DEVELOPMENT AND APPLICATION OF GC-MS/MS METHOD FOR DETERMINATION OF FOUR NITROSAMINE IMPURITIES IN TWO SARTAN DRUG PRODUCTS AS PER PROCEDURE 4, USP GENERAL CHAPTER <1469>

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Received April 21st, 2025
Accepted June 4th, 2025

Abstract: *This study demonstrates that the chromatographic, mass spectrometric, and sample preparation conditions outlined in Procedure 4, USP General Chapter <1469> are compatible with the available laboratory equipment at the National Institute of Drug Quality Control for the analysis of four nitrosamine impurities: NDMA, NDEA, NEIPA, and NDIPA. We also validated the method on two finished pharmaceutical product matrices: film-coated tablets containing Losartan potassium and tablets containing Telmisartan. The results showed that the validation criteria met the requirements for both sample matrices. The method validation achieved limits of quantification (LOQ) of 0.01 to 0.03 µg/g, linearity ranges from 0.5 - 15.0 ng/mL or 5 - 100/200 ng/mL, recovery of 99.3% and 114.5%, and precision with relative standard deviations (RSDs) of 2.5% to 12.8%, indicating suitability for determining four impurities in the two studied finished products. Throughout the experiments, it was observed that the sample matrix significantly affects the nitrosamine impurity analysis. The method was then applied to analyze the four nitrosamine impurities in 13 finished product samples containing Losartan potassium and Telmisartan available on the market.*

Keywords: *Nitrosamine, Sartan, GC-MS/MS*

1. INTRODUCTION

N-nitroso compounds are among the structural groups of high potency mutagenic carcinogens in several animal species, and some are classified as probable or possible human carcinogens referred to as the "cohort of concern" in ICH M7 [5]. In recent years, nitrosamines have been detected in various pharmaceutical products such as antihypertensive drugs, diabetes medications, and H2 histamine receptor antagonists, etc. Currently, several analytical methods using mass spectrometry techniques have been developed worldwide for the detection of nitrosamine impurities. Some pharmacopoeias have officially published general chapters (USP GC <1469>, BP Appendix VIII) [1,2] outlining procedures for testing four nitrosamine impurities (N-nitrosodimethylamine [NDMA], N-nitrosodiethylamine [NDEA], N-nitrosoethylisopropylamine [NEIPA], N-nitrosodiisopropylamine [NDIPA]) in Sartan active pharmaceutical ingredients, but no specific methods for finished products have yet been established.

Based on the currently available equipment, we conducted the study: "*Development and Application of GC-MS/MS Method for Determination of four Nitrosamine Impurities in two Sartan Drug Products as per Procedure 4, USP General Chapter <1469>*", in order to assess the suitability of applying this method to finished product matrices.

2. EXPERIMENTAL

2.1. Equipments, Instruments, Chemicals, and Reference Standards

2.1.1. Equipments and Instruments

Gas chromatographymass spectrometry system (GC-MS/MS triple-quad, Agilent 8890, MS/MS detector Agilent 7010B), Agilent VF-WAXms column (30 m x 0.25 mm, 1 µm) SN: NL007388327 or equivalent. Mettler Toledo analytical balance (devision 0.1 mg), Sigma 4 – 16 KS centrifuge, Velp scientifica vortex mixer, Eppendorf

micropipettes: 10 – 100 µL, 100 – 1000 µL, volumetric flasks, class A glass pipettes, etc.

All equipments and instruments were periodically calibrated according to ISO/IEC 17025 and GLP standards.

2.1.2. Chemicals and Reference Standards

- Reference standards: Information on reference standards is provided in Table 1.

Table 1. *Information on Reference Standards*

Name	Origin	Batch No.	Concentration
N-nitrosodimethylamine (NDMA)	USP	R15630	1.00 mg/mL
N-nitrosodiethylamine (NDEA)	USP	R15620	1.01 mg/mL
N-nitrosoethylisopropylamine (NEIPA)	USP	R155F0	1.00 mg/mL
N-nitrosodiisopropylamine (NDIPA)	USP	R155E0	1.01 mg/mL
NDMA (13C2 99%, d6 98%)	Cambridge Isotope Laboratories	SDJC05A	1 mg/mL

- Solvent (Methylen chloride): analytical grade (Merck).

2.2. Subjects and Research Methods

2.2.1. Research subjects

- *Test samples:* Finished products containing Losartan potassium or Telmisartan collected from the market. Each sample was from a different manufacturer or a different production batch.

Table 2. *List of Samples*

No.	Code	Dosage Form	Active ingredient	Concentration (mg)
1	M1	Film-coated tablet	Losartan kali	50
2	M2	Tablet	Telmisartan	80
3	M3	Film-coated tablet	Losartan kali	50
4	M4	Tablet	Telmisartan	40
5	M5	Tablet	Telmisartan	40
6	M6	Film-coated tablet	Losartan kali	50
7	M7	Tablet	Telmisartan	40
8	M8	Film-coated tablet	Losartan kali	50
9	M9	Tablet	Telmisartan	80
10	M10	Film-coated tablet	Telmisartan	40
11	M11	Film-coated tablet	Losartan kali	50
12	M12	Tablet	Telmisartan	40
13	M13	Film-coated tablet	Losartan kali	50

Note*: Sample M1 and M2 were used for method development and validation. Sample M3 to M13 were used to determine nitrosamine impurities.*

- *Spiked Samples*: prepared by adding nitrosamine standards at various concentrations.

2.2.2. Research Methods

Based on *Procedure 4, USP General Chapter <1469>* [2], chromatographic conditions, mass spectrometry parameters, and sample preparation procedures were investigated to optimize and adapt the method to the available laboratory conditions.

2.2.2.1. Analytical Procedure

** Chromatographic Conditions:*

- VF-WAXms column (30 m x 0.25 mm, 1µm)

- Temperature: Injector: 250°C, Transfer line to MS detector: 250°C, Ionization source: 250°C

Column: 40°C held for 0.5 minutes, ramped at 20°C/min to 200°C, then ramped at 60°C/min to 250°C, and held for 3 minutes

- Carrier gas: Helium

- Flow rate: 1.0 mL/min

- Injection volume: 2 µL

- Detector: MS/MS (triple quadrupole mass spectrometer)

Table 3. *Mass Spectrometry Conditions*

Ionization	Electron Ion, 70 eV
Temperature Source	230^0C
Temperature Quad	$Q_1 = Q_2 = 150^0$C
MRM	
Collision gas Flow	Nitrogen 1.5 mL/min
Quenching gas Flow	Helium 4 mL/min
NDMA	74 -> 44, CE 6, Dwell 150 ms 74 -> 42, CE 22, Dwell 150 ms
NDEA	102 -> 85, CE 4, Dwell 150 ms 102 -> 56, CE 18, Dwell 150 ms
NDMA: C13-d6	82 -> 48, CE 15, Dwell 100 ms
NEIPA	116 -> 99, CE 5, Dwell 150 ms 71 -> 56, CE 5, Dwell 150 ms
NDIPA	130 -> 88, CE 5, Dwell 150 ms 130 -> 42, CE 10, Dwell 150 ms

* Sample Preparation Procedure:

- Sample diluent (blank sample): Methylen chloride

- *Internal standard solution:* 50 ng/mL of NDMA: C13-d6 in methylene chloride (50 ppb).

- *Mixed stock standard solution:* Prepare a 1 µg/mL mixed standard solution of each of the four nitrosamines (NDMA, NDEA, NEIPA, NDIPA) by appropriately diluting USP standard solutions in methylene chloride (Accurately pipette 100 µL of each 1 mg/mL NDMA, NDEA, NEIPA, and NDIPA standard solution into a 100 mL volumetric flask. Make up to volume with methylene chloride and mix well).

- *Working standard solutions:* Prepare 12 mixed standard solutions of the 4 nitrosamines (NDMA, NDEA, NEIPA, and NDIPA) with concentrations ranging from 0.5 ppb to 200 ppb, and with the internal standard at a concentration of 50 ppb. Select appropriate concentrations depending on the targeted nitrosamine concentration in the sample.

- *Sample solution:* Accurately weigh an amount of the sample powder equivalent to 500 mg of drug substance (Losartan/Telmisartan) into a 15 mL centrifuge tube. Add 5.0 mL of the internal standard solution. Vortex for 1 minute, and centrifuge at 11,000 rpm for 10 minutes. Filter the supernatant through a 0.45 µm membrane filter into an HPLC vial.

2.2.2.2. Method Validation

The method was validated following USP guidelines for analytical procedures for impurities [3], including the following parameters: specificity/selectivity, limit of detection, limit of quantification, linearity, accuracy, repeatability, intermediate precision, and method robustness.

3. RESULTS AND DISCUSSION

3.1. Chromatographic Conditions

Chromatographic conditions were applied as specified in Procedure 4, USP General Chapter <1469>.

3.2. Method Validation

3.2.1. System Suitability

Inject the 40ppb mixed reference standard solution into chromatographic system repeatedly 6 times, record the chromatograms The result of system suitability evaluation are presented in the Table 4.

Table 4. *System Suitability Results*

Nitrosamine	Retention Time		Response Ratio		Ion intensity ratios (n = 6)	
	mean ± SD	RSD (%), n = 6	mean ± SD	RSD (%), n = 6	mean ± SD	RSD
NDMA	7.443 ± 0.0005	0.01	2.092 ± 0.047	2.26	0.51 ± 0.006	1.11
NDEA	8.038 ± 0.0005	0.01	1.114 ± 0.010	0.91	0.37 ± 0.002	0.41
NEIPA	8.317 ± 0.000	0.00	2.075 ± 0.038	1.86	0.62 ± 0.003	0.46
NDIPA	8.506 ± 0.000	0.00	1.210 ± 0.031	2.59	0.64 ± 0.009	1.42

Results showed that: All parameters met the acceptance criteria (RSD of retention time < 1.0%; RSD of response and ionic intensity ratios < 20.0%). Therefore, the system is stable and suitable for the quantification of nitrosamine impurities.

3.4.2. Specificity

Blank, samples, mixed standard, individual nitrosamine standards, and spiked solutions were analyzed following analytical procedure. The results showed:

- Blank chromatogram (without internal standards): No interfering peaks with the characteristic ion fragments of nitrosamines and internal standards were observed at retention times corresponding to those in the mixed standard chromatogram.

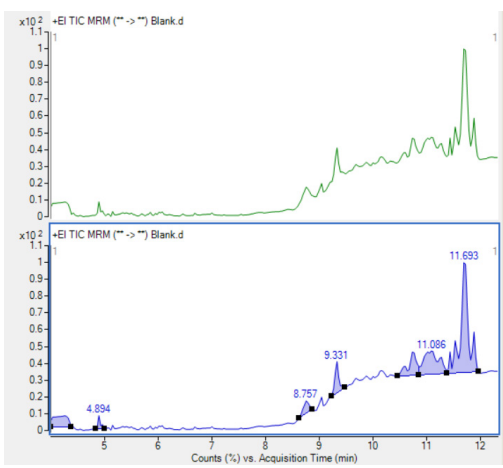
- Sample chromatogram (without internal standards): No interfering peaks with the characteristic ion fragments of nitrosamines and internal standards were observed at retention times corresponding to those in the internal standard chromatogram.

- Individual nitrosamine standard chromatogram: only one peak, with fragment ion ratios matching those expected for that specific nitrosamine.

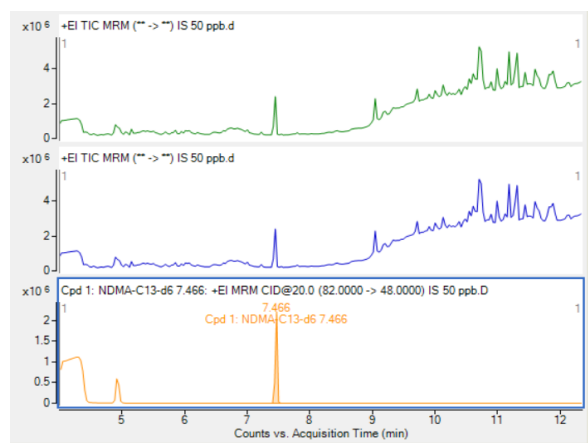
- Internal standard chromatogram: no peaks at retention times corresponding to those of the nitrosamines in the chromatograms of the individual nitrosamine standards.

- Spiked samples chromatogram: The nitrosamine and internal standard peaks were clearly identifiable, with retention times and ionic strength ratios consistent with those observed in the mixed standard chromatogram.

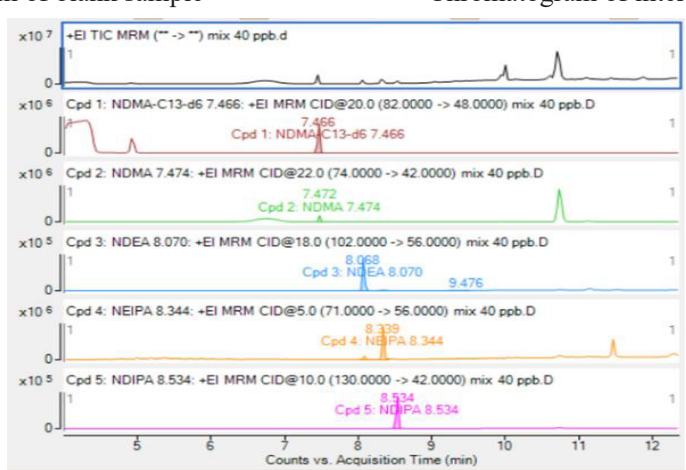
Therefore, the method is specific and selective for the nitrosamines and internal standard under investigation.



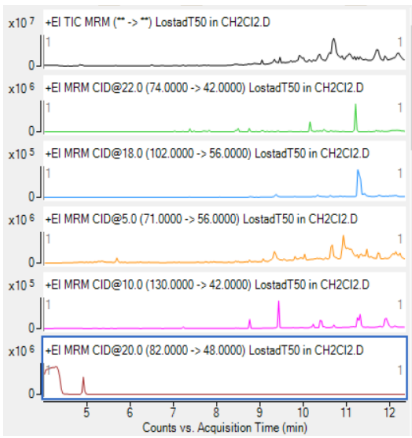
Chromatogram of blank sample



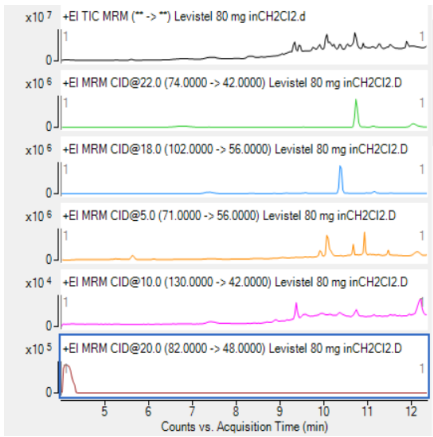
Chromatogram of internal standard sample



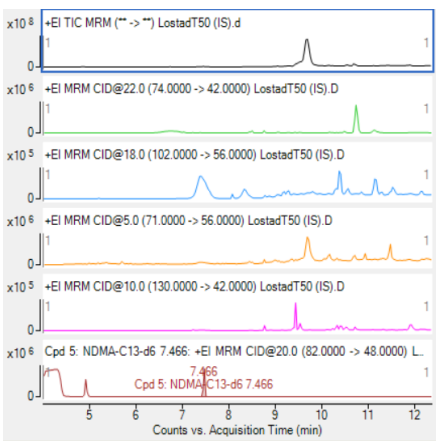
Chromatogram of 40 ppb standard sample



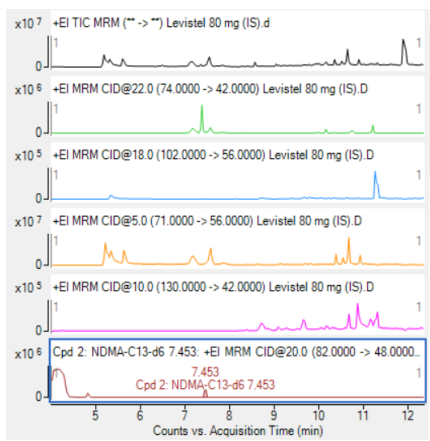
M1 sample without internal standard



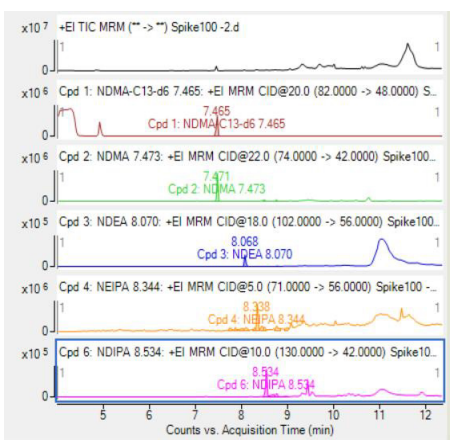
M2 sample without internal standard



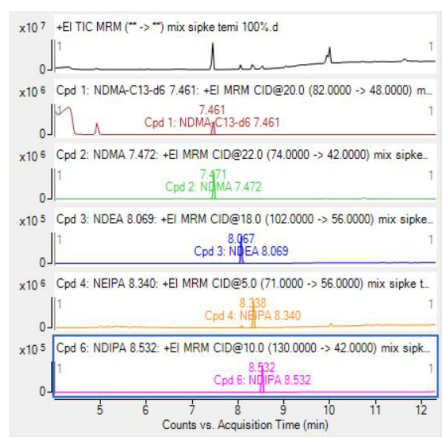
Chromatogram of film-coated tablet sample (M1)



Chromatogram of tablet sample (M2)



Chromatogram of M1 spiked sample



Chromatogram of M2 spiked sample

Figure 4. *Representative Chromatograms of Specificity*

(Blank sample; Internal standard sample; Mixed standard sample; M1 sample without IS, M2 sample without IS, M1 sample, M2 sample, M1 spiked sample, M2 spiked sample)

3.4.3. Limit of Detection (LOD) and Limit of Quantitation (LOQ)

Using standard solutions at concentrations of 0.5 ppb, 1 ppb, 2.5 ppb, 3.75 ppb, 5 ppb, and 10 ppb, a low-concentration linear range was established. From the slope (S) and intercept (a) of the calibration curve, the theoretical LOD and LOQ values of the method were calculated. Based on the determined LOD and LOQ values, spiked samples were prepared at the LOD and LOQ levels and injected into the chromatographic system. Results showed that: In the chromatograms of samples at the LOD concentration, the peaks of the four nitrosamine impurities were clearly identified, with retention times corresponding to those obtained from the standard solution. The RSD values of the response ratio of each impurity peak to the internal standard were ≤ 20.0%. At the LOQ concentration, in matrix M1, the recovery (107.8% - 119.6%) and repeatability (RSD < 20%) for the four nitrosamines met the criteria. In matrix M2, the recovery (105.0% - 122.8%) and repeatability (RSD < 20%) for the four nitrosamines also met the criteria.

Based on the experimental data, the LOD and LOQ values are summarized in Table 6:

Table 6. *LOD, LOQ of each nitrosamine*

Nitrosamine	M1 sample matrix		M2 sample matrix	
	LOD (ng/g)	LOQ (ng/g)	LOD (ng/g)	LOQ (ng/g)
NDMA	3.00	10.00	9.00	30.00
NDEA	3.03	10.10	9.09	30.30
NEIPA	9.00	30.00	9.00	30.00
NDIPA	3.64	12.12	3.64	12.12

3.4.4. Calibration Curve and Linearity

A series of 12 standard solutions with concentrations ranging from 0.5 ppb to 200 ppb of the 4 nitrosamine impurities were analyzed. The results of the linearity assessment for each nitrosamines are presented in Table 7.

Table 7. *Results of calibration curve and linearity*

Name	Concentration range (ng/mL)	Linear regression equation (y = ax + b)	Correlation coefficient (r)	% y-intercept
NDMA	0.5 – 15.0	y = 0.0526x + 0.005	0.9998	0.24
	5.0 – 200.0	y = 0.0512x + 0.025	1.0000	1.56
NDEA	0.5 – 15.0	y = 0.0285x – 0.002	0.9997	1.47
	5.0 – 100.0	y = 0.0288x – 0.016	0.9999	2.91
NEIPA	0.5 – 15.0	y = 0.0509x + 0.0022	0.9994	1.13
	5.0 – 100.0	y = 0.0511x + 0.0134	0.9999	1.77
NDIPA	0.5 – 15.0	y = 0.0293x – 0.0007	0.9996	0.59
	5.0 – 100.0	y = 0.0302x – 0.0087	1.0000	1.43

The experimental results showed that the correlation coefficients of all four nitrosamines were greater than 0.990, and the % y-intercept values were within the acceptable limits. This indicates a linear correlation between the analyte concentrations and the peak area ratios within the studied concentration range (For samples with low nitrosamine impurity levels, the linear range of 0.5 ng/mL to 15.0 ng/mL was used, for samples with higher nitrosamine impurity content, the linear range of 5.0 ng/mL to 100/200 ng/mL was applied).

3.4.5. Accuracy and Repeatability of the Method

The accuracy and repeatability of the method were evaluated using spiked samples, in which a known volume of stock standard solution was added to the test samples to achieve three spiking levels: low (LOQ), medium (acceptable nitrosamine limit), and high (approximately 120%/130% of the acceptable nitrosamine limit). At each level, six replicates were analyzed. The results are presented in Table 8 and Table 9.

Table 8. *Accuracy and Repeatability - M1 sample matrix*

Nitrosamine	LOQ concentration		Medium concentration		High concentration (120%)	
	Recovery (mean ± SD) (n = 6)	*RSD (%, n = 6)*	*Recovery (mean ± SD) (n = 6)*	*RSD (%, n = 6)*	*Recovery (mean ± SD) (n = 6)*	*RSD (%, n = 6)*
NDMA	107.8 ± 11.5	10.7	99.3 ± 2.0	2.1	99.8 ± 2.8	2.8
NDEA	119.6 ± 2.0	1.7	104.1 ± 4.0	3.8	111.2 ± 2.2	2.0
NEIPA	111.7 ± 6.0	5.4	106.4 ± 4.5	4.2	114.1 ± 3.5	3.1
NDIPA	113.9 ± 4.7	4.1	109.7 ± 7.0	6.4	114.5 ± 2.7	2.4

Table 9. *Accuracy and Repeatability – M2 sample matrix*

Nitrosamine	LOQ concentration		Medium concentration		High concentration (130%)	
	Recovery (mean ± SD) (n = 6)	*RSD (%, n = 6)*	*Recovery (mean ± SD) (n = 6)*	*RSD (%, n = 6)*	*Recovery (mean ± SD) (n = 6)*	*RSD (%, n = 6)*
NDMA	105.0 ± 10.9	10.4	99.8 ± 3.5	3.5	99.1 ± 3.3	3.4
NDEA	109.4 ± 3.1	2.8	105.4 ± 3.7	3.5	102.0 ± 4.0	4.0
NEIPA	118.8 ± 5.8	4.9	113.2 ± 3.4	3.0	111.7 ± 4.7	4.2
NDIPA	122.8 ± 9.4	7.7	96.8 ± 2.9	3.0	93.5 ± 3.1	3.3

The results in Table 8 and Table 9 show that at LOQ, medium, and high concentration levels of the spiked samples, the recovery of the four nitrosamines in both sample matrices were within the acceptable range (70% – 130%). The repeatability also met the USP requirements (RSD ≤ 20%).

3.4.6. Precision

Method precision includes both repeatability and intermediate precision. Intermediate precision was evaluated using spiked samples at the medium concentration level, with the analyses performed on different days by different analysts. The results are presented in Table 10.

Table 10. *Result of method precision*

Name	M1 sample matrix		M2 sample matrix	
	Recovery (%) (mean ± SD) (n = 12)	*RSD (%) (n = 12)*	*Recovery (%) (mean ± SD) (n = 12)*	*RSD (%) (n = 12)*
NDMA	101.1 ± 2.5	2.5	103.4 ± 10.0	9.6
NDEA	109.0 ± 5.9	5.4	112.2 ± 14.3	12.8
NEIPA	109.8 ± 4.8	4.4	111.3 ± 5.2	4.7
NDIPA	114.3 ± 7.1	6.2	114.0 ± 7.7	6.8

The results showed that the intermediate precision of the method met the criteria, with RSD values (n = 12) < 25% for all four nitrosamines in both sample matrices.

3.4.7. Robustness

The robustness of the method was assessed by examining system suitability and recovery of medium-concentration spiked samples under slight variations of chromatographic conditions: Column temperature: ± 4^0C, flow rate: ± 10%. Recovery results of nitrosamines were compared with recovery values in Section ***3.4.5. Accuracy and Repeatability of the Method***. Acceptance criteria required the RSD to be ≤ 20%. The results are summarized in Table 11.

Table 11. *Method robustness*

Nitrosamine		NDMA	NDEA	NEIPA	NDIPA
Flow rate 1.0 + 10% (mL/min)	**System suitability**	Accept	Accept	Accept	Accept
	Recovery (%)	99.9	105.2	106.6	110.1
	RSD (%)	2.3	3.4	3.4	5.2
Flow rate 1.0 - 10% (mL/min)	**System suitability**	Accept	Accept	Accept	Accept
	Recovery (%)	100.0	105.4	107.3	110.1
	RSD (%)	2.30	3.5	3.6	5.2
Column temperature ($40 + 4^0$C)	**System suitability**	Accept	Accept	Accept	Accept
	Recovery (%)	100.5	105.0	106.4	108.9
	RSD (%)	2.6	3.3	3.4	5.4
Column temperature ($40 - 4^0$C)	**System suitability**	Accept	Accept	Accept	Accept
	Recovery (%)	100.7	105.2	107.0	110.4
	RSD (%)	2.8	3.4	3.5	5.3

The results shown in Table 12 confirm the robustness of the method under changes. Thus, chromatographic conditions can change as follow: Column temperature: ± 4^0C, flow rate: ± 10%.

3.5. Application

Following the development and validation of the analytical method, Procedure 4, USP General Chapter <1469> was applied for the analysis of nitrosamine impurities in 13 commercially available finished pharmaceutical products containing Losartan potassium and Telmisartan. The results are presented in Table 12.

Table 12. *Analysis of nitrosamine impurities in several pharmaceutical products*

Sample	NDMA	NDEA	NEIPA	NDIPA
M1	Below LOQ (10.0 ppb)	ND	ND	ND
M2	**90.89 ppb**	ND	ND	ND
M3	Below LOQ (10.0 ppb)	Below LOQ (10.1 ppb)	ND	ND
M4	ND	Below LOQ (10.1 ppb)	ND	ND
M5	ND	ND	ND	ND
M6	Below LOQ (10.0 ppb)	Below LOQ (10.1 ppb)	ND	ND
M7	ND	ND	ND	ND
M8	ND	ND	ND	ND
M9	Below LOQ (30.0 ppb)	ND	ND	ND
M10	ND	ND	ND	ND
M11	ND	ND	ND	ND
M12	Below LOQ (50.0 ppb)	ND	ND	ND
M13	ND	ND	ND	ND

*** Note:** - *"ND": Not detected*
- *"Below LOQ": Below Limit of Quantitation*

The analysis results of 13 finished product samples on the market showed that some nitrosamines were not detected in the samples (denoted as "ND"). In some samples, nitrosamines were detected but below the limit of quantification (denoted as "Below LOQ"). Only sample M2 was found to contain NDMA at a concentration of 90.89 ng/g.

4. CONCLUSION

Through the course of experimentation, this study assessed the suitability of chromatographic and mass spectrometric conditions for applying Procedure 4, USP General Chapter <1469> under the available equipment at the National Institute of Drug Quality Control Vietnam. Essentially, the chromatographic conditions were implemented in accordance with Procedure 4, USP General Chapter <1469>. Regarding sample preparation, when applying the method described in Procedure 4, USP <1469>, we encountered several difficulties, such as: insufficient solvent volume led to incomplete wetting of powdered samples, difficulty in homogenization, low volume of extractable solution, and filtered sample solutions were prone to gelatinization, making injection into the chromatographic system difficult. Method validation was conducted in accordance with USP guidelines using two representative product matrices. Parameters evaluated included: specificity, LOD/LOQ, linearity, accuracy and precision (repeatability and intermediate precision), and robustness. The method met validation requirements for both matrices. Regarding LOD - LOQ, due to the good sensitivity of the equipment, the limit of detection (LOD) for the four nitrosamines ranged from 0.3 - 0.9 ng/mL in solution and from 3 - 9 ng/g in sample. The limit of quantification (LOQ) ranged from 1 - 3 ng/mL in solution and 10 - 30 ng/g in sample. These values are in accordance with FDA requirements (LOQ of analytical methods ≤ 0.03 ppm = 30 ng/g for drugs with daily doses ≤ 880 mg/day [4]).

ACKNOWLEDGMENT

This study was carried out as part of the collaborative efforts between the National Institute of Drug Quality Control (NIDQC) and the United States Pharmacopeia (USP) from 2021 to the present. We would like to express our sincere gratitude to Dr. Amanda Guiraldelli, Ms. Ruth Lee Choo Ai, and other members of the APAC USP team for their technical guidance, constructive feedback, and continued support, which have significantly contributed to the development and successful implementation of this research.

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