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Research Article

Zanamivir estimation using RP-HPLC

RP-HPLC Method for Estimation of Zanamivir in API and Pharmaceutical Formulation

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Abstract: The present work aimed to develop a new simple, accurate and reproducible RP-HPLC method for the analysis of Zanamivir. The method was validated according to ICH (Q2R1) guidelines. The chromatographic conditions were effectively monitored for the elution of analyte utilizing YMC C18 column (4.6 × 150 mm, 5µ); water: methanol (20:80 % v/v) as a solvent system with a 0.6 mL/min of flow at detection wavelength of 320 nm. The retention of analyte was achieved at 2.497 minutes. The tablet sample was assayed with 99.52 %±0.425 purity. The system suitability parameters such as plate count and tailing factor were found to be 4159.0 and 1.5, respectively. The linearity of the method achieved at the concentration range of 20-100 µg/mL with a correlation coefficient (r^2) of 0.998. The accuracy study showed 99.95%±0.126 of recovery of analyte. Precision in terms of repeatability was found within the limit (% RSD-0.159), while intermediate precision was shown % RSD of 0.15. LOD and LOQ were predicted at 3.04 and 10.14 µg/mL, respectively. In addition, the method found robust at a deliberate change of flow rate and solvent composition. Therefore, the results confirmed the suitability of the method for quantifying Zanamivir in their formulations.

Keywords: YMC C18 column; Zanamivir; RP-HPLC; methanol; validation, Analysis

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1. INTRODUCTION

The present work deals with the development and validation of an RP-HPLC method for the estimation of Zanamivir (ZAN) in API and tablet dosage forms.[1,2] Different formulations of ZAN are being commercially available in single and in combination with other drugs (Figure 1). ZAN is an antiviral agent that works as a neuraminidase inhibitor indicated for the remedy of simple acute infection due to influenza A and B virus in adults and pediatric patients. ZAN also proven it significant inhibition of the human sialidases NEU3 and NEU2.[3]

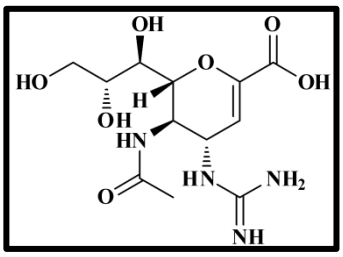

Fig 1: Chemical structure of ZAN.

Literature review suggests few spectrophotometric[4], HPLC[5,6], HPTLC[7], and mass spectroscopic [8,9] methods for estimation of ZAN in single or in mixtures in different dosage forms. As per the literature information, there was no well-established RP-HPLC method for the estimation of ZAN in pharmaceutical dosage forms. Therefore, it urges an improved version of the RP-HPLC method for the effective quantification and quality control of ZAN in their formulations. An attempt was made to develop a new simple, accurate, and reproducible RP-HPLC approach for the analysis of ZAN. Further, the method was validated as per ICH (Q2R1) guidelines to ensure agreement with acceptance criteria. The present communication explored the development, optimization, and validation of the RP-HPLC method.

2. MATERIALS AND METHODS

2.1. Materials

The solvents and chemicals employed such as water, methanol, acetonitrile, orthophosphoric acid, and buffers (HPLC grade) were purchased from Merck, India. API of ZAN was procured from Hetero Labs, Hyderabad. The pharmaceutical formulations were purchased from the local market.

2.2. Instrumentation

All the experiments were executed on Waters HPLC enabled with an auto sampler, separation module 2695, photodiode cluster identifier 996 and Empower 2 system. Quantification was accomplished on the C18 column (YMC; 4.6 ×150 mm, 5µ) with isocratic elution.

2.3. Method development

2.3.1. Optimization of the method

The analyte was eluted on the C18 column (YMC; 4.6 ×150 mm, 5µ) with 0.6 mL/min flow of methanol: water (80:20 % v/v) with PDA detection. The injection volume was maintained as 20 µL in all the experiments. The ambient temperature was maintained at the column and analyte sampler throughout the elution. Run time was maintained as 6.0 min as the analyte was eluted at 2.497 minutes.

2.3.2. Preparation of sample solution

Tablet powder equivalent to 10 mg of ZAN was taken in a 10 mL volumetric flask containing 2 mL of diluent and the volume was adjusted and sonicated. Pipette out 10 mL of the above stock solution into another volumetric flask (100 mL capacity) and the volume was adjusted to get a final concentration of 100 µg/mL of ZAN.[10,11]

2.3.3. Preparation of standard solution

10 mg (0.01 g) of ZAN standard was transferred into a 10 mL volumetric flask containing 2 mL of diluent, further dilutions were made to get a final concentration of 100 µg/mL of ZAN as per sample preparation.

2.4. Assay

Accurately weighed tablet material (equivalent to 10 mg of ZAN) was transferred into a 10 mL of the volumetric flask containing 2 mL of diluent and the volume was adjusted with diluent. Further dilutions were continued to get a final concentration of 100 µg/mL of ZAN. The resulted solution was injected into the HPLC system in triplicate. The % purity of ZAN was determined with the help of the peak area of sample and standard. [10,11]

2.5. Method validation

The method developed for quantification of ZAN was validated as per ICH guidelines.[12,13]

2.5.1. System suitability

System suitability parameters were evaluated by injecting the standard solution as per the optimized protocols. The standard solution was prepared by taking 10 mg of ZAN into a 10 mL volumetric flask containing 2 mL of diluent and sonicated. Further requisite dilutions were continued as per the optimized procedure. The key parameters like plate count and tailing factor, etc., were checked to assess the suitability of the system.[14]

2.5.2. Specificity

The specificity experiments were conducted to demonstrate

the interference of excipients with analyte retention time and peak area. The retention time and peak area of responses were checked by replicate injections of standard, sample, and blank solutions.[7]

2.5.3. Linearity and range

The 0.2, 0.4, 0.6, 0.8, and 1.0 mL of standard stock solution (1mg/mL) was transferred into five separate 10 mL volumetric flasks and the volume was adjusted to get the final concentrations of 20, 40, 60, 80 and 100 µg/mL of ZAN, respectively. The resulted solution at each concentration level was injected to get the respective peak areas. The correlation coefficient (r^2) of the calibration plot of peak area versus concentration was correlated with standard limits.

2.5.4. Accuracy

The accuracy of the method was determined by standard addition at 50, 100, and 150 % w/v level to the test sample.[15] The sample solutions were prepared by spiking 5 mg (50 % w/v), 10 mg (100 % w/v) and 15 mg (150 % w/v) of standard. The dilutions were made as per the procedure given under method optimization. The amount (% recovery) of standard recovered was determined by considering the peak area of triplicate injections. The mean % recovery was correlated with the standards of ZAN.

2.5.5. Precision

Repeatability

It was illustrated in terms of assay repeatability of the test sample.[16] The sample solution prepared from homogenous lots and injected for five determinations. The % RSD was determined for all replicate injections.

Intermediate precisions

The intermediate precision (ruggedness) of the method was determined by performing on different days by different analysts. The analysis was carried out by following repeatability protocols. The % RSD for the peak area of five replicate injections was calculated to check compliance with acceptance criteria.

2.5.6. Robustness

The solvent flow rate was monitored as 0.4 mL/min and 0.6 mL/min to assess the compliance of robustness of the optimized method. Also, the organic composition of the mobile phase was varied as 80% v/v ±5 (75 % v/v and 85 % v/v of methanol). Standard solution (100 µg/mL) was injected in triplicate with these changes and correlated with actual retention time and peak area. The % RSD of responses at these changes was assessed.

2.5.7. Limit of detection (LOD)

LOD of ZAN in the test sample was set at levels approximating the LOD according to the standard formula [LOD = 3.3 x S/ σ]. The standard deviation of the responses (S) and the slope of the calibration curve (σ) was obtained at 3:1 signal-to-noise ratio of the response.[17,18]

2.5.8. Limit of quantification (LOQ)

LOD of ZAN in the test sample was set at levels approximating the LOD according to the standard formula [LOD = 10x S/ σ]. The standard deviation of the responses (S) and the slope of the calibration curve (σ) were obtained at 10:1 signal-to-noise ratio of the response. [17,18]

3. RESULTS AND DISCUSSION

3.1. Method development

3.1.1. Optimization of the method

The present work was intended to develop a new approach for the estimation of ZAN with the help of RP-HPLC technique. To optimize the proposed method, several trials were performed using methanol, phosphate buffer (pH-6.8), acetonitrile, and water as a solvent system in different combinations in appropriate ratios. As per the chromatographic results, the methanol and water in the ratio of 80:20 % v/v proved to be an effective solvent system for elution of analyte. The absorption wavelength was found at 320 nm on PDA detection. The optimum flow rate was adjusted as 0.6 mL/min with an injection volume of 20 µL. The efficiency in elution was achieved at ambient temperature at the column with an auto sampling. As the analyte peak was eluted at 2.497 minutes, the run time was set as 6.0 minutes (Figure 2). The peak parameters were optimum for chromatogram in terms of asymmetric factor, plate count, tailing factor and height, etc.

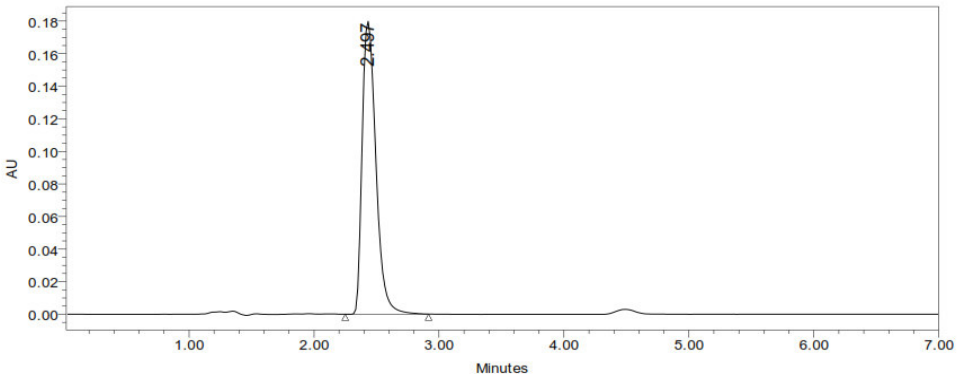

Fig 2: Chromatogram at optimized conditions.

3.2. Assay

The ZAN in tablet formulations was assayed with the help of optimized chromatographic conditions. Triplicate injections were made to get the percentage (%) purity of analyte in the formulations. As per the proposed method, the % purity of ZAN was found to be 99.52% ± 0.425.

3.3. Method validation

3.3.1. System suitability

The peak parameters results met the system suitability requirements. The retention time was detected at 2.497 minutes. The number of theoretical plates and tailing factor were noticed as 4159. 0 and 1.5, respectively (Table 1).

Table: System suitability data.

S. No.	Retention time (minutes)	Area	Height (µV)	Plate count	Tailing factor
1	2.425	695226	117709	4159.0	1.5
2	2.429	694341	115422	4059.1	1.4
3	2.426	694434	117204	4059.3	1.5
Mean±SD		694667±486.6			
% RSD		0.07			

3.3.2. Specificity

The specificity of the method was assured by the complete separation of analyte peak from other peaks originating from the sample matrix. Specificity evaluation was accomplished by injecting separate solutions of the standard, sample, and blank into the chromatographic system. The specificity results confirmed no interference of any excipients or/and impurities in the retention time of the analyte (Figure 3-5).

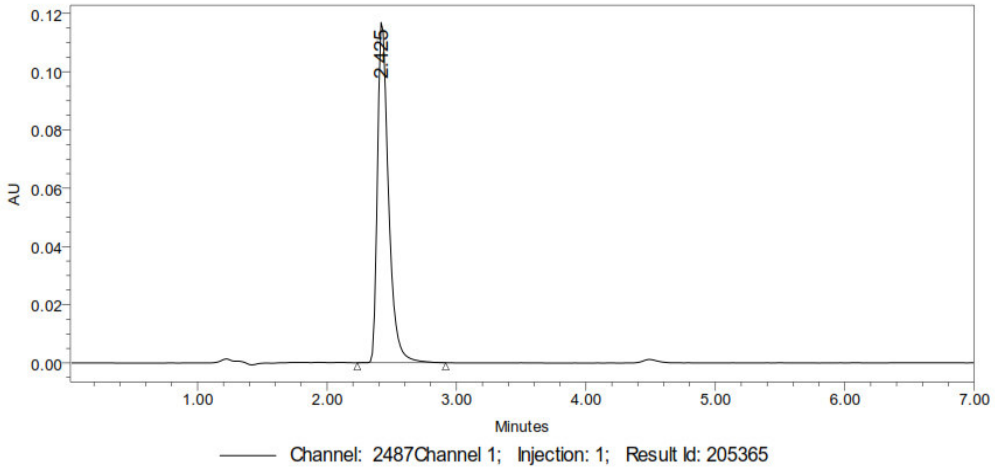

Fig 3: Chromatogram of the sample.

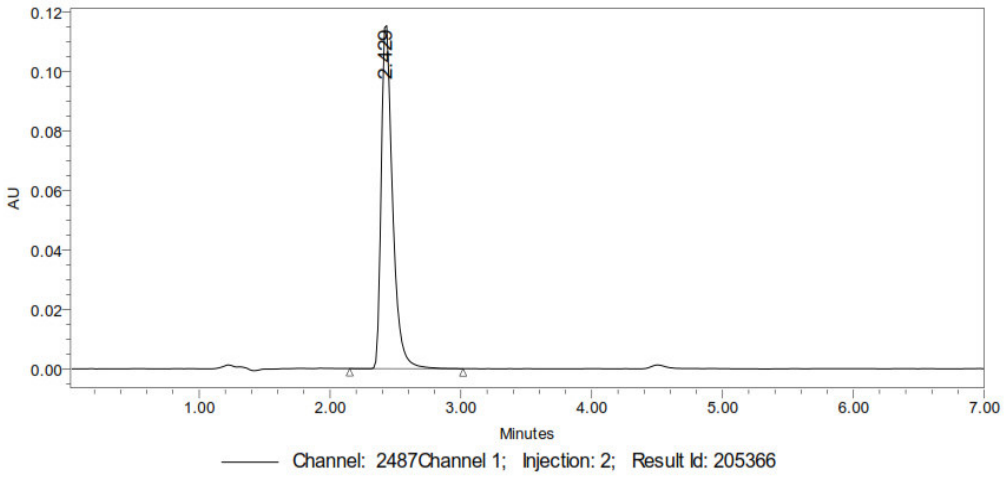

Fig 4: Chromatogram of the standard.

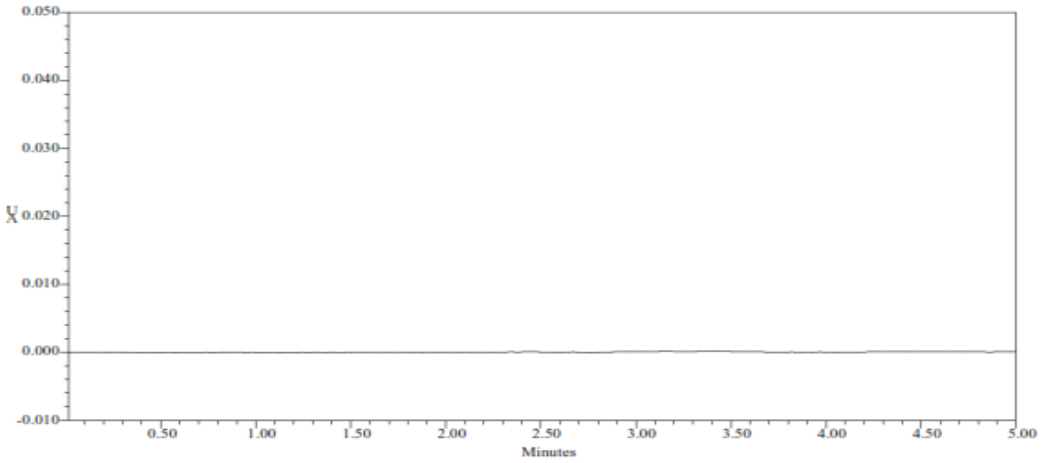

Fig 5: Chromatogram of the blank.

3.3.3. Linearity and range

The calibration curve was plotted with the peak area of ZAN obtained on elution versus corresponding concentrations to check the complying linearity (Figure 6). The linearity was attained over a concentration range of 20-100 µg/mL. The calibration plot at these concentrations expressed the correlation coefficient (r^2) of 0.998 with linear regression equation of y = 9750.5x + 86207 (Table-2).

Table 2: Linearity data for ZAN

S. No	Concentration (µg/mL)	Mean peak area ±SD
1	20	264840±128
2	40	491451±265
3	60	677620±654
4	80	873311±452
5	100	1048958±264
Correlation coefficient (r^2)		0.998

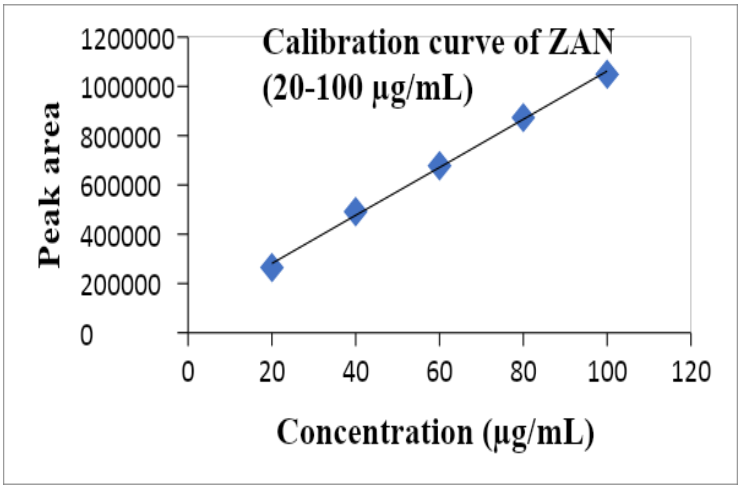

Fig 6: Calibration curve of ZAN at 20-100 µg/mL.

3.3.4. Accuracy

The accuracy of the method was carried out by injecting spiked sample solutions at 50%, 100 %, and 150 % w/v levels in triplicates. The mean peak area at each level was considered for knowing the % recovery of standard (Table 3). The % recovery of ZAN was found as 99.95 %±0.426 (98 - 102%).

Table 3: Accuracy data of ZAN (n=3)				
Spiked level	Mean area ±SD	Standard addition (mg)	% recovery	Mean recovery ±SD
50%	728287±264	5	99.91	99.95%±0.426
100%	1378202±762	10	99.18	
150%	2115480±824	15	99.60	

3.3.5. Precision

It was performed in terms of repeatability assay and intermediate precision.

Repeatability

Assay repeatability was analyzed by a single analyst with a varied number of samples. The standard solution was injected five times and measured the retention time and peak area. The % RSD for the area of replicate injections was within specified limits (Table 4).

Table.4: Repeatability data (% RSD) results for ZAN (n=5)			
S. No.	Retention time (minutes)	Peak Area	% Assay
1	2.423	693877	99.11
2	2.424	696531	99.26
3	2.424	693977	99.52
4	2.424	695278	99.16
5	2.423	697676	99.24
Mean		695468	99.25
SD		1642.7	0.158
%RSD		0.24	0.159

Intermediate precision

The intermediate precision was carried out by five replicate injections of ZAN standard solution. Each standard solution was injected into the chromatographic system and peak area of each injection was considered for % RSD calculations. The %RSD of intermediate precision was observed within specified limits (% RSD- 0.15).

Table.5: Intermediate precision data (n=5)		
S. No.	Retention time (minutes)	Peak Area
1	2.423	693078
2	2.424	693338
3	2.424	695080
4	2.424	694843
5	2.423	695336
Mean		694335
SD		1047.5
%RSD		0.15

3.3.6. Robustness

The robustness was determined by varying the flow rate as 0.4 mL/min and 0.8 mL/min and the mobile phase ratio. The method was found robust even with a slight change in flow rate (±0.2 mL/min) and mobile phase ratio (±5% v/v). The results were depicted in Table 8 & 9.

Table 8.: Robustness data by a varying flow rate of mobile phase ratio (n=3)				
S. No.	Flow rate (mL/min)	Retention time (minutes)	Mean peak area ±SD	% RSD
1	0.4	2.448	692036±257	0.826
2	0.6	2.428	692844±285	0.921
3	0.8	2.411	692076±267	0.124

Table 9: Robustness data by varying mobile phase ratio (n=3)				
S. No.	Methanol composition (% v/v)	Retention time (minutes)	Mean peak area ±SD	% RSD
1	75	2.418	691076±127	0.821
2	80	2.428	692334±215	1.023
3	85	2.409	693081±134	0.962

3.3.7. LOD

The standard deviation of y-intercepts of the regression equation and slope of curve at signal-to-noise ratio (3:1) was considered for LOD assessment (Figure 7). The detection limit for ZAN was found as 3.04 µg/mL.

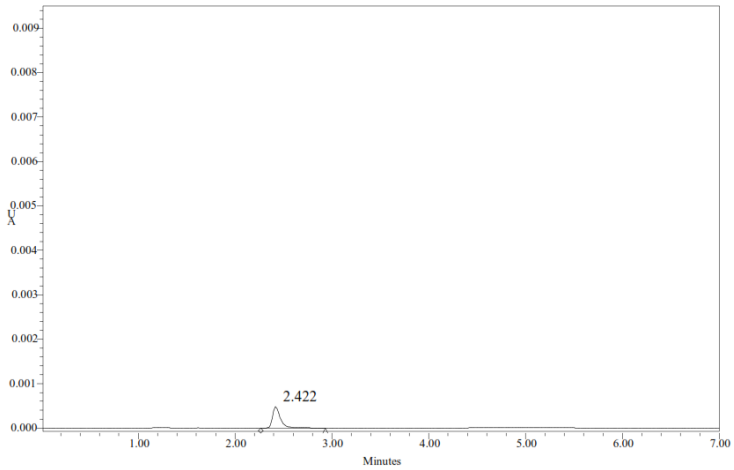

Fig. 7: LOD chromatogram.

3.3.8. LOQ

The LOQ was carried out by determining the standard deviation of y-intercepts of the regression equation and slope of the curve at a signal-to-noise ratio (10:1) (Figure 8). The quantitation limit for ZAN was found as 10.14 µg/mL.

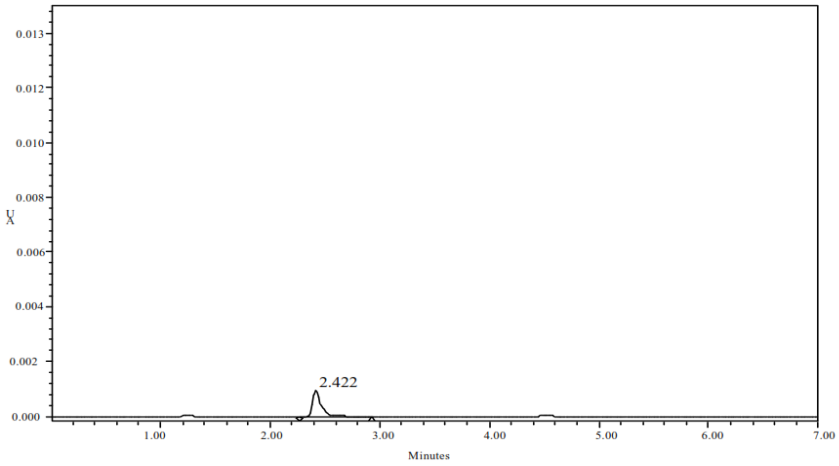

Fig. 8: LOQ chromatogram.

4. CONCLUSION

A RP-HPLC approach was established for the estimation of ZAN and it was fulfilled to all the validation criteria as per ICH guidelines. The analyte separation was achieved with YMC C18 (4.6 × 150 mm, 5µ) column by water and methanol in the ratio of 20:80 % v/v at 0.6 mL/min flow. The eluted analyte was monitored at 320 nm on PDA detector. The analyte peak was eluted at 2.497 mins. The results were within the acceptable range for accuracy, precision, linearity, and specificity. The results of LOD and LOQ confirm sensitivity of method. Therefore, the proposed method could be applied for the determination of analyte drug in bulk and marketed tablet formulations.

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6. AUTHORS CONTRIBUTION STATEMENT

Mrs. Amani. P has contributed her efforts in collecting and hypothesizing the investigative and experimentation work. Dr. Narender M guided the project and reviewed the analysis data. Dr. Karunakar and Mrs. Ramya Sri were helped in the process of analyzing the data. All the authors were equally involved in development of methodology, analysis of results and contributing the collection of final data for preparing the manuscript.

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7. ABBREVIATIONS

RP-HPLC: Reverse Phase-High Performance Liquid Chromatography. **API:** Active Pharmaceutical Ingredient **SD**: Standard Deviation% **RSD**: Percent relative standard deviation. **ICH**: International Conference on Harmonization

8. CONFLICT OF INTEREST

Conflict of interest declared none

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