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Analysis and risk assessment of nitrosamines in sartans using GC-MS and Monte Carlo simulation

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The precise detection of nitrosamines at very low levels in various substances is crucial because of their known toxicity and potential to cause genetic mutations. As a result, regulations have been established to evaluate the risk of nitrosamines and manage their presence in pharmaceuticals. In this study, we developed an analytical method involving solvent extraction and gas chromatography-mass spectrometry for the determination of N-nitrosodimethylamine and N-nitrosodiethylamine based on the internal standard method. According to validated results based on ICH guidelines, calibration curves spanned from 2.5 to 40 ng mL^{-1} with a limit of quantitation of 0.015 µg g^{-1} for N-nitrosodimethylamine and 0.003 µg g^{-1} for N-nitrosodiethylamine, meeting the US FDA requirements. The method's relative recovery was 80–120%, with a relative standard deviation of ≤ 12%. Then, the levels of both nitrosamines were determined using the validated method in 84 samples of active pharmaceutical ingredients or finished products, including valsartan, valsartan methyl ester, sacubitril/valsartan, losartan, and telmisartan. Additionally, we utilized Monte Carlo simulation to assess the potential health risks associated with the use of sartans. According to the obtained results, the Hazard index values were found to be less than 1 indicating that children and adult users may not be at risk of health problems.

Keywords Nitrosamines, Risk assessment, Quality control, Pharmaceutical, GC-MS

Nitrosamines (NAs) represent a category of organic impurities that pose a substantial challenge to the pharmaceutical industry[1]. Of particular concerns are Nitrosodimethylamine (NDMA) and Nitrosodiethylamine (NDEA), both identified as members of a high-risk cohort by the International Agency for Research on Cancer (IARC) of the World Health Organization (WHO)[2]. The carcinogenic properties of nitrosamines have prompted widespread concerns, particularly due to evidence suggesting that exposure to N-nitrosodimethylamine (NDMA) may be linked to the development of liver, lung, stomach, respiratory, and kidney cancers in multiple animal studies[3]. Similarly, NDEA is a known cause of liver cancer and has been shown to increase tumor risk in animals and humans through its effects on DNA after bioactivation[4]. These findings highlight the critical need for vigilant in the detection and management of NAs in products to mitigate cancer risks associated with their use.

Researchers have verified that nitrosamines can be present in active pharmaceutical ingredients (APIs) or finished products due to exposure to equipment, materials, or solvents contaminated with nitrosamine compounds[5]. In 2018, research revealed that certain pharmaceuticals, such as those containing tetrazole groups, may cause the formation of NDMA from their constituents. In particular, aprotic polar solvents, such as zinc chloride ($ZnCl_2$) and sodium azide (NaN_3), are utilized in the preparation of tetrazole groups in pharmaceuticals, which may form DMF upon exposure to sodium nitrite ($NaNO_2$). These groups are found in the structure of pharmaceuticals such as valsartan and losartan, classified as angiotensin II receptor antagonists or blockers (ARBs)[6]. The U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) have acknowledged the presence of carcinogenic NDMA and NDEA impurities in the raw materials and final products of sartan pharmaceuticals[7]. As a result, they have recalled valsartan products due to NDMA contamination[8].

Various techniques have been utilized to establish acceptable intake (AI) thresholds for impurities present in various pharmaceuticals. Historically, the International Council for Harmonization of Technical Requirements

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for Pharmaceuticals for Human Use (ICH) method, based on a 50% tumor incidence (TD_{50}), has been the conventional approach. Additionally, the Threshold of Toxicological Concern (TTC) concept was developed to determine the acceptable intake (AI) levels for compounds demonstrating low carcinogenic potential and lacking comprehensive research[5]. This method involves a simple linear extrapolation from a dose resulting in a TD_{50} of 1 in 10^6, representing a highly conservative approach. Another approach is based on the most potent N-nitrosamines and a limit TTC of 18 ng/day[6]. According to the research, the AI for NDMA and NDEA is also considered to be 96 and 26.5 ng/day, respectively; consuming this amount of these nitrosamines increases the probability of getting cancer by 1:100,000 after 70 years[7]. Nevertheless, the pharmaceutical industries are undertaking numerous N-nitrosamine impurity risk assessments, given their potential to form complex and diverse structures during the reaction between nitrosating agent impurities or degradant compounds. Therefore, a practical and science-based approach is required to identify the hazards associated with long-term consumption. One such approach is risk assessment based on Monte Carlo simulation[8–10].

Numerous analytical methods are developed for analyzing various compounds present in pharmaceuticals[11–17]. In the early stages of field analysis, gas chromatography-tandem mass spectrometry (GC-MS/MS)[15,18] was widely used. Moreover, liquid chromatography-tandem mass spectrometry (LC-MS/MS) or liquid chromatography-high-resolution mass spectrometry (LC-HRMS) was applied to sartans and other pharmaceutical products[12–14,19]. Despite the continued implementation of these methods in recent years[20–37], the global scale of the issue has compelled most laboratories to employ simpler and more routine methodologies, such as gas chromatography-mass spectrometry (GC-MS)[38–42] and high-performance liquid chromatography (HPLC-UV)[43–47]. Additionally, to further accelerate research, advanced methods such as supercritical fluid chromatography (SFC)[48] and electrophoresis-nano-electrospray ionization-mass spectrometry (CE-nanoESI)[49], have been introduced.

In this research, we developed an analytical method according to gas chromatography-mass spectrometry (GC-MS) technique. The proposed method's figures of merit in terms of selectivity, linearity, limit of quantification, and system suitability were obtained. Using full scan and selected ion recording (SIR) modes, we simultaneously identified two volatile nitrosamines (NDMA and NDEA) in sartans that conform to ICH Topic Q2(R1) recommendations[50]. Additionally, we employed multiple reaction monitoring (MRM) mode to confirm the presence or absence of these impurities. For monitoring nitrosamines in active pharmaceutical ingredients or finished products of sartan family drugs, NDMA and NDEA in 84 real samples were determined by the validated method. Notably, previous studies have not been addressed the health risks of NAs. Therefore, we used our results to assess the health risks of nitrosamines through Monte Carlo simulation.

Methods

Materials

The NDMA solution, with a concentration of 1000 μg mL^{-1} in methanol (reference: 31427), NDEA standard (99.0% purity), and deuterated N-nitrosodimethylamine (NDMA-d6) in methanol at 1000 μg mL^{-1} were sourced from Restek (Bellefonte, Pennsylvania). Dichloromethane was obtained from Millipore Sigma (Burlington, Massachusetts). Active pharmaceutical ingredients (APIs) and commercially available finished products (tablets) were provided by 11 pharmaceutical companies in Iran. Samples were analyzed as soon as receive and were stored at refrigerator in airtight containers to limit exposure to environmental conditions that could lead to nitrosamine formation.

Standard solution preparation

A stock solution of 50.00 μg mL^{-1} NDMA and 49.50 μg mL^{-1} NDEA was prepared in a mixed solution. To generate calibration standards, appropriate dilutions of stock solutions in dichloromethane were made at concentrations of 2.50, 5.00, 7.50, 10.00, 15.00, 20.00, 25.00, and 40.00 ng mL^{-1} of NDMA, and 2.48, 4.95, 7.42, 9.90, 14.85, 19.80, 24.75, and 39.60 ng mL^{-1} of NDEA. For calibration curves, fresh solutions are required, and a concentration for the internal standard NDMA-d6 was maintained at 15.00 ng mL^{-1}.

Sample preparation

In this study, sample preparation follows the method proposed by FDA[15]. Two types of samples, APIs and tablets, were analyzed. For tablets, the cover, if present, was removed entirely, and five tablets were finely ground into a homogenous substance. 500 mg of each sample (either the API or the ground tablet) was weighed and transferred to a 15 mL centrifuge tube. Then 50 μL of an appropriate concentration of NDMA-d6 was added, which resulted in an internal standard concentration of 15.00 ng mL^{-1}, following the incorporation of the extraction solvent. Subsequently, 5 mL of dichloromethane, the optimal extraction solvent for NDMA and NDEA analysis using GC-MS, was added to the tube. The mixture was then vortexed for 2 min and centrifuged at 4000 rpm for 2.5 min. Afterward, 2 mL of the supernatant was filtered through a 0.45-μm nylon syringe filter and then subjected to GC-MS analysis.

GC-MS analysis

A gas chromatograph with a tandem mass spectrometer (GC-MS/MS, Agilent 6890 N Quattro Micro™ GC, Waters Micromass MS Technologies) was used to analyze NDMA and NDEA. Separations were performed using a Stabilwax®-MS capillary column (30 m × 0.25 mm, film thickness 0.25 μm; Restek Corp., Bellefonte, PA, USA). The ultra-high purity helium as a carrier gas was held at a constant flow rate of 1 mL/min. A 2.0 μL of each sample was injected in splitless mode with an inlet temperature of 250 °C. The oven temperature was programmed from 40 °C to 200 °C at a ramp rate of 20 °C/min, followed by a ramp from 200 °C to 245 °C at 60 °C/min, with initial and final hold times of 2 and 3 min, respectively. The transfer line temperature was held at 280 °C and ionization occurred through electron ionization (EI, ionization energy, 70 eV) mode. The mass spectrometer was operated in full scan, selected ion recording mode (SIR), and multiple reaction monitoring

(MRM). NDMA and NDEA were identified and quantified in full scan and SIR mode, respectively. The mass-spectral library (NIST 98 version 1.6d) was used to identify the NDMA and NDEA in scan mode. Analyses were conducted in triplicate to ensure precision and reliability of measurements.

Method validation

To validate the proposed method for GC-MS, several parameters were considered, including specificity, linearity, limits of detection (LOD), limits of quantification (LOQ), range, accuracy, precision, and system suitability. These parameters were determined in accordance with the guidelines provided by the ICH Topic Q2(R1)[51]. Calibration curves were constructed by plotting the area ratio of each analyte to NDMA-d6 versus the concentration ratio of each analyte to NDMA-d6, in the concentration ranges of 2.50–40.00 ng mL^{-1} and 2.47–39.60 ng mL^{-1} for NDMA and NDEA, respectively. The linear equation, determination of coefficient (R^2), y-intercept, and slope were obtained by using the least squares linear regression analysis method. The LOD and LOQ were determined based on the signal-to-noise ratio of 3.3 and 10, respectively. To assess the intra- and inter-day accuracy and precision, the recovery and relative standard deviation (RSD%) were calculated in three levels of NAs (low, medium, and high). The system suitability of the system was evaluated by determination of the R^2, RSD% of six replicate injections of the 40.00 and 39.60 ng mL^{-1}, and S/N ratio for the 5.00 and 4.95 ng mL^{-1} standards of NDMA and NDEA, respectively. In addition, control charts for the peak areas of NAs at specific concentrations that were repeatedly injected during the research period (approximately 18 months), were plotted. Finally, the validated proposed method was applied to analyze NDMA and NDEA in API of valsartan, losartan, telmisartan, valsartan methyl ester, and sacubitril/valsartan and their finished products, as listed in Table 1. To quantify the amount of selected NAs using internal standard method, a single point calibration was used.

Health risk assessment

Estimated daily intake (EDI, mg/day) of different nitrosamine impurity forms from two main drug groups as well as valsartan and losartan were calculated based on the following equation:

$$\mathrm{EDI} = \mathrm{C} \times \mathrm{IR}, \tag{1}$$

C is the concentration of nitrosamines (mg kg^{-1}); IR is, the daily ingestion rate (kg day^{-1}); After EDI calculation by Eq. 1, the chronic daily intakes (CDI) (mg/kg.day) of nitrosamines were determined according to the below equation:

Sample	Type	Number of samples	NDMA		NDEA	
			Code	Concentration found (μg g^{-1})	Code	Concentration found (μg g^{-1})
Valsartan	API	35	Vals.A.01-Vals.A.28	N.D.[a]	Vals.A.01-Vals.A.13	N.D.
					Vals.A.14	0.0064
			Vals.A.29-Vals.A.35	N.A.[b]	Vals.A.15-Vals.A.35	N.D.
Valsartan	Tablet	8	Vals.T.01-Vals.T.05	N.D.	Vals.T.01-Vals.T.08	N.D.
			Vals.T.06	N.A.		
			Vals.T.07	1.67		
			Vals.T.08	1.79		
Valsartan methyl ester	API	10	VME.A.01-VME.A.05	N.D.	VME.A.01-VME.A.07	N.D.
			VME.A.06-VME.A.07	N.A.	VME.A.08	0.058
			VME.A.08	0.106	VME.A.09	0.022
			VME.A.09	N.D.	VME.A.10	N.A.
			VME.A.10	0.108		
Losartan	API	16	Los.A.01-Los.A.08	N.D.	Los.A.01-Los.A.05	N.D.
					Los.A.06	6.62
					Los.A.07	0.026
					Los.A.08	N.A.
			Los.A.09-Los.A.16	N.A.	Los.A.09	4.47
					Los.A.10	1.68
					Los.A.11	1.01
					Los.A.12	1.41
					Los.A.13-Los.A.16	N.D.
Losartan	Tablet	8	Los.T.01-Los.T.08	N.D.	Los.T.01-Los.T.08	N.D.
Telmisartan	API	3	Tel.A.01-Tel.A.03	N.D.	Tel.A.01-Tel.A.03	N.D.
Sacubitril/Valsartan	API	4	VS.A.01-VS.A.04	N.D.	VS.A.01-VS.A.04	N.D.

Table 1. Types of pharmaceuticals used in the present study and the obtained concentration of NAs in them. [a]Not detected. [b]Not analyzed.

$$\mathrm{CDI} = \frac{\mathrm{EDI} \times \mathrm{AF} \times \mathrm{LFC} \times \mathrm{ED}}{\mathrm{BW} \times \mathrm{TL}}, \tag{2}$$

AF is the portion dose of ingested nitrosamines that are physiologically absorbed, ED is the exposure duration (year), LFC is the consumption dose, TL is the typical lifetime (year), and BW is the human body weight (kg); 15 kg for children and 70 kg for adults; ED, exposure duration for adults and children.

The hazard quotient (HQ) due to drug consumption is evaluated by the below equations[9]

$$\mathrm{HQ} = \frac{\mathrm{CDI}}{\mathrm{RfD}}, \tag{3}$$

where RfD is the reference oral dose (mg/kg day).

The hazard index (HI) was evaluated:

$$\mathrm{HI} = \mathrm{HQ1} + \mathrm{HQ2} + \cdots + \mathrm{HQn} \tag{4}$$

HI ≤ 1 means risk is not notable, but if HI > 1, health risk concern is considerable.

Cancer risk was calculated based on the United States Environmental Protection Agency (US-EPA) ≤ 1×10^{-6} criteria. By Eq. (4), Carcinogenic risk (CR) was estimated:

$$\mathrm{CR} = \mathrm{CDI} \times \mathrm{CSF} \tag{5}$$

Numerous ambiguities can arise during health risk assessment estimates. High uncertainty is typically associated with using single-point values to calculate the health risk of nitrosamine exposure. To reduce these uncertainties, Monte Carlo simulation (MCS) a probabilistic method was employed. Using MCS, Crystal Ball (version 11.1.2.4.600 (32-bit) Oracle, Inc., USA) was utilized for assessing both non-carcinogen and carcinogen risks. The simulation involved at least 100,000 trials, and the criterion for consumers' health was set at 95%.

Results and discussion

Method development

The determination of NDMA and NDEA impurities in sartans has recently come under scrutiny. Among the FDA-reported methods for the impurity analysis of NDMA and NDEA in sartans, the GC-MS technique was utilized with a headspace injection mode to reduce matrix interference[17]. This approach, however, has certain limitations, such as unsuitability for thermolabile samples, the inability to reinject samples, and the requirement for careful method development to ensure robust determinations. To overcome these limitations, a GC-MS technique with direct injection to a classical split/splitless inlet was developed and validated in this study for the analysis of trace concentrations of NDMA and NDEA in API and finished products of sartans. Additionally, the capabilities of MRM mode in MS/MS instrument were leveraged to clear the ambiguity in the identification of analytes in special conditions.

The choice of stationary phase employed has a significant impact on the quality of retention and peak shape of analytes in GC-MS techniques. As per the polarity of NDMA and NDEA, which are demonstrated in Table S1, a high polarity stationary phase with carbowax was employed to accomplish separation. This material was also utilized in the FDA's recently published method [10]. Given the low solubility of non-polar sartans, particularly valsartan, in dichloromethane (DCM), and the excellent solubility of NDMA and NDEA in this solvent (Table S1), employing this sample preparation technique reduces matrix interference, an extension of column lifespan, and a decline in machine contamination.

Considering that the injection in the developed method is direct, there is a possibility that the injected matrix or solvent peak may co-elute with the NDMA. To achieve optimal separation, an initial constant temperature of 40 °C was maintained for 2 min, followed by a ramp at a rate of 20 °C to reach 200 °C. Furthermore, the inlet temperature was adjusted between 160 and 250 °C, with the highest abundance for selected mass-to-charge ratios of two analytes obtained at 250 °C (data not shown). The optimization of certain parameters of the EI ion source, including extraction lens voltage, focus lens voltage 1, and focus lens voltage 3, was conducted to enhance the sensitivity of the detector and to obtain better ionization of the analytes. Details of the ion source parameters are listed in Table S2.

Identification of the components was carried out by analyzing their retention times and interpreting their mass spectra, obtained in the EI full scan mode (Fig. S1), using a National Institute of Standards and Technology (NIST) library search. For quantitative analysis, the NDMA, NDMA-d6 as an internal standard, and NDEA were monitored in SIR mode by using the m/z 74, 80, and 102 parent ions, respectively. The SIR chromatograms, overlaid for NDMA, NDEA, and NDMA-d6 on different days, have been presented in Fig. 1. The retention times, exhibiting a good degree of repeatability, for NDMA, NDEA, and NDMA-d6 were found to be 6.69 ± 0.01, 7.31 ± 0.03, and 6.69 ± 0.01 min, respectively. In cases where the presence of nitrosamines is ambiguous, the product ions of NDMA (m/z 44 and 42 due to the loss of NO and deprotonation after the loss of NO from the molecular ions) and NDEA (m/z 85 due to protonation after the loss of O from the molecular ions and 56) have been utilized in MRM mode. The instrumental variables such as dwell time, collision energy and delay time are mentioned in Table S2. This method provides a reliable way to resolve uncertainties concerning the presence of nitrosamines.

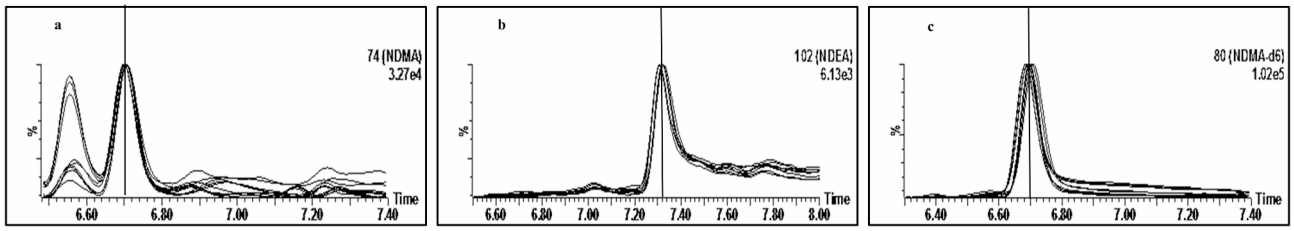

Fig. 1. The overlaid selected ion recording (SIR) chromatograms of the standard solution on different days of (**a**) NDMA with a concentration of 2.50 ng mL^{-1}, (**b**) NDMA-d6 with a concentration of 15.00 ng mL^{-1}, and (**c**) NDEA with a concentration of 2.47 ng mL^{-1}.

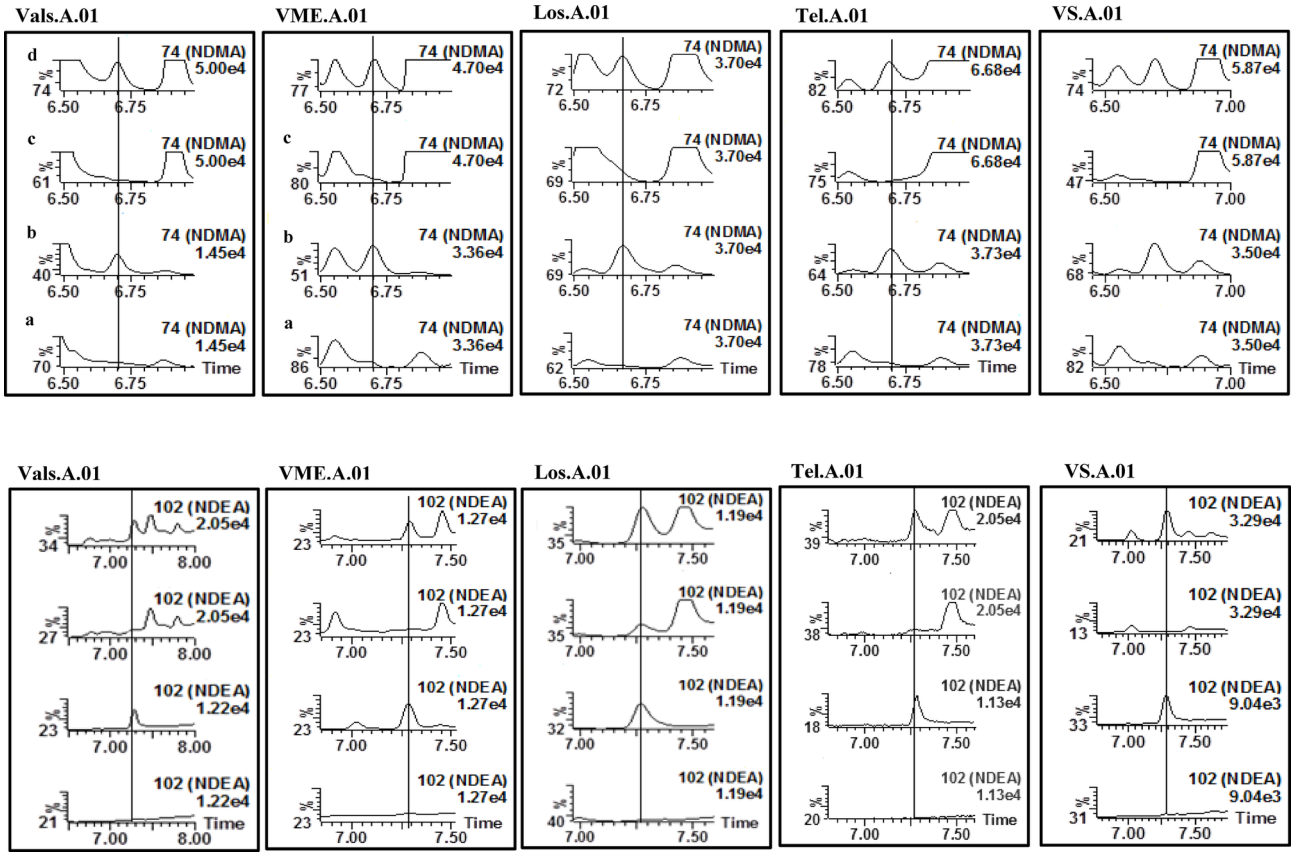

Fig. 2. Selected ion recording (SIR) chromatograms of the NDMA (first row) and NDEA (second row) in the real samples shown in Table 1. (**a**) DCM, (**b**) standard solution with a concentration of 2.50 ng mL^{-1} for NDMA and 2.47 ng mL^{-1} for NDEA, (**c**) blank sartans' matrices, (**d**) and spiked sartans' matrices at 2.50 and 2.47 ng mL^{-1} of NDMA and NDEA, respectively.

Method validation

Specificity

The specificity of the proposed method was evaluated in SIR mode. Since the interferences may include solvents, this was achieved by separately injecting DCM as the solvent and an authentic nitrosamine mixture standard solution with a concentration of 2.50 ng mL^{-1} for NDMA, 2.47 ng mL^{-1} for NDEA, and 15.00 ng mL^{-1} for NDMA-d6. Furthermore, blank matrices of sartans (API and tablet of valsartan, valsartan methyl ester, losartan, telmisartan, and sacubitril/valsartan listed in Table 1 and corresponding matrices spiked with both nitrosamines and internal standard were also injected. The results for Vals.A.01, Tel.A.01, VME.A.01, Los.A.01, and VS.A.01 samples in SIR mode for NDMA and NDEA are shown in Fig. 2. Similar results for the finished products are also shown in Fig. S2. As illustrated in these figures, no interferences were detected with the analytes' peak at their respective retention times (6.7 and 7.3 min). Thus, the ability of the proposed method to exclusively recognize and measure the target analytes in the selected sample matrixes was confirmed. If an additional peak was observed near the target retention time, MRM mode was employed to differentiate potential overlapping peaks from the target analytes. For instance, in VME.A.03, an interfering peak at 7.2 min, corresponding to NDEA,

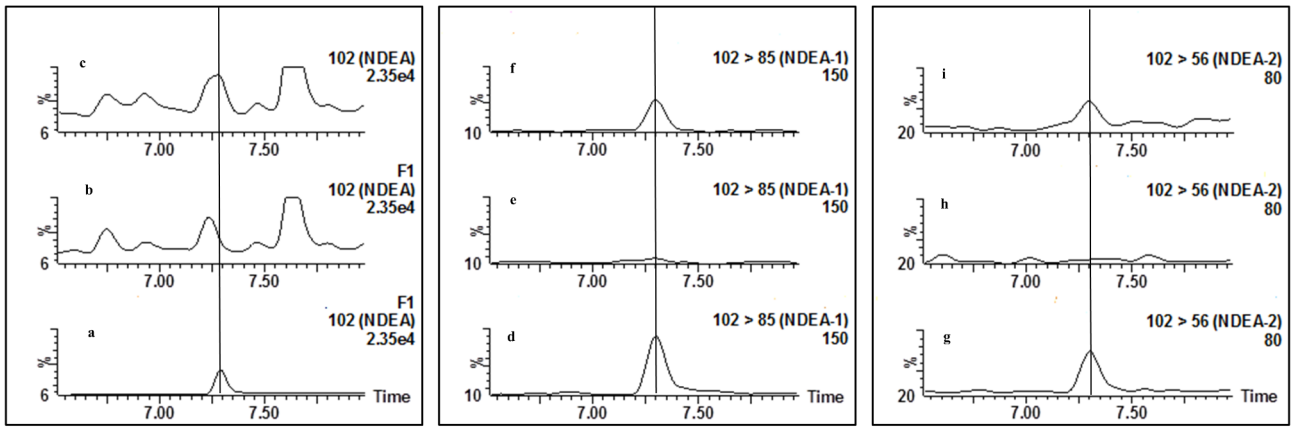

Fig. 3. Selected ion recording (SIR) chromatograms for (**a**) standard solution with a concentration of 9.9 ng mL^{-1}, (**b**) VME.A.03, (**c**) spiked VME.A.03 at 9.9 ng mL^{-1} of NDEA, MRM chromatograms with the mass transition of m/z 120 > 85 for (**d**) standard solution with a concentration of 9.9 ng mL^{-1}, (**e**) VME.A.03, (**f**) spiked VME.A.03 at 9.9 ng mL^{-1} of NDEA, and mass transition of m/z 102 > 56 for (**g**) standard solution with a concentration of 9.9 ng mL^{-1}, (**h**) VME.A.03, and (**i**) spiked VME.A.03 at 9.9 ng mL^{-1} of NDEA.

Sample	Line equation	Linear range (ng mL^{-1})	R^2	LOD[a] (ng mL^{-1})	LOQ[b] (ng mL^{-1})	Concentration (ng mL^{-1})	Intra-day ($n = 3$)		Inter-day ($n = 3$)	
							RR (%)	RSD[c] (%)	RR (%)	RSD (%)
NDMA	Y = 1.3419 x + 0.0331	2.50–40.00	0.9982	0.5	1.5	4.00	102	6	103	1
						18.00	107	4	120	7
						30.00	80	2	118	6
NDEA	Y = 0.7476 x − 0.0109	2.47–39.60	0.9980	0.1	0.3	3.96	110	8	102	3
						17.80	103	2	117	7
						29.70	87	5	120	12

Table 2. Analytical performance of the proposed GC-MS method for determination of NDMA and NDEA in sartans. [a]Limit of detection. [b]Limit of quantification. [c]Relative standard deviation.

was observed. Since the mass transition of NDEA includes m/z 102 > 85 and m/z 102 > 56 as quantifier and qualifier, respectively, both m/z values need to be observed in MRM mode. As depicted in Fig. 3, in VME.A.03, neither the first mass transition (102 > 85) nor the second mass transition (102 > 56), were detected. Based on the MRM chromatograms of the standard solution, which display both mass transitions, it can be inferred that the peak is not associated with NDEA.

Linearity range

To assess the linearity within the working standard range of the analyte, a set of solutions of the standard mixture of NDMA, NDEA, and NDMA-d6 in DCM were prepared, with concentrations ranging from 2.50 to 40.00 ng mL^{-1} for NDMA, and 2.47 to 39.60 ng mL^{-1} for NDEA, along with a concentration of 15.00 ng mL^{-1} for NDMA-d6. These solutions were subsequently injected directly into the GC-MS and the related results as shown in Fig. S3. Calibration curves were constructed based on the peak area ratio of NDMA (at m/z = 74) to NDMA-d6 (at m/z = 80) and NDEA (at m/z = 102) to NDMA-d6 (at m/z = 80) against the concentration ratio of each nitrosamine to NDMA-d6. The residual plots and ANOVA tests were then used to evaluate the validation characteristics. As shown in Fig. S4, the residual plots did not display any specific pattern, and the ANOVA results for both analytes (Tables S3, S4) indicated significant regression (F-value 4403 and 2470 for NDMA and NDEA, respectively), and nonsignificant bias (p-value 0.26 and 0.62 for NDMA and NDEA, respectively) at the 95% confidence limit. Additionally, the coefficient of determination (R^2) was greater than 0.998 for each nitrosamine. Table 2 provides analytical performance of the proposed GC-MS method for determination of NDMA and NDEA in sartans samples.

Limit of detection and quantification

Based on the description given in Sections "Method" and "Method validation", the LOD and LOQ for NDMA were determined to be 0.5 and 1.5 ng mL^{-1} respectively, while the corresponding values for NDEA were 0.1 and 0. 3 ng mL^{-1}, respectively (Table 2). As previously mentioned, the sample preparation involved adding 5 mL of

DCM to 500 mg of real sample. Based on this method, the LOD and LOQ values can be reported as 0.005 and 0.015 μg g^{-1} for NDMA and 0.001 and 0.003 μg g^{-1} for NDEA. These values are less than the acceptable limits of these impurities in sartans, which are reported as 0.3 and 0.083 μg g^{-1} in valsartan, 0.96 and 0.27 μg g^{-1} in losartan, and 1.2 and 0.33 μg g^{-1} in telmisartan, for NDMA and NDEA, respectively[51].

Accuracy and precision

The accuracy and precision of the proposed method were assessed by determining the relative recovery (%RR) and relative standard deviation (%RSD). Standard mixture solutions containing the analytes were prepared at three concentration levels (4.00, 18.00, and 30.00 ng mL^{-1} for NDMA and 3.96, 17.80, and 29.70 ng mL^{-1} for NDEA) along with a concentration of 15.00 ng mL^{-1} for NDMA-d6. The experiment was conducted on one day and over three different days. As illustrated in Table 2, the %RRs of NDMA and NDEA ranged from 80 to 120% and 87–120%, respectively, with %RSD of less than 12.

System suitability

In a report by the FDA[15], specific criteria were outlined to evaluate the suitability of a validated method. These criteria include a coefficient of determination of 0.998, a relative standard deviation (RSD %) of 5% for the peak area of six replicate injections of the 40.00 ng mL^{-1} standard, and a signal-to-noise ratio of 10 for the nitrosamines' standard of 5.00 ng mL^{-1}. The proposed method and the results obtained were evaluated against these parameters. As demonstrated in Table 3, the obtained data confirms that the method is suitable.

Furthermore, in accordance to the ICH protocol[52], control charts were used as an additional statistical tool for quality risk management during the analysis. In this study, control charts were plotted for the peak area of NDMA at a concentration of 2.50 ng mL^{-1} and NDEA at a concentration of 2.47 ng mL^{-1}, which were frequently injected, were plotted, as shown in Fig. S5. Most of the areas fell between the Upper Control Limit (UCL) and the Lower Control Limit (LCL) and exhibited a normal deviation. These findings suggest that the proposed method is suitable for the simultaneous estimation of two nitrosamine impurities in APIs and finished products of sartans. Also, the use of the internal standard has reduced these changes as well.

Analysis of real samples

To evaluate the effectiveness of the proposed GC-MS method for routine analysis in the pharmaceutical industry, an investigation was conducted to detect the presence of NDMA and NDEA (one or both) in the APIs and finished products of sartans manufactured by 11 different companies. The results of the analysis of real samples have been presented in Table 1 with each value determined through three repetitions. In most of the samples examined, the NAs were not detectable (ND). Among the 84 samples examined, NDMA was detected in two API samples and two finished products at concentrations ranging from 0.106 to 0.108 μg g^{-1} and 1.67–1.79 μg g^{-1}, respectively. Additionally, NDEA in some of API samples (are related to 2019 when monitoring of these contaminants in the sartans family began) was found to be in the range of 0.0064–6.62 μg g^{-1} and was not seen in any finished product. The chromatograms of two samples, VME.A.08 for NDMA and Vals.A.14 for NDEA, are presented in Fig. S6 as an example. These findings provide valuable insights into the quality control of sartans in the pharmaceutical industry.

Comparison with other methods

Finally, a comparison was made between the method presented in this study and previously published methods for the analysis of nitrosamine impurities in pharmaceuticals, as listed in Table 4. The obtained LOQ using the proposed method is better than some of published reports based on GC-MS or GC-MS/MS method and covers the acceptance limits for these two nitrosamines[18,35,41,42]. The LODs of FDA's report for direct injection with GC-MS/MS (0.005 and 0.008 μg g^{-1} for NDMA and 0.001 and 0.002 μg g^{-1} for NDEA in drug substance and finished product, respectively)[15], are comparable with the obtained results in this study. The scope of the proposed method for analysis of two main nitrosamines (in API and finished products of valsartan, losartan, telmisartan, valsartan methyl ester and Sacubitril/Valsartan) is greater than the other published reports using GC-MS or GC-MS/MS method[18,35,41,42]. Moreover, the proposed method does not require special equipment such as headspace accessory or solid phase extraction cartridges with different sorbents.

Although the LOQ in reports that used LC-MS/MS[20,30,53] as analysis method are similar to the obtained results in this study, in practice the results of the proposed GC-MS method are less dependent on instrumental variables and more robust than LC-MS/MS method. For example, in LC-MS/MS, the presence of any contamination or matrix causes ion suppression, decreases intensity and lacks LOQ. Thus, the proposed approach, utilizing GC-MS, proves to be a more simple, cost-effective, and convenient alternative for analyzing of NDMA and NDEA in the large number of API and pharmaceutical formulations containing sartans and its implementation in the quality control laboratories of pharmaceutical manufacturers enables.

Parameters	NDMA	NDEA	FDA acceptance criteria[10]
Coefficient of determination (R^2)	0.9982	0.998	≥ 0.998
% RSD[a] of six replicate injections of the 40.00 ng mL^{-1} NDMA and 39.60 ng mL^{-1} NDEA ($n = 6$)	3.0	2.8	≤ 5
The S/N ratio of the 5.00 and 4.95 ng mL^{-1} linearity standard of NDMA and NDEA, respectively ($n = 3$)	34 ± 4[b]	70 ± 3	≥ 10

Table 3. System suitability of the proposed GC-MS method for determination of NDMA and NDEA in sartans. [a]Relative standard deviation. [b]Standard deviation.

Drug	Real sample type	Impurity	Sample preparation method	Analysis method	LOQ*	Linear range*/Recovery	Ref
Valsartan	API[a] and FPs[b]	NDMA[c]	LSE[d]	GC-MS[e]	0.5 μg g^{-1}	0.005–0.2 μg mL^{-1}/99%	41
Losartan	API	4 NAs	LSE	HS-GC-MS[f]	25 ppb (ng g^{-1})**	25–5000 ng mL^{-1}/Accuracy deviation: – 6.47–7.25%	42
Valsartan	API	4 NAs	LSE	GC-MS/MS	0.06–0.09 ppm (μg g^{-1})**	0.093–0.464 and 0.062–0.461 ppm/101–103 and 88–102%	35
Candesartan cilexetil, Olmesartan medoxomil Valsartan, Irbesartan	API	4 NAs[g]	LSE	GC-MS/MS[h]	0.008–0.500 ppm (μg g^{-1})**	3-60 and 0.8–16 ng mL^{-1}/88–124%	18
Valsartan, Irbesartan	APIs and FPs	11 NAs	LSE	LC-APCI-MS/MS[i]	0.008–0.05 ppm (μg g^{-1})**	0.5–50 ng mL^{-1}/73–115 and 80–128%	30
Losartan, Hydrochlorothiazide	FPs	8 NAs	LSE	UPLC-TQ-MS/MS[j]	0.5 ng mL^{-1} 0.005 μg g^{-1}	1–100 ng mL^{-1}/95–97 and 99–103%	53
Valsartan, Losartan, Irbesartan	APIs and FPs	12 NAs	LSE	LC-TQ-MS/MS[k]	20–50 ng g^{-1}	2.5–50 ng mL^{-1}/80–120%	20
Sartans	API	8 NAs	LSE	LC-APCI-MS/MS	1.07–1.27 ng mL^{-1}	2–100 ng mL^{-1}/93–103%	53
Valsartan, Losartan, Telmisartan Valsartan sacubitril Valsartan methyl ester	API and FPs	NDMA and NDEA	LSE	GC-MS	0.015 and 0.003 μg g^{-1} or 1.5 and 0.3 ng mL^{-1}	1.5–40 and 0.3–40 ng mL^{-1}/80–120 and 87–120%	Current study

Table 4. Comparison of the proposed GC-MS method with other reported methods for analysis of NAs in pharmaceutical products. *The units are exactly the numbers given in the articles, **It has been changed for uniformity. [a]Active pharmaceutical ingredients (API), [b]Finished products (FPs), [c]N-Nitrosodimethylamine (NDMA), [d]Liquid solid extraction (LSE), [e]Gas chromatography–mass spectrometry (GC-MS), [f]Headspace (HS), [g]N-Nitrosamines (NAs), [h]Gas chromatography-tandem mass spectrometry (GC-MS/MS), [i]liquid chromatography-atmospheric pressure chemical ionization-tandem mass spectrometry (LC-APCI-MS/MS) and [j]Ultra-performance liquid chromatography-triple quadrupole-mass spectrometry (UPLC-TQ-MS/MS) and [k]Liquid chromatography-triple quadrupole-mass spectrometry (UPLC-TQ-MS/MS).

Monte Carlo simulation analysis for Health risk assessment

Input parameters in simulation for risk assessment calculation are AF and LFC with 1 ratio, ED based on the treatment period is 2 months, and typical life time (LF) is 70 and 6 for adults and children, respectively. C is the average nitrosamine impurity in the form of sartans (Table 1).

Non-carcinogenic risk

The rank order of estimated HQ based on Section "Health risk assessment" description for nitrosamines in adult and children consumers in valsartan consumption was 4.92×10^{-9} and 1.10×10^{-4}, respectively (Fig. 4a and b). These amounts in losartan consumption were 9.71×10^{-6} for adults, and 1.94×10^{-4} for children (Fig. 4c and d). Based on the risk assessment results (HI < 1) in investigated drugs, it can be concluded that the consumption of these drugs in both age groups may not be exposed to health risks.

Carcinogenic risk

A cancer dietary assessment was conducted for the Iranian population to estimate the carcinogenic risk from nitrosamine exposure. The assessment multiplies the average exposure over 70 years by the cancer potency factor (Q1*), yielding a non-unit number that represents the excess cancers potentially attributed to lifelong exposure to nitrosamine impurities. To dietary cancer risk assessment aims to estimate risk at a level typically below the EPA's concern threshold of one in million (1×10^{-6}) ; however, the agency generally considers risks up to 10^{-6} to fall within the low-risk category and beneath the agency's criteria for action. A Q1* represents an upper bound estimate of cancer risk. As illustrated in Fig. 4e and f, the risk levels for valsartan at 1.15×10^{-8} and for losartan at 2.04×10^{-8} suggest they are not likely carcinogens to humans.

Conclusion

The developed GC-MS method is rapid (preparation time less than 5 min and analysis time under 14 min), selective (no interfering matrix coelution at the retention time of two impurities), accurate (relative recovery ranging from 80 to 120%), and precise (relative standard deviation ranging from 1 to 12%) for simultaneous analysis of NDMA and NDEA in the sartan APIs and finished products. Additionally, the acceptance criteria of system suitability have been confirmed for the proposed method. Moreover, control chart of NDMA and NDEA peak has shown good reproducibility over about 18 months. Thus, this method can serve as an additional option for regulatory-purpose analysis for NDMA and NDEA in the drug substances and finished products of sartans for pharmaceutical companies and researchers. Based on the levels of nitrosamine impurities in five types of sartan samples, including valsartan, losartan, telmisartan, valsartan methyl ester, and sacubitril/valsartan in 84 real samples, a Monte Carlo simulation-based human health risk assessment was conducted and indicated no significant risk of adverse health effects from these impurities in the tested drugs for adult and child consumers.

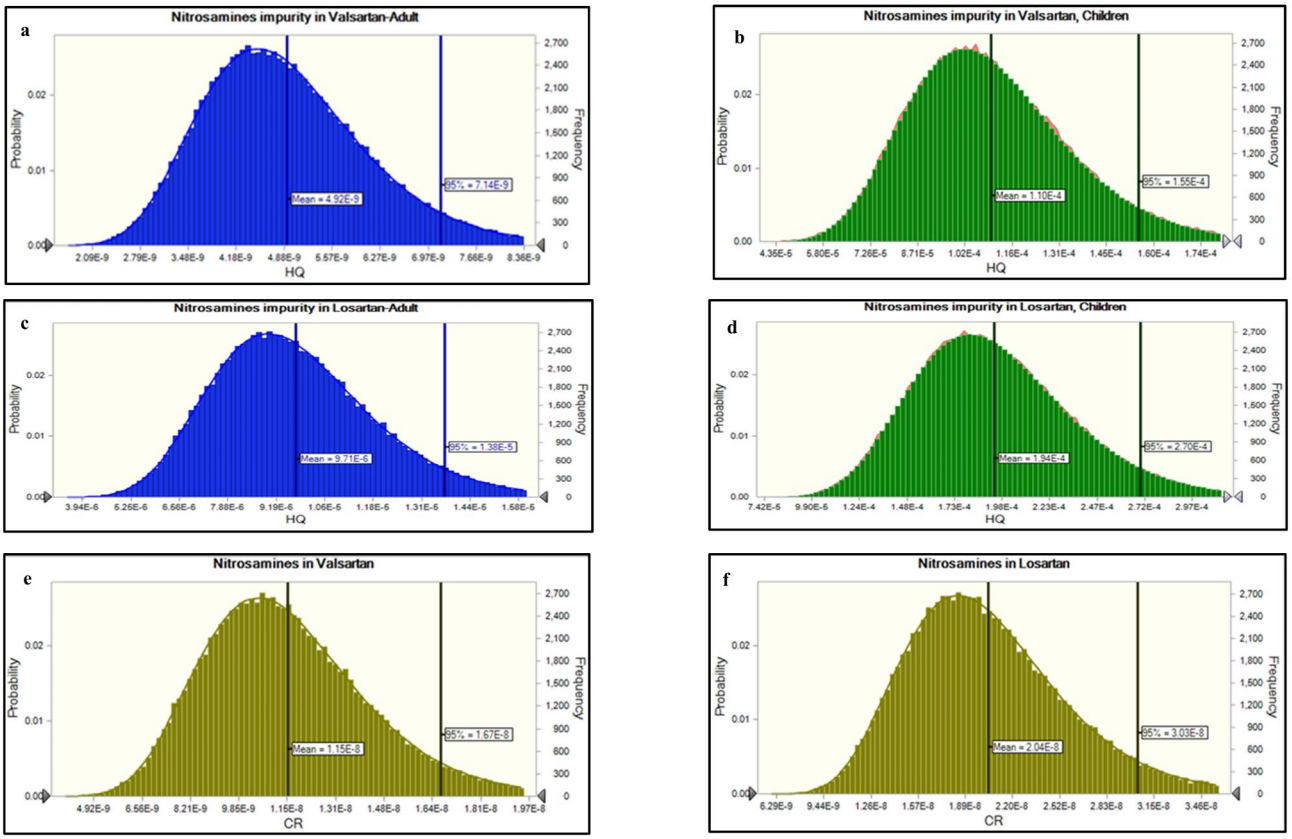

Fig. 4. HQ for (**a**) and (**c**) adults and (**b**) and (**d**) children in Iran due to nitrosamine impurity in valsartan and losartan drug samples, respectively. CR due to nitrosamine impurity in (**e**) valsartan and (**f**) losartan drug samples for consumers in Iran.

Data availability

All data generated or analyzed during this study are included in this published article and its supplementary information files.

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Author contributions

Z.T. and N.A. conceived the study and contributed to conceptualization. Z.T. administered the project and supervised the work. F.S. performed formal analysis and wrote the original draft. G.A. performed formal analysis, data processing, and contributed to writing, review, and editing. N.M. and S.M. performed formal analysis. M.K. contributed to review and editing. V.M. conducted risk assessment analysis. All authors reviewed the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

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