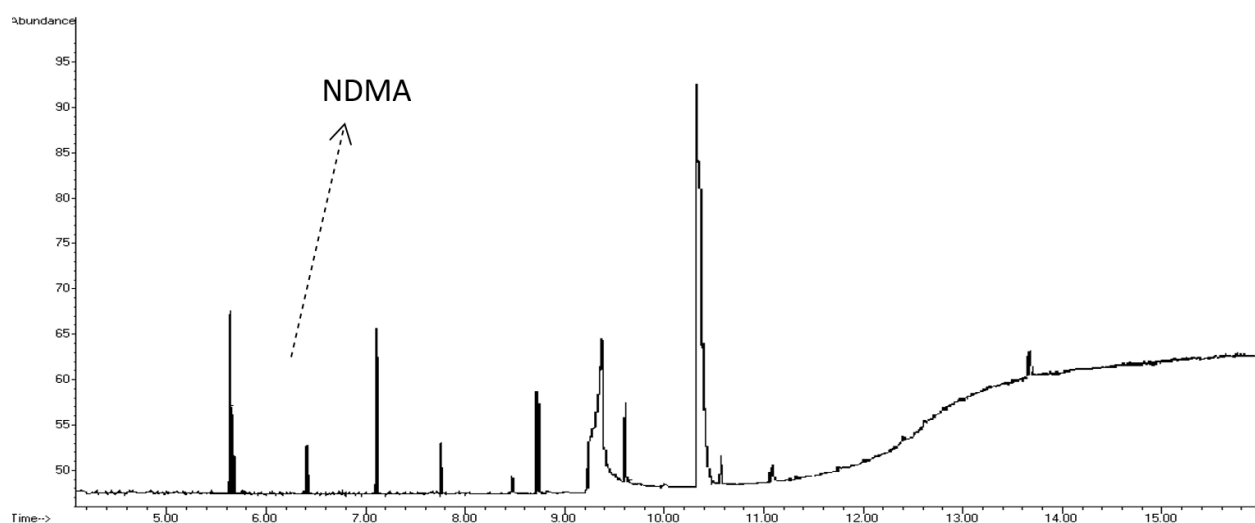
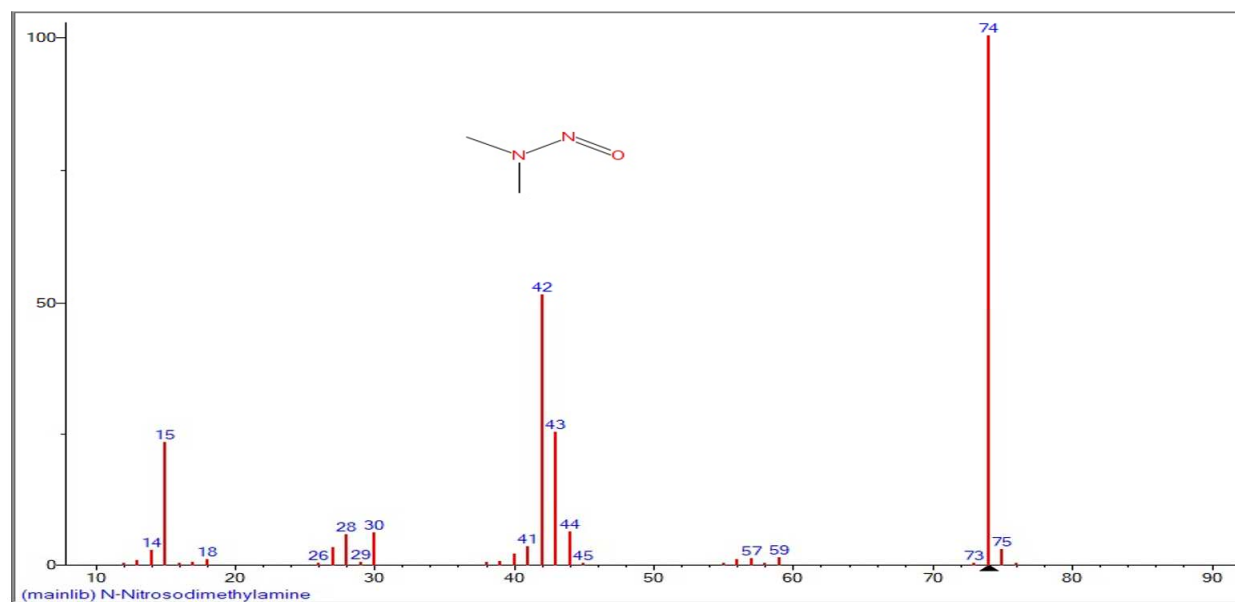
Linearity data of NDMA standard solution concentration of 0.25µg/mL, 0.50µg/mL, 1.0µg/mL, 5.0µg/mL, 10.0 µg/mL, to 20µg/mL has shown linearity (fig. 8)

**Table 4:** Repeatability data of 1.0µg/mL NDMA standard solution.

S No	Replicates	Response/Area	% Assay
1	1	17291	102.02%
2	2	17585	100.36%
3	3	17308	101.92%
4	4	17491	100.88%
5	5	17587	100.35%
6	6	17452	101.10%
	Average	17484.33	101.106
	SD	129.685	0.729
	% RSD	0.743	0.722

**Table 5:** LOD and LOQ determination of NDMA standard

Regression equation	Y-intercept	Slope	SD of standard	LOD (3.3SD/m)	LOQ (10SD/m)
Y=19848x-7456.4	7456.4	19848	57.138	0.0103	0.0314

**Fig. 4:** Chromatogram (TIC) of 0.1µg/mL NDMA standard solution.**Fig. 5:** Mass Spectra of standard NDMA matched with NIST 2020 library.

**Table 6:** System suitability data of six replicates of 0.50µg/mL NDMA reference solution (n=6)

Injection No=6	Avg. Retention time	Avg. Peak response	Avg. Tailing factor	Avg. Plate count
Mean	6.526	9183.333	1.408	2032.5
SD	0.003	57.138	0.005	17.155
%RSD	0.043	0.622	0.389	0.844

**Table 7:** Percent recovery of NDMA in spiked sample of lisinopril (Sample A-01, A-02, A-03).

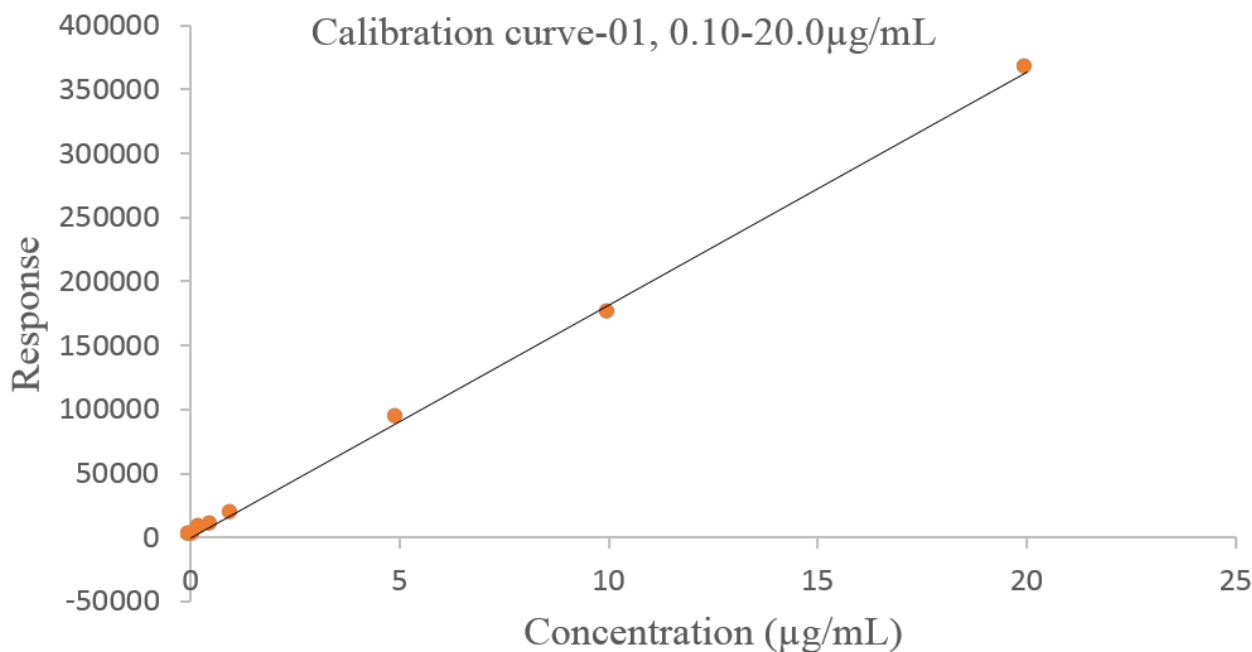
Conc. of NDMA (0.25µg/mL) spiked with sample (n=3)	Sample replicate (n=3)	Average Peak response (n=3)	Avg. concentration. obtained = (area-intercept)/slope	Average % recovery
01	1	4408.67	0.2732	107.51%
02	1	4411.67	0.2684	107.57%
03	1	4375.67	0.2664	106.78%

**Table 8:** Percent recovery of NDMA in spiked sample of diltiazem (Sample B-01).

Concentration of NDMA standard spiked (µg/mL)	Sample replicate	Peak response	Conc. obtained = (area-intercept)/slope	% recovery	Average % recovery
0.25	1	4477	0.2725	109.01%	
0.25	2	4505	0.2741	109.63%	108.66%
0.25	3	4401	0.2683	107.34%	

**Table 9:** Percent recovery of NDMA in spiked sample of diltiazem (Sample B-02).

Concentration of NDMA standard spiked (µg/mL)	Sample replicate	Peak response	Conc. obtained = (area-intercept)/slope	% recovery	Average % recovery
0.25	1	4590	0.2787	111.50%	
0.25	2	4450	0.2710	108.42	109.66%
0.25	3	4480	0.2727	109.08	



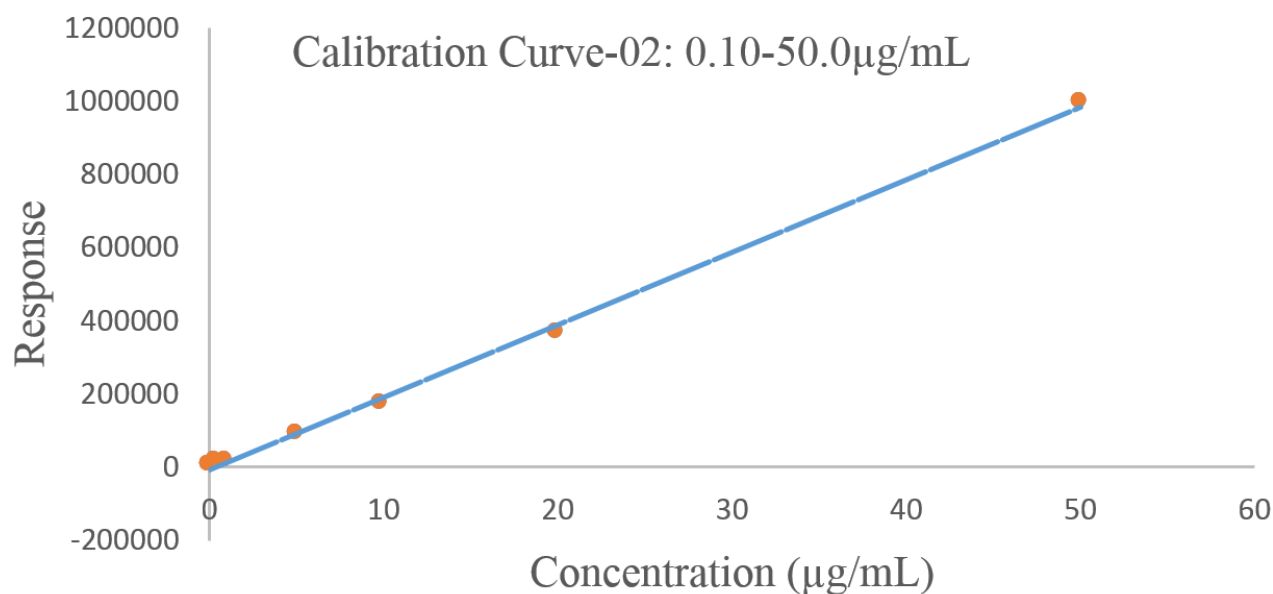
Regression coefficient ( $R^2$ ) of calibration curve-01 = 0.9993

Y-intercept of calibration curve-01 = 330.27, Slope of Calibration curve-01= 18178

**Fig. 6:** Calibration curve-01 plotted against NDMA standard concentrations of 0.1µg/mL to 20.0µg/mL and their respective response.

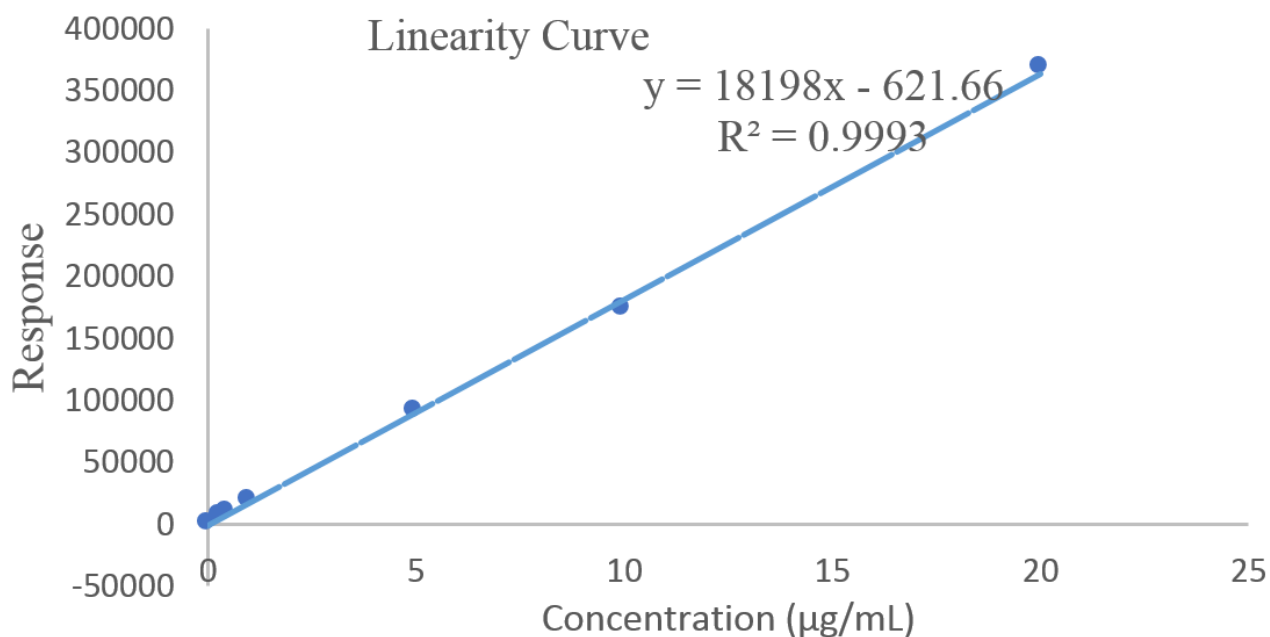
**Table 10:** Percent recovery of NDMA in spiked sample of diltiazem (Sample B-03)

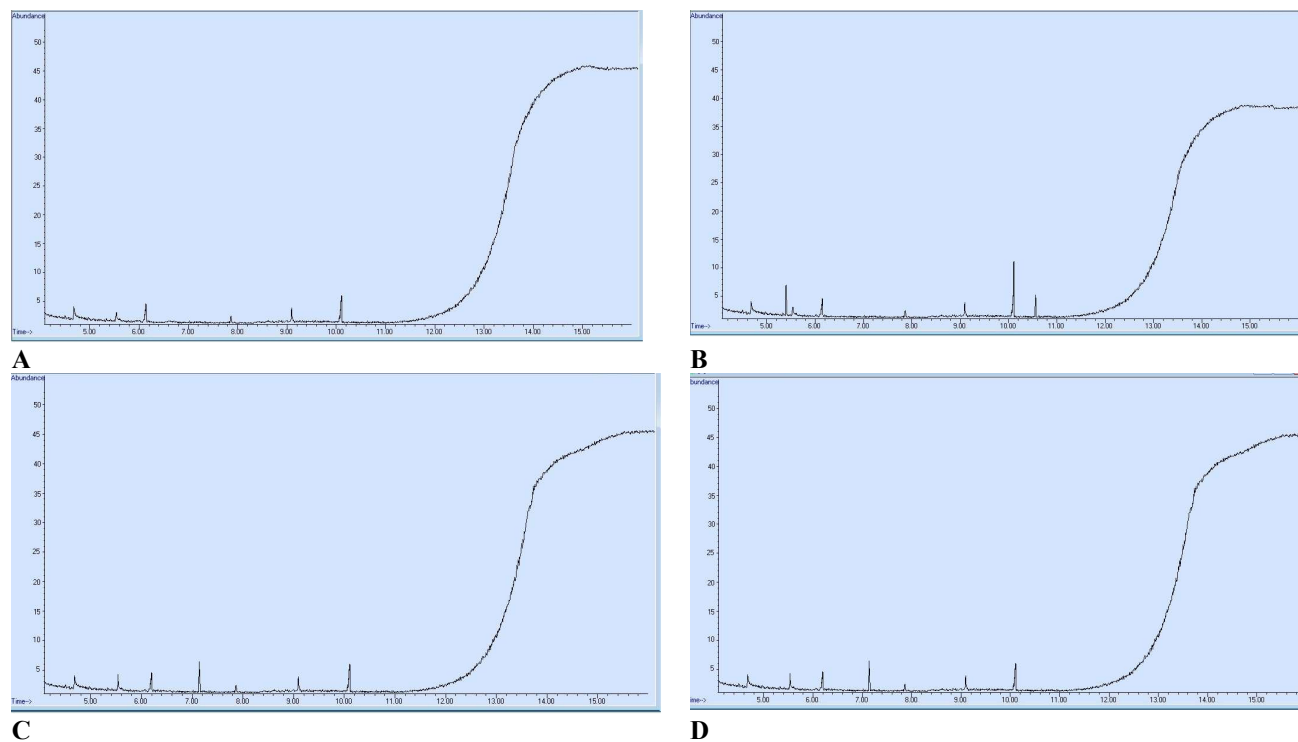
Concentration of NDMA standard spiked ( $\mu\text{g/mL}$ )	Sample replicate	Peak response	Conc. obtained = (area-intercept)/slope	% recovery	Average % recovery
0.25	1	4403	0.268	107.38%	108.45%
0.25	2	4490	0.273	109.29%	
0.25	3	4461	0.2716	108.65%	



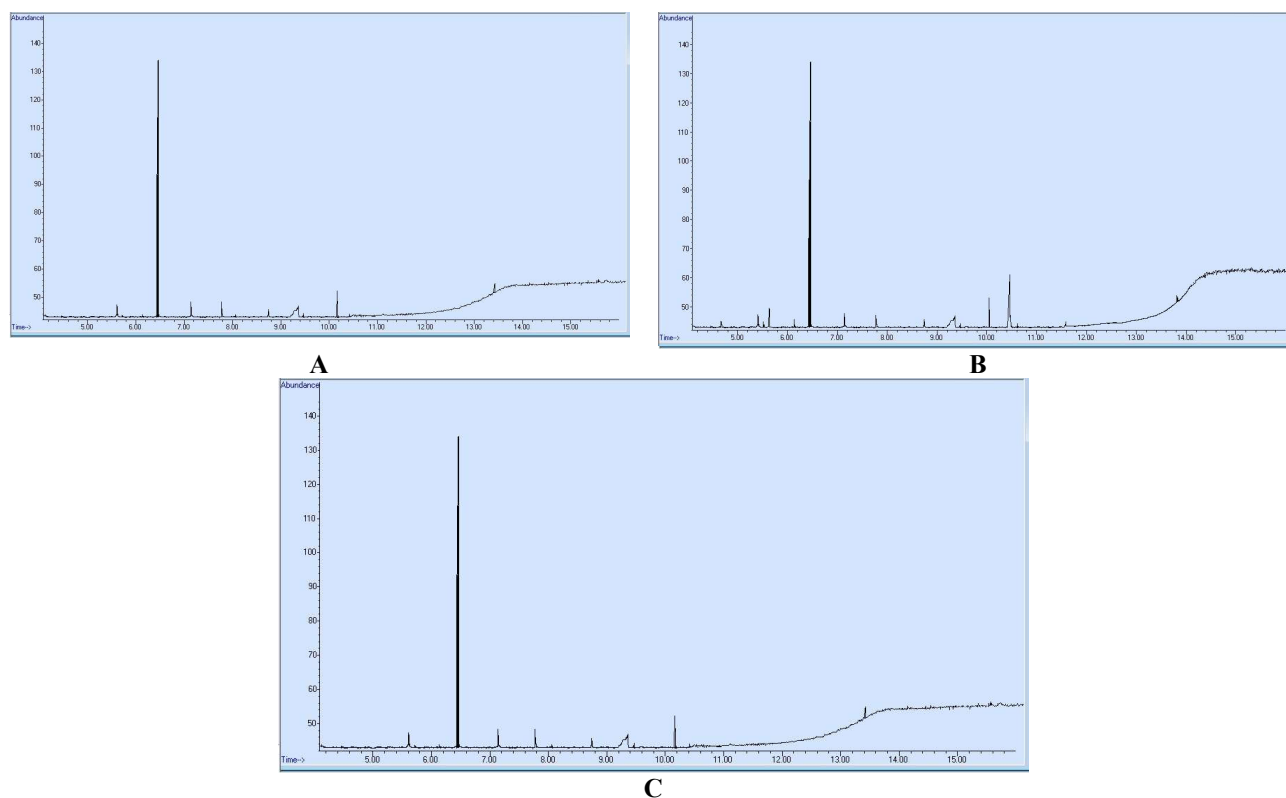
Regression coefficient ( $R^2$ ) of calibration curve-02 = 0.9986

Y-intercept of calibration curve-02 = 6310.6, Slope of Calibration curve-02 = 19815

**Fig. 7:** Calibration curve-02 obtained from different concentrations of NDMA standard solution.**Fig. 8:** Linearity curve obtained by plotting NDMA standard solution concentration of 0.25  $\mu\text{g/mL}$  to 20  $\mu\text{g/mL}$  against their respective response.

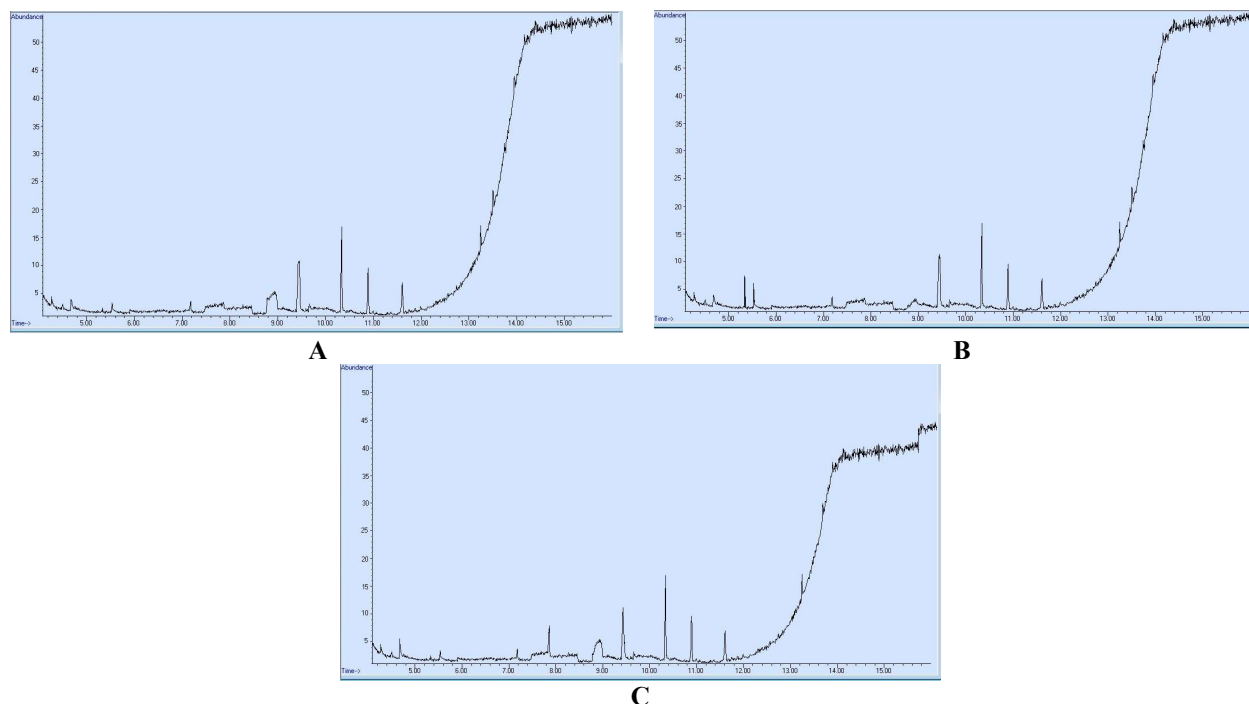


**Fig. 9:** Chromatogram (TIC) of A: 0.1 $\mu$ g/mL NDMA standard solution B: different brands of Lisinopril tablet A-01, C: Lisinopril tablet A-02, D: Lisinopril tablet A-03



**Fig. 10:** Chromatograms (TIC) of different brands of Lisinopril tablet spiked with NDMA standard A: A-01, B: A-02, C: A-03





**Fig. 11:** Gas Chromatogram (TIC) of Diltiazem Tablet Samples for the presence of NDMA, A: B-01, b: B-02, C: B-03  
\*On X-Axis, Time and on Y-Axis absorbance

### Repeatability

#### Limit of detection and quantification

#### System suitability

#### Screening of samples

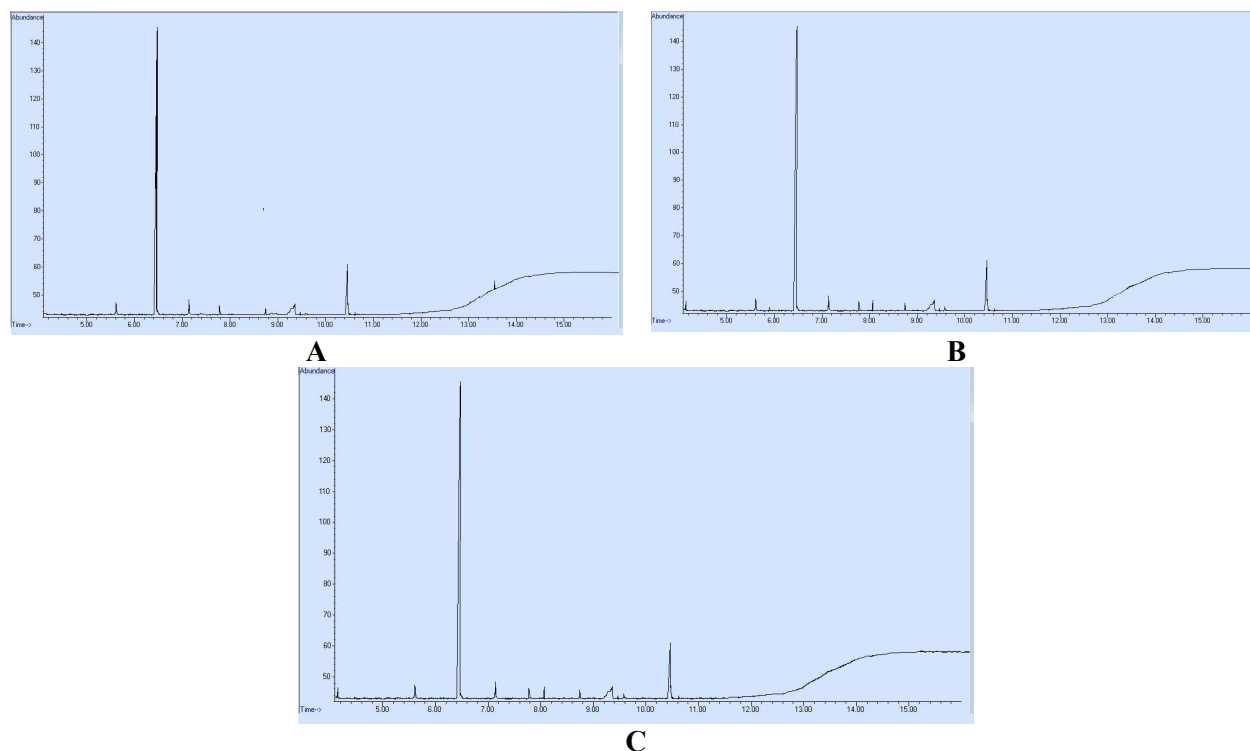
Samples of three different brands of each of diltiazem tablets and lisinopril tablets were screened for detection of NDMA impurity above the FDA allowable limit. The results of GCMS-HS scan of lisinopril and diltiazem revealed the absence of NDMA (table 8-10) as shown in fig. 9 and 11 while the samples spiked with the NDMA standard have shown good recovery (table 7, fig.10 and 12). The response was measured and percent recovery was calculated.

## DISCUSSION

Nitrosamine, A carcinogenic moiety is reported to be present in drugs containing secondary and tertiary amines in their chemical structure. Various mechanism for formation of NDMA in drugs have been discussed in the literature but the exact pathway by which these nitrosamines forms is still unknown (Cioc *et al.*, 2023). It is hypothesized that these NAs are formed in presence of secondary, tertiary or quaternary amines and a nitrite salt. Under acidic conditions these nitrites can interact with amines to form nitrosamines while most probably, it can be formed in manufacturing process, nitrous acid is used to quench the azides in the presence of amines whether as active pharmaceutical ingredient (API), degradants of API, intermediate of a manufacturing process, starting material or added intentionally as reagents. In addition to this, amide solvents e.g N, N-dimethyl formamide,

recovered solvents, catalysts and reagents may contain nitrosamines and can be the source of contamination of the product but the presence of this moiety in multiple drugs especially being administered for longer duration is being associated with risk of developing complications including cancer.

To ensure the safety of most commonly used anti-hypertensive drugs, GCMS-HS analysis was performed by FDA approved method. From the remote times, it was considered the sensitive method for the detection of nitrosamines in microgram amount at high temperature as compared to other methods (Alshehri *et al.*, 2020a,b) as it enables efficient separation and detection of small quantities of analyte up to the levels of parts per billion. The adopted method was verified as per FDA and ICH guidelines for selected parameters (FDA 2019) like accuracy, linearity, repeatability, LOQ and LOD. Results of all these parameters confirmed the validity of the method as they were within specified range, compare the peaks of samples with standard peak of NDMA (fig. 4,5). The linearity data produced straight line with regression coefficient ( $R^2$ ) of 0.9993 whereas, repeatability data obtained from 6 replicates of 1.0 µg/mL standard solution had %RSD of 0.722. The signal to noise ratio of 0.25 µg/mL standard solution was evaluated via Mass Hunter software and result obtained was 11.7 which was within the limit as mentioned in method that standard solution should be higher than 10 for validity of method. To ensure the validity and accuracy of results obtained, standard solution was spiked with the three different concentrations of 0.25 µg/mL, 0.5 µg/mL and 1.0 µg/mL



**Fig. 12:** Mass spectra of Diltiazem sample spiked with NDMA standard A: B-01, B: B-02, C: B-03

\*On X-Axis. Time and on Y-Axis absorbance

standard solution and percent recovery was 107.099%, 100.379% and 92.591% respectively while LOD 0.0103 and LOQ of 0.0314 PPM confirmed that the method is capable to detect nitrosamines level above 96ng/mL. Method verification parameters were found within defined criteria. After the verification, two calibration curves having different concentrations were drawn as already mentioned, The regression coefficient of first curve was 0.9993 while 0.9986 was value of second calibration curve. According to given criteria in FDA method, if the peak area of NDMA peak in sample under investigation is less than the peak area of 20.0µg/mL standard solution than calibration curve-01 should be used otherwise calibration curve-02 should be used for peak responses greater than 20.0µg/mL (FDA 2019).

By using the verified method samples of selected brands were analyzed in triplicates on GC-MS-HS for NDMA detection, no peak response obtained which proves the absence of NDMA which make these brand safe to use. To verify the response of method parameters or suppression of response by interaction of excipients in dosage form or probable degradation of standard by high temperature (Alshehri *et al.*, 2020a), the three replicates of each of three selected brands of both lisinopril and diltiazem were spiked with 0.25µg/mL NDMA standard, calibration curve-01 was used for the calculation of percentage recovery of spiked amount of NDMA in sample. The average percentage recovery of spiked samples was between 100% to 110% which indicate the specificity and accuracy of the method.

Results have shown that amines can be the source of formation of nitrosamines but not in all drugs containing them. The authenticity of the method was proven by study of Wichitnithad *et al.*, 2021 developed method on GCMS-HS for detection of four nitrosamine moieties having calibration curve in the range of 25 to 5000µg/ml while in this study calibration curve was drawn for concentration ranging from 0.10µg/mL to 50µg/mL which proves that the LOQ and accuracy of this method is much better than the previous method.

## CONCLUSION

The results indicate that opted method is simple, precise, accurate, sensitive and can be used further for NDMA determination at microgram level in lisinopril and diltiazem formulations. No NDMA peak was detected in both formulations after evaluating their respective chromatogram and spectra which makes these drugs safe to be used. This method provides the possibility to analyze other amine based drugs available in market for the screening of these nitrosamine impurities so that their safety profile can be establish.

## ACKNOWLEDGEMENT

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