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Development and Validation of a Robust Analytical Method for the Quantification of NDMA and NDEA Impurities in Ranitidine Hydrochloride API by HPLC and LC-MS/MS

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ABSTRACT

The detection of N- nitrosodimethylamine (NDMA) and N- nitrosodiethylamine (NDEA), classified as probable human carcinogens, has raised serious safety concerns regarding ranitidine hydrochloride (HCl), a widely used H2-receptor antagonist. This study focuses on the development and validation of sensitive and robust analytical methods using high-performance liquid chromatography (HPLC) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) for the quantification of NDMA and NDEA in ranitidine hydrochloride active pharmaceutical ingredient (API). A reversed-phase HPLC method was optimized for preliminary screening, followed by LC-MS/MS for confirmatory quantification. Validation parameters such as specificity, linearity, accuracy, precision, limit of detection (LOD), and limit of quantification (LOQ) were evaluated as per ICH Q2 (R1) guidelines. The LC-MS/MS method achieved LOD as low as 0.25ng/ml for NDMA and 0.5ng/ml for NDEA. The proposed method is highly suitable for routine ranitidine API quality control analysis and can be extended to related nitrosamine investigations.

KEYWORDS: *NDMA, NDEA, Nitrosamine, Ranitidine HCl, LC-MS/MS, HPLC, Method validation, Impurity quantification.*

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1. INTRODUCTION

Ranitidine hydrochloride, a histamine H2- receptor antagonist, has been widely used in the management of peptic ulcers and gastroesophageal reflux disease (GERD). It gained popularity due to its efficacy and favorable safety profile. However, in 2019, the global pharmaceutical community was alerted by regulatory agencies such as the USFDA (US Food and Drug Administration) and EMA (European Medicines Agency) regarding the unexpected presence of N- nitrosodimethylamine (NDMA) and N- nitrosodiethylamine (NDEA)- both probable human carcinogens- in several ranitidine-containing products [1-6].

NDMA and NDEA belong to the class of nitrosamines, which are known to be genotoxic and potentially carcinogenic at the trace levels. They are typically formed through secondary and tertiary amine interactions with nitrosating agents during chemical synthesis, storage or degradation [7-10]. In the case of ranitidine, it has been hypothesized that the drug's molecular structure- which contains a dimethylamine group- may render it susceptible to nitrosation, especially under the conditions of elevated temperature, pH variation, or oxidative stress [11-12].

In response to the detection of these impurities, regulatory agencies established strict acceptable daily intake (ADI) limits:

- NDMA: 96ng/day
- NDEA: 26.5ng/day

These limits necessitate the development of robust, sensitive, and validated analytical methods capable of detecting nitrosamines at parts-per-billion (ppb) or nanogram-per-gram (ng/g) levels.

Traditional analytical methods such as gas chromatography (GC) often require derivatization steps and may lack specificity when applied to complex pharmaceutical matrices. High- performance liquid chromatography (HPLC), when coupled with

mass spectrometric detection (LC-MS/MS), offers superior sensitivity, selectivity. While HPLC with UV detection may still used for preliminary screening of degradation products, LC-MS/MS is currently considered the gold standard for trace-level quantification of nitrosamines in drug substances and drug products. The current study focuses on the development and validation of a dual- method approach [13-15]:

- HPLC with UV detection for preliminary screening.
- LC-MS/MS for accurate quantification of NDMA and NDEA in ranitidine HCl API.

Both methods are optimized for specificity, linearity, sensitivity (LOD/LOQ), precision, accuracy and robustness, in accordance with ICH Q2(R1) guidelines. In the study, we report the development and validation of a robust, specific, and sensitive analytical method for the quantification of NDMA and NDEA in ranitidine hydrochloride active pharmaceutical ingredient (API) using both HPLC and LC-MS/MS techniques. The methods were optimized and validated by evaluating the parameters such as specificity, linearity, accuracy, precision, detection limit, quantification limit, and robustness. Additionally, forced degradation studies were conducted under various stress conditions (acidic, basic, oxidative, thermal, and photolytic) to investigate potential nitrosamine formation and to assess the method's capability in stability-indicating assays. The dual method approach not only enhances the reliability of impurity detection but also provides a comprehensive framework for quality control, regulatory compliance, and risk assessment of ranitidine and similar amine-containing drug substances [16-19].

2. MATERIALS & METHODS

Chemicals and standards

For method development and validation, analytical grade active pharmaceutical ingredients (APIs) were used, each supported by a certificate of analysis confirming a purity greater than 99% and compliance with specified quality standards. Sigma-Aldrich provided the ranitidine hydrochloride reference standard, NDMA standard solution, and TIC provided NDEA standard solution. SD Fine Chemicals provided LC-MS/MS solvents such as acetonitrile, ammonium hydroxide solution, ammonium acetate, and methanol. The Milli-Q RO water purification system provided ultrapure water for mobile phase preparation and sample processing, which was then filtered through a 0.22 µm PVDF membrane. All additional chemicals and reagents were analytical grade and purchased from reputable commercial providers.

Preparation of Standard Solutions and Buffer

A precise quantity of 10 mg of RAN HCl was precisely weighed and diluted in 10 mL of methanol to yield a primary stock solution with a concentration of 100µg/mL. To achieve full dissolution, the solution was sonicated for 5 minutes. To create a secondary solution of 100 µg/mL, dilute 1 mL with 10 mL of methanol (Solution A).

The 10mM ammonium acetate buffer was prepared by dissolving 0.256 g of ammonium acetate in 200 mL of distilled water. To achieve full dissolution, the solution was sonicated for 10 minutes. The buffer pH was initially measured and found to be 6.3; thus, it was raised to 7.0 using a 25% ammonium hydroxide solution. To remove particles, the buffer was filtered through a 0.45 µm membrane filter before being used in the mobile phase [20-23].

Chromatographic Conditions for RAN HCl by using HPLC

A Phenomenex C18 column (150 mm × 4.6 mm, 5 µm particle size) was used for chromatographic separation with a mobile phase of acetate buffer and acetonitrile in an 85:15 (v/v) ratio. The flow rate was kept constant at 1.0 mL/min, and the column temperature was fixed at 25°C. A 20 µL sample volume was injected over a 15-minute timeframe. The mobile phase was prepared by mixing 150 mL (15%) of HPLC-grade acetonitrile with 850 mL (85%) of the prepared acetate buffer. The mixture was degassed by ultrasonication for 15 minutes before use. The typical chromatographic profile obtained under these optimized conditions is illustrated in **(Figure 1)**, showing the standard chromatogram of RAN HCl with well-resolved peaks and satisfactory symmetry [21-22].

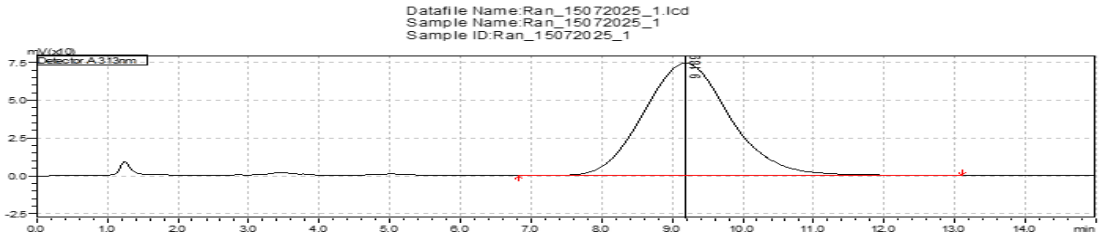


Figure 1: Typical standard chromatogram of Ranitidine Hydrochloride (RAN HCl)

Chromatography and tandem mass spectrometry

The RAN HCl was separated using an Atlantis C18 column (75 × 4.6 mm, 5 µm particle size) at room temperature. The mobile phase, which was made up of 10 mM ammonium acetate and acetonitrile in a 10:90 (v/v) ratio, was given at a constant flow rate of 0.7 mL/min under isocratic conditions. A 10 µL sample volume was injected, with a run time of 4 minutes. RAN HCl eluted in 1.993 minutes **(Figure 2)**, with distinct, symmetrical peaks suitable for quantitative analysis. A precise LC-MS/MS approach was created for the quantification of RAN HCl, using optimised chromatographic and mass spectrometric conditions that lead to high sensitivity and selectivity. Negative ion mode using ESI was employed and full scan spectra revealed prominent protonated molecular ions at m/z 313.05, 349.05 and 359.10 **(Figure 3)**. The optimized MRM transitions selected for quantification were m/z 349.05 → 170.00 and 313.10 → 325 **(Figure 4)**. These transitions were chosen based on sensitivity and signal stability. The molecular weight of RAN HCl is 350.86 g/mol, with [M+H] + as the major adduct ion and 349.05 as the precursor ion. The developed method ensured good peak shape, sensitivity and short run time, making it suitable for routine analysis on standard LC-MS/MS systems (**Table 1**).

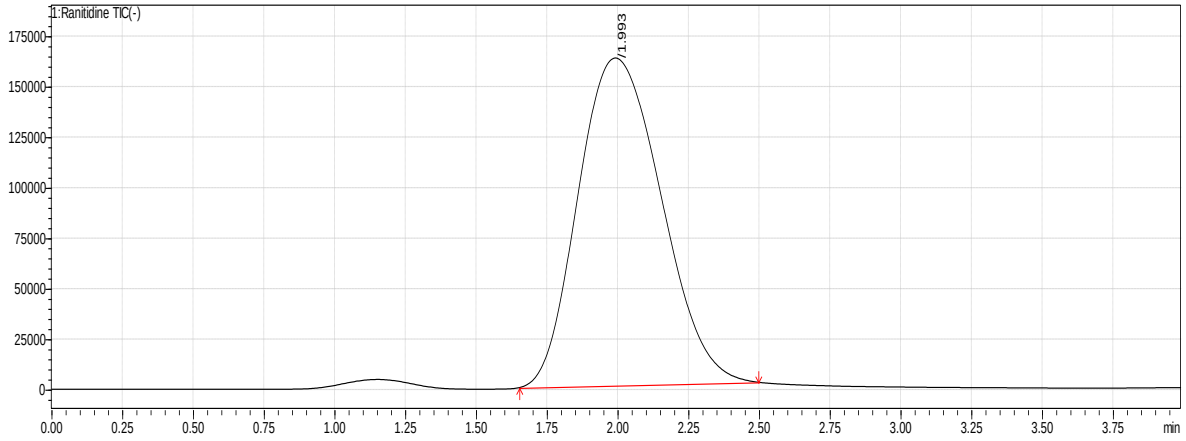
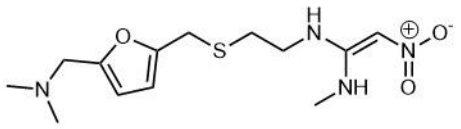


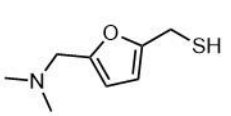
Figure 2: Typical standard chromatogram of Ranitidine Hydrochloride

Table 1: Mass parameters optimised for the identified ranitidine hydrochloride using LC-MS/MS

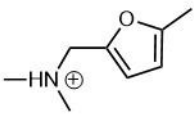
Analyte	Molecular weight (g/mol)	Adduct Ion	Precursor Ion	Product Ion	Dwell time (m sec)	Q1Pre Bias (V)	Q3 Pre Bias (v)	Collision energy (eV)	Retention time (min)	ESI mode opted
Ranitidine HCl	350.86	$[M-H]^+$	349.05	313.05	100.0	20.0	24.0	12.0	1.993	Negative
				142.05	100.0	20.0	20.0	20.0		
				170.00	100.0	20.0	24.0	22.0		



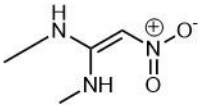
Molecular Weight: 314.40



Molecular Weight: 171.26



Molecular Weight: 140.21



Molecular Weight: 131.14

Figure: MRM Structures with product ions.

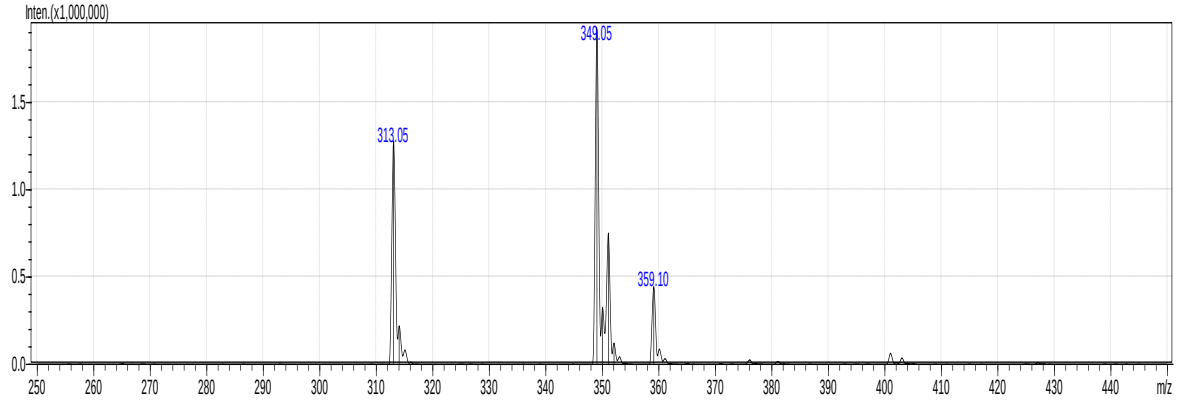


Figure 3: Negative scan of Ranitidine Hydrochloride

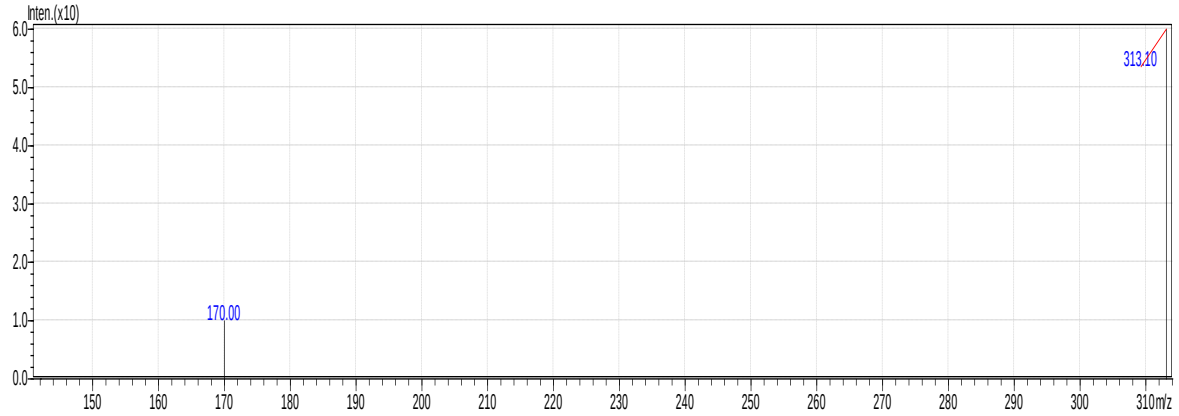


Figure 4: MRM scan of Ranitidine Hydrochloride

Method Development for quantification of NDMA and NDEA Standard Impurities by using LC-MS/MS:
For recovery experiments, standard stock solutions of NDMA and NDEA **(Figures 5& 6)** were prepared at a concentration of 100µg/ml in methanol and stored at 2-.8˚C in amber vials to prevent degradation. Sample solutions were prepared by accurately weighing 10mg of ranitidine hydrochloride API into a 10 mL volumetric flask, and dissolving it in methanol, sonicating for 10 minutes, and filtering using a 0.22µm PVDF syringe filter. **Table 2** and **Table 3** show the optimized parameters for NDMA and NDEA, respectively.

To evaluate method accuracy, spiking was carried out at three concentration levels- 50ng/ml, 100ng/ml, and 150ng/ml- by adding 5, 10, and 15µL, respectively, of each NDMA and NDEA stock solution into 10ml of API solution. The spiked solutions were mixed thoroughly and analyzed using both HPLC and LC-MS/MS to determine the recovery of each impurity. A blank (unspiked) API sample was also prepared to confirm the absence of native NDMA and NDEA and to rule out matrix interference [17, 21, 24].

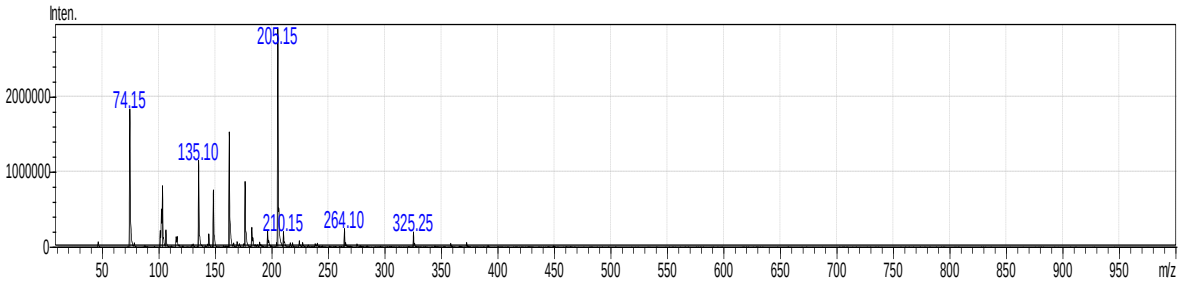


Figure 5: MRM scan of NDMA

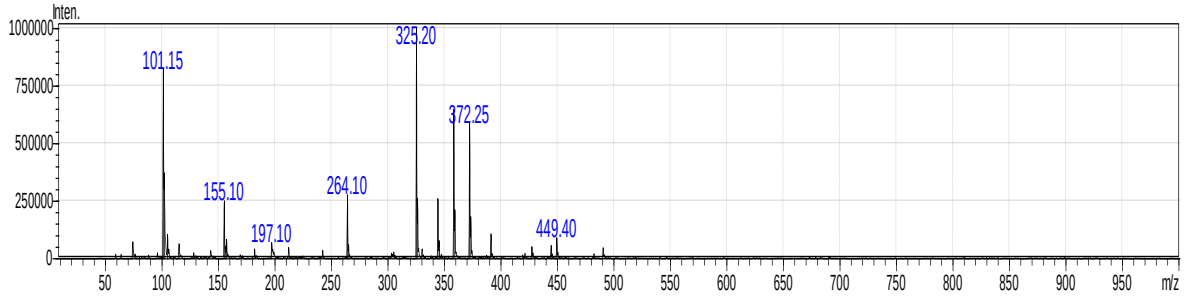


Figure 6: MRM scan of NDEA

Table 2: Mass parameters optimised for the identified NDMA using LC-MS

Analyte	Molecular weight (g/mol)	Adduct Ion	Precursor Ion	Product Ion	Dwell time (m sec)	Q1Pre Bias (V)	Q3 Pre Bias (v)	Collision energy (eV)	ESI mode opted
NDMA	74.08	$[M-H]^+$	74.00	29.15	100.0	-20.0	-28.0	-21.0	Positive
				18.10	100.0	-20.0	-20.0	-15.0	
				46.05	100.0	-20.0	-20.0	-18.0	

Table 3: Mass parameters optimised for the identified NDEA using LC-MS

Analyte	Molecular weight (g/mol)	Adduct Ion	Precursor Ion	Product Ion	Dwell time (m sec)	Q1Pre Bias (V)	Q3 Pre Bias (v)	Collision energy (eV)	ESI mode opted
NDEA	102.13	$[M-H]^+$	101.15	58.10	100.0	-28.0	-22.0	-25.0	Negative
				74.15	100.0	-26.0	-28.0	-19.0	
				46.15	100.0	-28.0	-20.0	-22.0	

3. RESULTS and DISCUSSION

System suitability parameters:

System suitability assessment is an important part of analytical method validation since it ensures that the chromatographic system is capable of performing accurate and reliable investigations. The optimised chromatographic settings were used to evaluate system compatibility metrics such as theoretical plates, tailing factor, LOD, and LOQ **(Table 4)**. The computed findings revealed that the system met the pre-established acceptance requirements, assuring the accuracy of the analytical approach for RAN-HCl [25-28].

Table 4: System Suitability by HPLC

Sl. No.	Parameters	Observation	Limit
1	Theoretical Plate	6781	N > 2000
2	Tailing Factor	1.02	T < 2
3	LOD	1 µg/mL	
4	LOQ	3 µg/mL	

System suitability by LC-MS/MS

System suitability parameters were evaluated using a representative LC-MS/MS chromatogram of RAN HCl. The retention time was recorded at 1.993 minutes, with 6098 theoretical plates reflecting good column efficiency. The peak symmetry value of 1.37 indicated an acceptable peak shape. The % RSD for replicate RAN HCl injections was 0.87%, demonstrating excellent precision of the method [25-28].

Specificity

No interfering peaks were observed at the retention time of RAN HCl (9.199 min & 1.993 min respectively) in the chromatogram **(Figures 7 & 8),** confirming the method's selectivity and sensitivity, with no contribution from endogenous components [25-28].

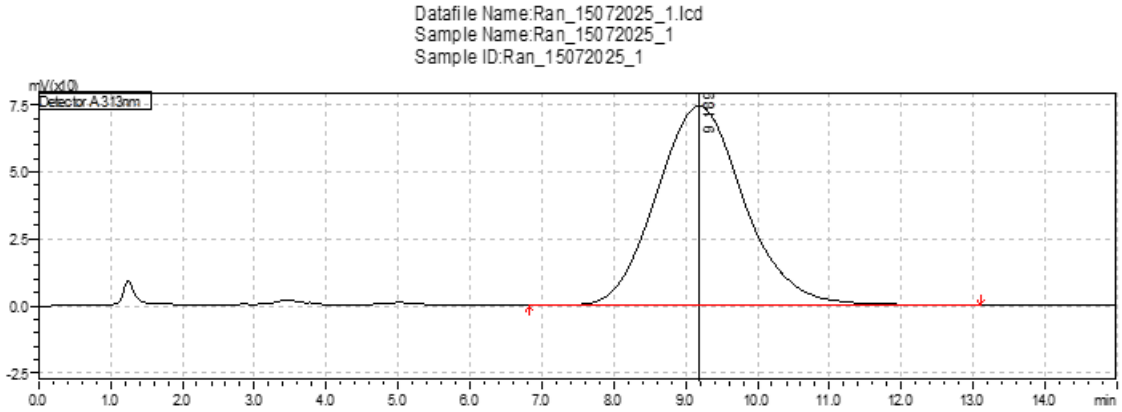


Figure 7: Typical standard chromatogram of Ranitidine Hydrochloride (HPLC)

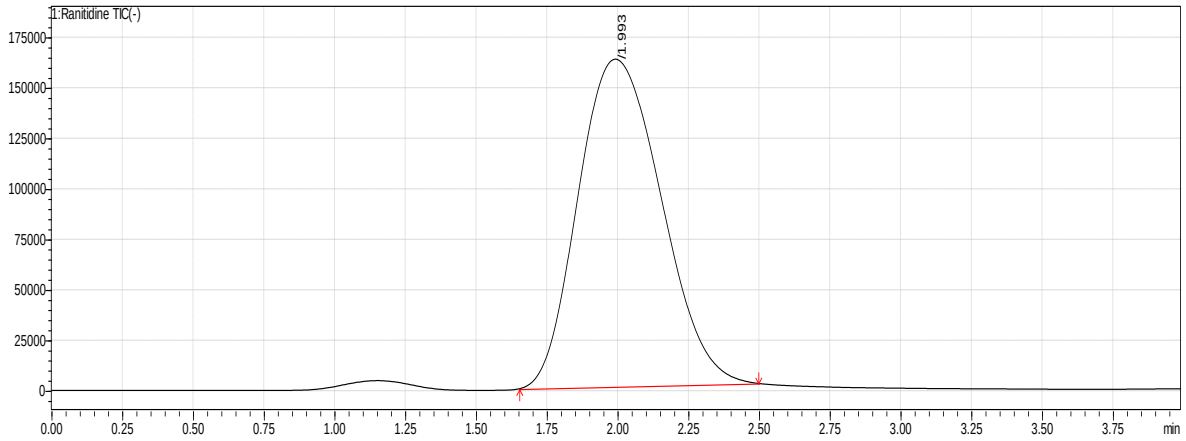


Figure 8: Typical standard chromatogram of Ranitidine Hydrochloride (LC-MS/MS)

LC-MS/MS analysis was performed on a triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source operated in a positive mode. The analytes were quantified using the Multiple Reaction Monitoring (MRM) mode. The optimized MRM transitions were as follows:

- NDMA: m/z 75→ 45, where 75 represents the protonated molecular ion $[M+H]^+$ and 45 is the main product ion.
- NDEA: m/z 101→ 74, corresponding to the protonated molecular ion and its characteristic fragment.

Method Validation of Ranitidine HCl API by HPLC

Linearity range:

RAN HCl demonstrated linearity at 7 concentration levels (1-30 µg/mL). Calibration curves were generated by graphing the peak area (Y-axis) against the corresponding concentration (X-axis), and the R2 value was 0.9985, demonstrating excellent linearity. The regression equation derived from the calibration curve was y= 24,613x + 39,554 **(Figure 9)**. After injecting the sample into the chromatographic apparatus, the peak areas for each component were determined. The linearity data is summarised in **Table 5**, and the results demonstrate that the approach is appropriate for accurate RAN HCl quantification across the investigated range [25-28].

Table 5: Linearity Table

Sl. No.	Concentration (µg/mL)	Area
1	1	18843
2	2	34687
3	4	68653
4	5	126306
5	10	243612
6	20	504224
7	30	735836

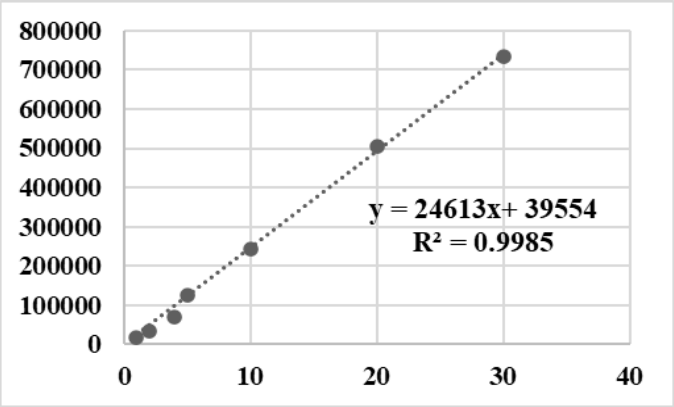


Figure 9 Linearity Plot for RAN HCl

LOD & LOQ

The limit of detection (LOD) for RAN HCl was 1 µg/mL, with a signal-to-noise ratio of 3:1. The limit of quantification (LOQ) was set at 3 µg/mL, with a signal-to-noise ratio of 10:1. These values suggest that the developed technique has good sensitivity for reliable detection and quantification of the analyte [25-28].

Accuracy and Precision

The method's accuracy and precision were examined at three levels of quality control: LQC, MQC, and HQC. The intraday accuracy was between 94.81% and 100.79%, with precision values (%RSD) ranging from 0.17% to 0.55%. Interday accuracy ranged between 93.24% and 99.40%, with percentage RSD values ranging from 0.17% to 0.56% **(Table 6)**. These results demonstrate that the approach is consistently accurate and precise over the measured concentration range, making it reliable for routine quantitative determination of RAN HCl [25-28].

Table 6: Accuracy studies

Sample (µg/ml)	Recovery (µg/ml) ± SD	Recovery (%)	Intraday		Interday	
			Accuracy (%)	Precision (% RSD)	Accuracy (%)	Precision (% RSD)
LQC- 2.5	2.3704± 0.004126	94.81	93.93	0.170	93.24	0.175
MQC- 10	9.8425± 0.04447	98.42	97.65	0.550	97.31	0.562
HQC- 20	20.1584± 0.09411	100.79	99.45	0.501	99.40	0.490

Method Validation of Ranitidine HCl API by LC-MS/MS

Linearity:

Calibration curves for RAN HCl were constructed using 7 different conc. levels ranging from 5 to 250 ng/mL **(Table 7)**. A high degree of linearity was observed for the method, as indicated by an R^2 value of 0.9998. The regression equation derived from the calibration curve was Y = 1965.3x + 3253.3, reflecting a clear and direct correlation between analyte concentration and peak area **(Figure 10)** [25-28].

Table 7: Linearity concentration ranges of Ranitidine HCl.

Sl. No.	Conc. (ng/mL)	Peak area
1	5	9843
2	30	54687
3	75	140653
4	125	240986
5	175	342906
6	200	390674
7	250	487620

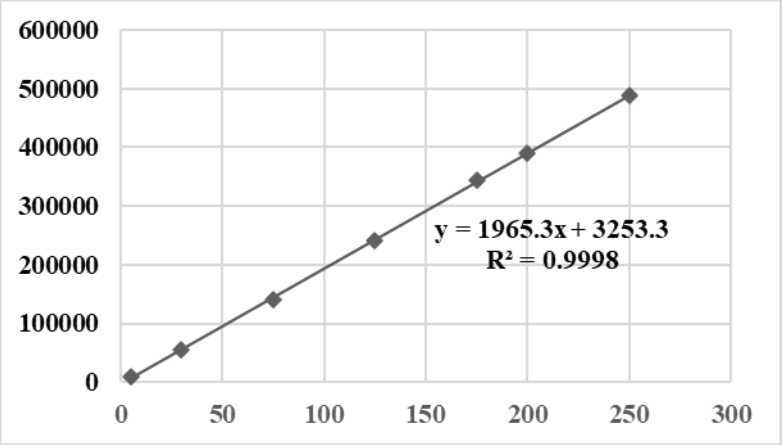


Figure 10: Linearity plot for Ranitidine

Accuracy and precision

Three quality control levels were used to measure accuracy: low (LQC), medium (MQC), and high (HQC) at concentrations of 5,125 and 225 ng/mL, respectively. Accuracy was determined by computing the mean percentage recovery at each QC level, which ranged from 95.33% to 97.93%. Repeated study confirmed the method's reliability and reproducibility, with % RSD values ranging from 0.23% to 0.69% [25-28].

Table 8: Accuracy & Precision table

Sample	Recovery (%)	Recovery (ng/ml) ± SD	Intraday		Interday	
			Accuracy (%)	Precision (%RSD)	Accuracy (%)	Precision (%RSD)
LQC-5	95.33	4.766 ± 0.033	94.86%	0.57	95.44%	1.80
MQC-125	97.07	121.346 ± 0.285	97.04%	0.12	95.96%	0.04
HQC-225	97.93	220.354 ± 0.562	98.04%	0.05	97.77%	0.13

LOD and LOQ

The LOD for RAN HCl was established at 1 ng/mL, based on a signal-to-noise ratio of 3;1, while the LOQ was determined to be 3 ng/mL, corresponding to a signal-to-ratio of 10:1. These values may vary depending on the reagents used and the purity of solvents, particularly when non-LC-MS/MS grade solvents are employed which can affect the signal clarity. The low LOD & LOQ values indicate that the developed method exhibits high sensitivity, making it suitable for trace-level detection of RAN HCl [25-28].

Solution stability

The working calibration standards and internal standard solutions of RAN HCl were stable for up to 24 hours under refrigerated conditions (2–8°C), showing no significant changes in retention time or peak area. However, when stored at room temperature, both the RAN HCl and internal standard solutions showed stability only up to 12 hours. Beyond this duration, noticeable changes in peak area and chromatographic response were observed, indicating degradation or loss of analyte integrity upon prolonged room temperature exposure **(Figure 11)** [25-28].

Forced Degradation Studies of Ranitidine HCl API:

Sample Preparation: Weighed accurately 10mg of ranitidine HCl API in a 10 mL (1mg/mL concentration) volumetric flask and further made up the volume using methanol (diluent). The solution was sonicated for 10minutes to dissolve completely. Further, 1ml from the above solution was dissolved in 10ml volumetric flask using methanol, which gives a 100µg/mL concentration (Solution A) **(Figure 12)**.

Acidic Condition: Form above-mentioned Solution A, 1ml of sample was dissolved in a 10ml volumetric flask, which was further made up using 0.1N HCl solution to yield an acidic solution.

Basic Condition: Form above-mentioned Solution A, 1ml of sample was dissolved in a 10ml volumetric flask, which was further made up using 0.1N NaOH solution to yield a basic solution.

Neutral Condition: Form above-mentioned Solution A, 1ml of sample was dissolved in a 10ml volumetric flask, which was further made up using water to yield a neutral solution.

Oxidative Condition: Form above-mentioned Solution A, 1ml of sample was dissolved in a 10ml volumetric flask, which was further made up using 3% H2O2 solution to yield an oxidative solution.

Thermal Condition: About 10mg of the API was kept in a dry petri dish and exposed to 60˚C in a hot air oven for 24 hours. After degradation, the sample was dissolved in in 10ml volumetric flask using methanol, which is a diluent. Further 1ml was dissolved in 10ml, yielding 100µg/mL concentration.

Photolytic Condition: About 10mg of the API was kept in a dry petri dish and exposed to UV light in UV chamber for 24 hours. After degradation, the sample was dissolved in in 10ml volumetric flask using methanol, which is a diluent. Further 1ml was dissolved in 10ml, yielding 100µg/mL concentration [29-30].

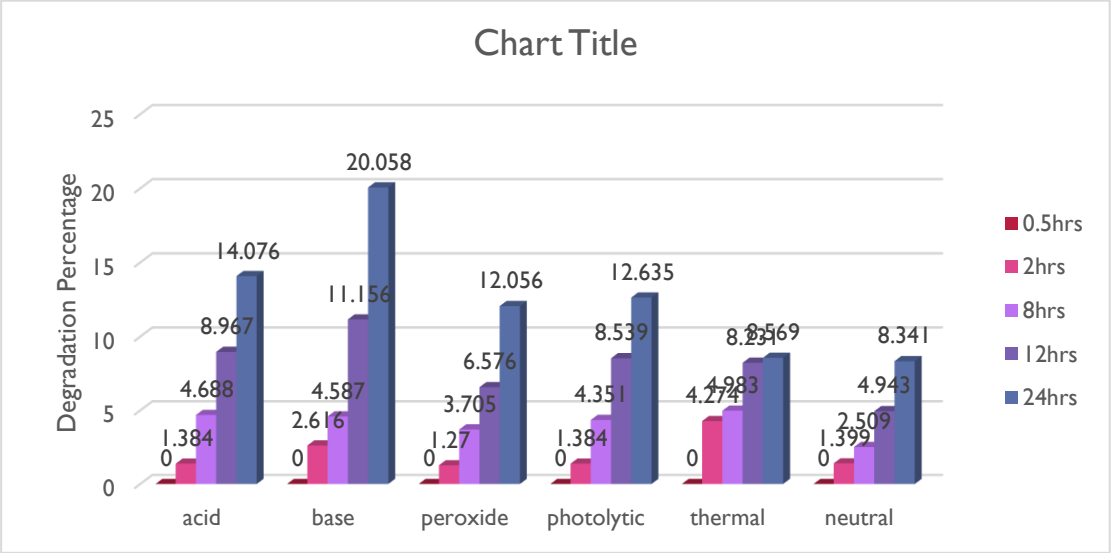


Figure 11: Degradation percentage chart for RAN HCl

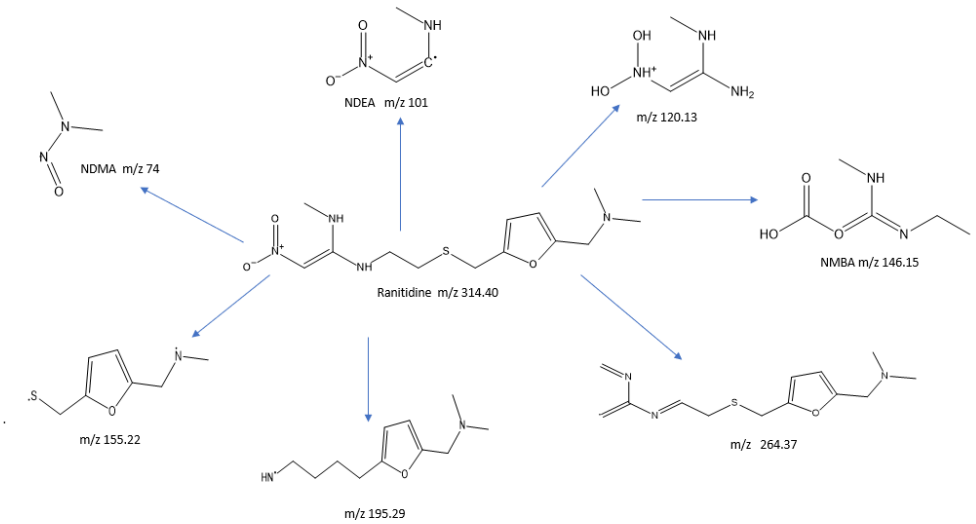


Figure 12: Degradation pathway for RAN HCl

Quantification calculation for NDMA

Linearity:

Calibration curves for RAN HCl were constructed using 7 different conc. levels ranging from 5 to 250 ng/mL **(Table 9)**. A high degree of linearity was observed for the method, as indicated by an R^2 value of 0.9998. The regression equation derived from the calibration curve was Y = 1965.3x + 3253.3, reflecting a clear and direct correlation between analyte concentration and peak area **(Figure 13)** [31-32].

Table 9: Linearity concentration ranges of NDMA.

Sl. No.	Conc. (ng/mL)	Peak area
1	0.25	2157
2	1	9956
3	5	51690
4	10	109808
5	25	268690
6	50	528917
7	100	701829

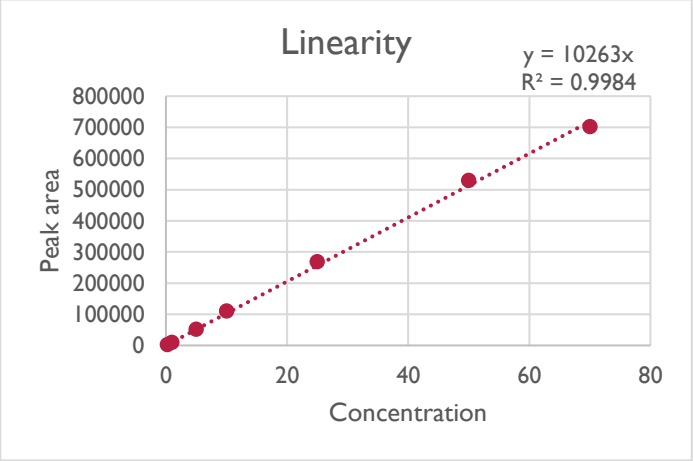


Figure 13: Linearity plot for NDMA

Calculation Formula:

Amount of analyte (ng/ml) = (Area of analyte peak in sample/ slope of standard calibration curve)

Therefore, the quantification amount for NDMA was found to be as follows (**Table 10)**.

Table 10: Quantification of NDMA

Sl. No.	Conc. (ng/mL)	Quantification amount
1	0.25	0.210ng/ml
2	1	0.970ng/ml
3	5	5.036ng/ml
4	10	10.699ng/ml
5	25	26.180ng/ml
6	50	51.536ng/ml
7	100	68.384ng/ml

Quantification calculation for NDEA

Linearity:

Calibration curves for RAN HCl were constructed using 7 different conc. levels ranging from 5 to 250 ng/mL **(Table 11)**. A high degree of linearity was observed for the method, as indicated by an R^2 value of 0.9998. The regression equation derived from the calibration curve was Y = 1965.3x + 3253.3, reflecting a clear and direct correlation between analyte

concentration and peak area **(Figure 14)** [31-32].

Table 11: Linearity concentration ranges of NDEA.

Sl. No.	Conc. (ng/mL)	Peak area
1	0.25	2188
2	0.5	4113
3	1	5182
4	2	10900
5	5	18053
6	10	33642
7	15	45231
8	20	65820
9	25	79409

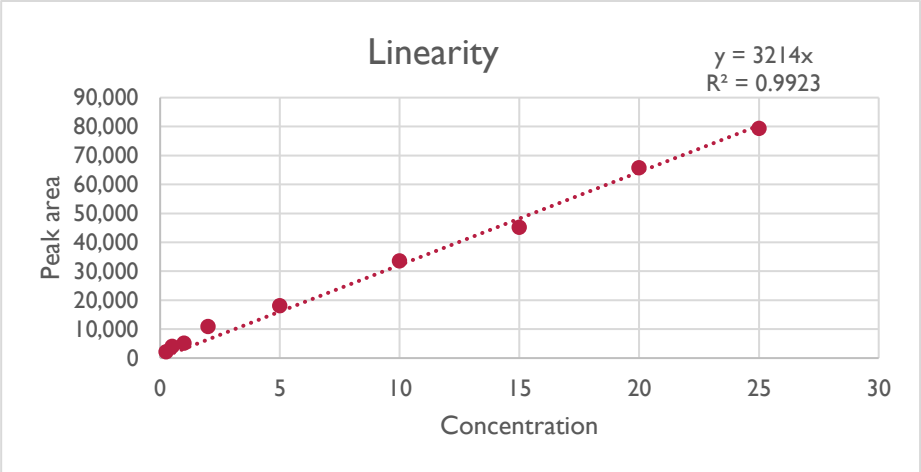


Figure 14: Linearity plot for NDEA

Calculation Formula:
Amount of analyte (ng/ml) = (Area of analyte peak in sample/ slope of standard calibration curve)
Therefore, the quantification amount for NDMA was found to be as follows (**Table 12)**.

Table 12: Quantification of NDMA

Sl. No.	Conc. (ng/mL)	Quantification amount
1	0.25	0.680ng/ml
2	0.5	1.279ng/ml
3	1	1.612ng/ml
4	2	3.391ng/ml
5	5	5.616ng/ml
6	10	10.467ng/ml
7	15	14.073ng/ml
8	20	20.479ng/ml
9	25	24.707ng/ml

4. DISCUSSION:

The developed HPLC and LC-MS/MS methods demonstrated strong capability for the precise and accurate quantification of NDMA and NDEA impurities in RAN HCl at trace levels. Quantification was performed using standard solutions prepared in the same diluent. The calibration curves showed excellent linearity across the tested range, and the method's

sensitivity allowed reliable quantification of both nitrosamines even below regulatory thresholds. The quantified levels of NDMA and NDEA indicate that the method provides robust quantitation with negligible instrument or operator-induced variability. The low LOQ values make this method suitable for routine analysis and stability testing, where even minor increases in nitrosamine levels over time must be detected and quantified.

5. CONCLUSION:

A simple, robust and highly sensitive HPLC and LC-MS/MS method was successfully developed and validated for the quantification of genotoxic impurities NDMA and NDEA in RAN HCl API. The method showed excellent sensitivity, specificity, linearity, precision, and accuracy, and complied with ICH validation requirements. Its ability to detect NDMA and NDEA at trace levels makes it a reliable tool for routine monitoring of nitrosamine impurities, ensuring the safety and quality of ranitidine-containing pharmaceutical products.

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