

## Development and Validation of a Stability-Indicating RP-HPLC Method for Quantification of Rotigotine in Polymeric Microsphere Formulations

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### ABSTRACT

A robust and stability-indicating reversed-phase high-performance liquid chromatography (RP-HPLC) method was developed and validated for the quantification of rotigotine in polymeric microsphere formulations in accordance with International Council for Harmonisation (ICH) guidelines. Chromatographic separation was achieved on a C8 column (150 mm × 4.6 mm, 5 μm) using a gradient mobile phase consisting of orthophosphoric acid and triethylamine in water (1:1:1000, v/v/v) as mobile phase A and acetonitrile as mobile phase B. The method was operated at a flow rate of 1.0 mL/min with detection at 278 nm and a total run time of 15 min.

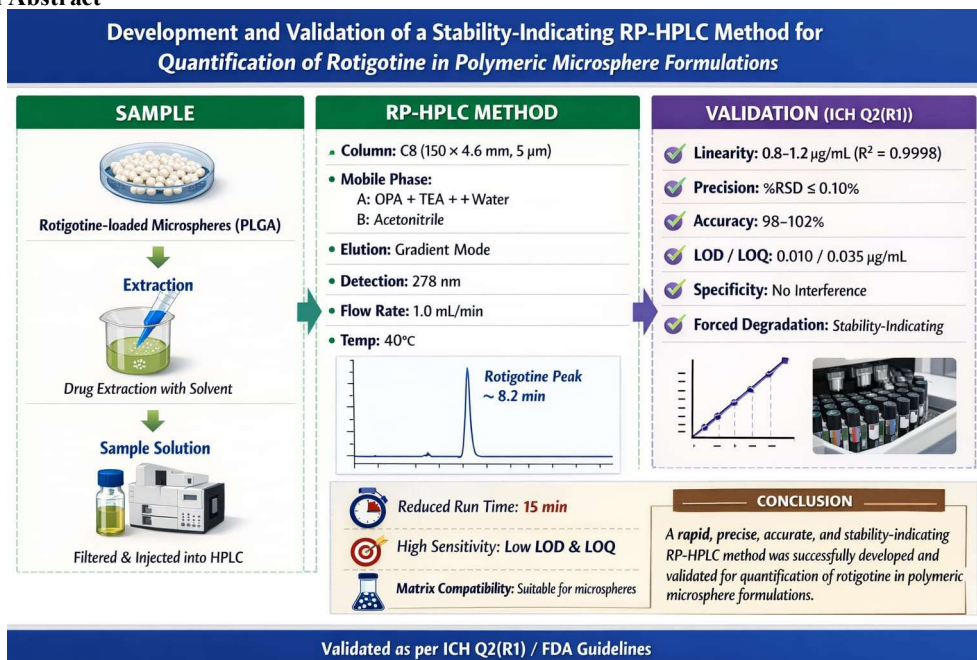
The method demonstrated excellent linearity over the concentration range of 80–120% ( $R^2 = 0.9998$ ), with high precision (%RSD < 2%) and accuracy (98–102%). The limits of detection and quantification were found to be 0.010 μg/mL and 0.035 μg/mL, respectively. Forced degradation studies confirmed the specificity and stability-indicating capability of the method, with no interference from degradation products or excipients.

Compared with previously reported methods, the proposed method demonstrates reduced analysis time, enhanced sensitivity, and improved applicability for complex polymeric matrices. The validated method is suitable for routine quality control, stability studies, and formulation development of rotigotine-loaded microspheres.

**Keywords:** Rotigotine, microspheres, ICH guideline, RP-HPLC, stability indicating.

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### Graphical Abstract



## INTRODUCTION

Rotigotine (ROG) is a non-ergoline dopamine receptor agonist that has garnered significant attention in the field of neurology due to its unique pharmacodynamic profile and therapeutic potential (van der Palen et al. 2018; Pahwa et al. 2017). Unlike ergoline-derived dopamine agonists, ROG exhibits a lower risk of adverse effects such as fibrosis and valvulopathy, making it a safer alternative for long-term treatment of Parkinson's disease (PD) and restless legs syndrome (RLS) (Pahwa et al. 2017; Poewe and LeWitt 2010).

The Rotigotine Transdermal System (RTS), approved by the U.S. Food and Drug Administration (FDA) in May 2007, represents a significant advancement in the delivery of ROG (Grimes et al. 2012; U.S. Food and Drug Administration 2007). The transdermal route offers several advantages over traditional oral administration, including bypassing first-pass metabolism, providing consistent plasma concentrations, and reducing gastrointestinal side effects (Raeder et al. 2021). The formulation is offered in six dosage levels, with strengths between 1 mg/24 h and 8 mg/24 h, allowing for individualized dosing based on disease severity and patient response. In Parkinson's disease, the advised dose lies between 2–8 mg/24 h, whereas for RLS, a lower range of 1–3 mg/24 h is generally used (National Center for Biotechnology Information 2023). This flexibility in dosing underscores the importance of RTS in achieving optimal therapeutic outcomes while minimizing adverse effects. Chemically, ROG is a complex molecule with the chemical name (6S)-6-(propyl [2-(2-thienyl) ethyl] amino)-5,6,7,8-tetrahydro-1-naphthalenol with the molecular formula  $C_{19}H_{23}NOS$  and a molecular weight of 315.48 g/mol. Its crystalline structure plays a key role in determining both solubility and stability, which are critical factors in its formulation and delivery (Elshoff et al. 2015). The physicochemical properties of ROG, including its functional groups, play a pivotal role in its analytical detection and quantification. These attributes are particularly relevant when developing novel drug delivery systems, such as microspheres, which aim to provide controlled and sustained release of the active ingredient (Elshoff et al. 2015).

The innovator formulation of ROG, marketed as NEUPRO™, is a transdermal system that comprises a drug matrix layer containing ROG along with excipients including ascorbyl palmitate, povidone, silicone adhesive, sodium metabisulfite, and dl- $\alpha$ -tocopherol (Bunten and Happe 2006). These excipients enhance the formulation's stability, adhesion, and overall efficacy. While NEUPRO™ has proven effective in delivering ROG through the skin, exploring alternative delivery systems, such as microspheres (MS), has introduced new analytical challenges. Microspheres enable the development of extended-release formulations, thereby enhancing patient adherence and therapeutic efficacy (Li et al. 2022). However, the absence of established analytical methods for quantifying ROG in such novel delivery systems represents a significant gap in the current literature (de Carvalho Mendes et al. 2021; Yi et

al. 2023; Saha and Pandey 2022; de Carvalho Mendes et al. 2024).

The development of a robust, simple, and selective analytical method for the quantification of ROG in Rotigotine-microspheres (ROG-MS) formulations is therefore of paramount importance. RP-HPLC was selected due to its high resolution, reproducibility, and suitability for analyzing compounds with diverse physicochemical properties (Yabr e et al. 2018). RP-HPLC is a widely used technique in pharmaceutical analysis, offering the ability to separate, identify, and quantify complex mixtures with high precision and accuracy (Nyarko et al. 2024; Ramireddy and Behara 2022; International Council for Harmonisation 2005). In compliance with ICH guidelines, the method underwent optimization and validation, ensuring accuracy, reproducibility, and relevance for both research and pharmaceutical development (U.S. Food and Drug Administration 2022).

Despite the availability of several analytical methods for the quantification of rotigotine in bulk drug substances and transdermal systems, there remains a significant gap in the literature regarding its estimation in advanced polymeric delivery systems such as microspheres. These systems introduce unique analytical challenges, including complex polymeric matrices, drug-polymer interactions, and potential interference from degradation products. Therefore, the development of a robust, stability-indicating analytical method capable of accurately quantifying rotigotine in microsphere formulations is critically important. In this context, the present study aims to develop and validate a sensitive, reproducible, and stability-indicating RP-HPLC method in accordance with ICH guidelines, specifically tailored for rotigotine-loaded polymeric microspheres, thereby addressing an unmet analytical need in controlled drug delivery research.

## MATERIALS AND METHODS

### Materials

Rotigotine (ROG) was generously provided by MSN Labs, Hyderabad, India. HPLC-grade solvents were used in this study, obtained from MERCK/RANKEM (India), while all other chemicals and reagents were of analytical grade.

### Instrumentation

Chromatographic separation was performed on a Waters Alliance 2695 RP-HPLC system, equipped with a 2489 Photodiode Array detector, quaternary pumps, and an autosampler. Data acquisition and processing were carried out using the Waters Empower workstation.

### Method Used to Prepare Microspheres

Microspheres containing Rotigotine were prepared using the emulsion solvent evaporation technique, a widely used method for polymeric controlled-release formulations. In this method, Rotigotine and the selected biodegradable polymer (e.g., PLGA or PLA) were dissolved in an organic solvent such as ethyl acetate. The organic phase was emulsified into an aqueous phase containing polyvinyl alcohol (PVA) under high-speed

homogenization to form an oil-in-water emulsion. The solvent was subsequently evaporated under continuous stirring, resulting in the formation of solid microspheres, which were collected by centrifugation, washed with distilled water, and dried.

#### **Preparation of Rotigotine-Loaded Polymeric Microspheres**

Rotigotine-loaded microspheres were prepared using biodegradable polymers such as **poly(lactic-co-glycolic acid) (PLGA)** to achieve controlled drug release. Rotigotine and PLGA were dissolved in an organic solvent to form the internal phase. This solution was emulsified in an aqueous phase containing stabilizer (PVA) using a high-speed homogenizer. The emulsion was stirred to evaporate the solvent, leading to precipitation of polymeric microspheres encapsulating Rotigotine. The microspheres were collected by centrifugation, washed repeatedly to remove residual stabilizer, and dried under vacuum. The resulting microspheres were stored in desiccators until further analysis.

#### **Sample Preparation for HPLC Analysis**

Accurately weighed Rotigotine-loaded microspheres equivalent to the required drug concentration were transferred into a volumetric flask. The microspheres were dissolved in the diluent consisting of purified water, acetonitrile, and triethylamine (500:500:1 v/v/v). The mixture was sonicated for 15–20 minutes to ensure complete drug extraction from the polymeric matrix. The solution was then diluted to volume and filtered through a 0.45  $\mu\text{m}$  PVDF or Nylon membrane filter before HPLC injection. The filtered sample solution was injected into the RP-HPLC system for quantitative analysis.

#### **Development and Optimization of Chromatographic Conditions**

Various combinations of aqueous and organic mobile phases were investigated to optimize the chromatographic separation and estimation of ROG.

##### **Chromatographic conditions**

Chromatographic separation was performed on a Hypersil BDS C8 column (150 mm  $\times$  4.6 mm, 5  $\mu\text{m}$  particle size). The analysis employed a gradient elution method (Table 1) and was completed within 15 minutes at a flow rate of 1.0 mL/min. The column temperature was maintained at 40  $^{\circ}\text{C}$ , with detection carried out using a UV-Vis detector set at 278 nm. A 20  $\mu\text{L}$  injection volume was used, and all experiments were conducted at ambient temperature (25  $^{\circ}\text{C}$ ).

**Preparation of Mobile Phase A:** A mixture of orthophosphoric acid, triethylamine, and purified water was prepared in a 1:1:1000 (v/v/v) ratio and thoroughly blended.

**Preparation of Mobile Phase B:** Acetonitrile.

**Preparation of Diluent:** Purified water, acetonitrile, and triethylamine were combined in a 500:500:1 (v/v/v) ratio and mixed thoroughly.

**Preparation of Needle Wash:** Purified water and methanol were combined in a 20:80 (v/v) proportion and mixed well.

The retention time was observed at about 8.2 minutes for ROG.

#### **RESULTS AND DISCUSSION**

An RP-HPLC method, capable of indicating stability, was developed in accordance with ICH guidelines to determine ROG in pharmaceutical formulations.

##### **Development and Optimization of Chromatographic Conditions**

Various combinations of aqueous and organic mobile phases, along with systematic adjustments in flow rate, column temperature, and detection wavelength, were evaluated to refine the chromatographic conditions for accurate quantification of ROG. Considering the physicochemical characteristics of ROG—particularly its moderate lipophilicity and potential for interaction with residual silanol groups on the stationary phase, a gradient elution approach was initially adopted. Gradient elution was selected to ensure efficient elution, improved peak resolution, and reduced analysis time, especially in anticipation of possible impurities, degradation products, or formulation excipients with varying polarities. Compared to isocratic elution, gradient mode enhances peak focusing at the column head, minimizes peak tailing, and improves overall chromatographic efficiency for compounds exhibiting broad polarity distribution. The preliminary optimized chromatographic method employed an aqueous mobile phase (A) containing triethylamine, trifluoroacetic acid, and water in a 1:1:1000 (v/v/v) composition, together with an organic phase (B) comprising methanol and acetonitrile in a 700:300 (v/v) ratio. Separation was performed at a flow rate of 0.8 mL/min with UV detection at 230 nm and a column temperature of 30  $^{\circ}\text{C}$ . Under these gradient conditions, ROG exhibited a distinct peak; however, the peak shape was irregular, possibly due to suboptimal pH control and secondary interactions between the analyte and the stationary phase. To address peak asymmetry and enhance chromatographic performance, the mobile phase composition was further optimized. Trifluoroacetic acid was replaced with orthophosphoric acid to achieve improved buffering capacity and more stable pH control. Additionally, methanol was eliminated from the organic phase to reduce viscosity-related band broadening and improve mass transfer kinetics.

The finalized optimized gradient method consisted of:

**Mobile Phase A:** Triethylamine, orthophosphoric acid, and water (1:1:1000, v/v/v)

**Mobile Phase B:** Acetonitrile

**Flow rate:** 1.0 mL/min

**Detection wavelength:** 278 nm (selected based on  $\lambda_{\text{max}}$  for enhanced sensitivity and specificity)

**Column temperature:** 40  $^{\circ}\text{C}$

These modifications significantly improved peak symmetry, resolution, and reproducibility. ROG exhibited a well-defined, sharp, and symmetrical peak with acceptable tailing factor, theoretical plate count, and retention time consistency. The optimized method satisfied all system suitability criteria in accordance with the recommendations of the U.S. Food and Drug Administration guidelines for analytical method validation. A representative chromatogram of the ROG standard solution is presented in Fig. 2.

#### Analytical Method Validation

The analytical method for quantifying ROG was validated in accordance with ICH guideline Q2 (R1), with the validation parameters including system suitability, system precision, specificity, method precision, ruggedness (intermediate precision), linearity, sensitivity, accuracy, range, robustness, filter validation, and forced degradation to assess stability-indicating capability (International Council for Harmonisation 2005).

#### System suitability

System suitability was evaluated by preparing a standard ROG solution and injecting it five times. The results are presented in the Supplemental File (Tables S1 & S2). Theoretical plates for the ROG peak in the standard solution were 3604 (acceptance criterion  $\geq 2000$ ), and the tailing factor was 1.18 (acceptance criterion  $\leq 2.0$ ). The % relative standard deviation (RSD) for five replicate injections was 0.10% (acceptance criterion  $< 2.0\%$ ). All system suitability parameters met FDA requirements (U.S. Food and Drug Administration 2022).

#### System Precision

To evaluate system precision, the standard ROG solution was prepared and injected six times according to the analytical method. The results are provided in the Supplemental File (Table S3). The % RSD of the ROG peak areas for the six replicate injections was 0.09% (acceptance criterion  $< 2.0\%$ ).

#### Specificity

Solutions of individual impurities, ROG sample, ROG placebo, and spiked ROG were prepared and analyzed, with results summarized in Table 2. Peak purity of ROG in the spiked sample met the required criteria, and all process-related impurities were well resolved from the ROG peak. Representative chromatograms of the blank and spiked samples are shown in Figures 3 and 4, respectively. The absolute differences in % assay during specificity testing are provided in the Supplemental File (Table S4).

#### Method Precision

Method precision was determined by analyzing six independently prepared sample solutions of the ROG formulation at the working concentration.

#### Ruggedness (Intermediate Precision)

Ruggedness of the method was evaluated by analyzing six independently prepared sample solutions of the ROG formulation at the working concentration, using different analysts, instruments, reagents, laboratories, columns, and on different days. Detailed information on variations, system suitability parameters, peak areas, and results for method precision and intermediate precision are provided in the Supplemental File (Tables S5–S8). The % assay values from the six preparations were within the specified range (95–105%), and the % RSD for these assay values was below 2%. The overall % RSD for all 12 results from method precision and intermediate precision remained within the acceptance criterion of less than 3%.

#### Linearity

ROG composite solutions at five concentrations (80%, 90%, 100%, 110%, and 120%) were prepared from the standard stock solution. Each concentration was analyzed in triplicate to assess linearity. The linearity curves were plotted between concentrations and corresponding peak areas (mAU) for ROG solutions ranging from 0.8 to 1.2  $\mu\text{g/mL}$ . Linearity results are summarized in Table 3, and the calibration curve is provided in the Supplemental File (Figure S1). The correlation coefficients for ROG were  $\geq 0.999$ , indicating a strong linear relationship between peak area and concentration, consistent with the calibration curve regression data.

#### Sensitivity

The method's sensitivity was evaluated by determining the limit of detection (LOD) and limit of quantification (LOQ) using the standard deviation of the intercept ( $\sigma$ ) and the slope (S) of the calibration curve (U.S. Food and Drug Administration 2022), according to the following equations:

$$\text{LOD} = 3.3 \times \sigma / S \text{ (U.S. Food and Drug Administration 2022).}$$

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

The LOD and LOQ for ROG were determined to be 0.010  $\mu\text{g/mL}$  and 0.035  $\mu\text{g/mL}$ , respectively.

#### Accuracy or Recovery

ROG composite solutions at 80%, 100%, and 120% concentrations were prepared in triplicate and analyzed to assess method accuracy. Recovery was determined using the component recovery approach, comparing the amount added (spiked) with the amount measured (recovered). Results are presented in Table 4. Individual recoveries ranged from 97.0% to 103.0%, with mean recoveries between 98.0% and 102.0%. The %RSD at each level was below 2%, confirming the method's accuracy.

### Range

Method validation was performed over the 80–120% concentration range of ROG, covering precision, accuracy, and linearity. All system suitability and validation parameters met the acceptance criteria as per ICH guidelines (International Council for Harmonisation 2005).

### Robustness

The robustness of the method was evaluated by varying the flow rate and column oven temperature. For each condition, standard and sample solutions were analyzed, altering one parameter at a time while keeping the other constant. Retention times and assay results are summarized in Table 5, while details of the varied parameters, system suitability metrics, and observed peak areas during robustness testing are provided in the Supplementary Information (Tables S9–S11). The assay results for ROG remained within the acceptable range of 95–105%.

### Bench Top Solution: Stability of Standard Solution and Sample Solutions

The stability of standard and sample solutions containing ROG was evaluated under room temperature conditions. Analyses were performed at the initial time point and after 48 hours to monitor any potential changes in the solutions. Both the standard and sample solutions were found to be stable over the 48-hour period, with no significant degradation observed. The difference in % assay between the initial and 48-hour samples did not exceed  $\pm 2.0\%$ , indicating minimal variation. Additionally, the similarity factor for the standard solution ranged from 0.98 to 1.02. Detailed results of the solution stability study are presented in the Supplementary Information (Table S12).

### Bench Top Stability of Mobile Phase

The mobile phase was stored at room temperature, and its stability was assessed by analyzing the standard solution at the initial time point and after 48 hours. No haziness or particulate matter was observed during this period, indicating that the mobile phase remained physically stable for up to two days. A summary of the results is provided in the Supplementary Information (Table S13).

### Filter Validation

The method was validated for filter compatibility using two types of 0.45  $\mu\text{m}$  filters: Nylon and Polyvinylidene fluoride (PVDF). A homogeneous sample solution of ROG was prepared, and separate portions of this solution were either centrifuged or passed through each filter type before being injected into the HPLC system. Comparative analysis showed that the difference between the filtered samples did not exceed 2%, and all assay values remained within the specified range of 95–105%, demonstrating consistent quantification. No significant interference or variation was observed

between the Nylon and PVDF filters, indicating that both are suitable for sample preparation. Comprehensive details of the filter specifications and corresponding assay results are presented in the Supplementary Information (Tables S14 and S15).

### Forced Degradation Study

The stability-indicating property of the HPLC assay for ROG in the microsphere formulation was thoroughly evaluated by exposing the samples to a range of stress conditions, including acid and base hydrolysis, oxidative degradation with hydrogen peroxide, aqueous hydrolysis, elevated temperature, high humidity, white fluorescent light, and UV light. Each stressed sample was analyzed using the chromatographic system, and the results are summarized in Table 6. Only minimal degradation of ROG was observed under the applied stress conditions, indicating good stability of the drug in the microsphere formulation. The ROG peak was found to be pure and spectrally homogeneous, confirming that no degradation products co-eluted with the main peak. These findings establish that the method is reliable, specific, and fully capable of indicating stability and detecting potential degradants in the formulation. The minimal degradation observed may be attributed to the protective effect of the polymeric matrix, which limits drug exposure to external stress conditions and enhances physicochemical stability.

### Comparison of reported analytical methods for Rotigotine with the proposed method

Parameter	Reported Methods	Present Method
Dosage form	Bulk / Transdermal Patch	Polymeric Microspheres
Run time	20–30 min	15 min
Detection Wavelength	UV/HPLC	RP-HPLC (278nm)
LOD ( $\mu\text{g/mL}$ )	$\sim 0.05\text{--}0.10$	0.010
LOQ ( $\mu\text{g/mL}$ )	$\sim 0.15\text{--}0.30$	0.035
Stability indicating	Limited	Yes
Matrix complexity	Low	High (Polymeric System)

The comparative analysis clearly demonstrates that the proposed method offers improved sensitivity, reduced run time, and enhanced applicability for complex microsphere-based delivery systems.

The analytical method developed in this study is particularly relevant for polymeric drug delivery systems, where accurate quantification is often hindered by matrix interference and incomplete drug extraction. The validated method not only ensures precise quantification of rotigotine but also supports its application in critical pharmaceutical studies such as *in vitro* drug release profiling, stability assessment, and formulation optimization. Furthermore, the stability-

indicating capability of the method makes it highly suitable for regulatory and quality control environments, where detection of degradation products is essential.

### CONCLUSION

The findings of this study indicate that the RP-HPLC method with gradient elution is highly specific, accurate, precise, linear, and rugged for the quantification of ROG in pharmaceutical formulations. The method exhibited robust performance, maintaining consistent results despite deliberate variations in column oven temperature and flow rate. The method showed both precision and accuracy at the defined limits of detection and quantification, demonstrating reliable performance even at the LOQ. Stability assessments confirmed that both standard and sample solutions remained stable for up to 48 hours at room temperature. Similarly, mobile phase stability was maintained for the same duration. Filter validation confirmed the suitability of 0.45 µm Nylon and Polyvinylidene fluoride filters for standard and sample solutions. Forced degradation studies were conducted on the ROG formulation, confirming the method's stability-indicating capability.

The method validation was carried out in accordance with the ICH guidelines, yielding results supporting its reliability for ROG measurement via RP-HPLC. To the best of our knowledge, this is the first validated RP-HPLC method reported for the quantification of Rotigotine in polymeric microsphere formulations. With a short run time of 15 minutes, the method is well-suited for routine analysis of ROG across different pharmaceutical formulations and for stability testing. Furthermore, this method has potential applications in additional studies, including assay development, stability assessments, in vitro release testing, and in vitro permeation testing for future quantitative analyses.

### DECLARATIONS

#### FUNDING

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#### COMPETING INTERESTS

The authors have no relevant financial or non-financial interests to disclose.

#### CONFLICT OF INTEREST

The authors declare that no potential conflicts of interest exist for this article's research, authorship, and/or publication.

#### AUTHOR CONTRIBUTIONS

**M. Himabindu:** Conceptualization, experimental work, data analysis, and manuscript drafting.

**Dr. Remya P. N.:** Study design, supervision, and manuscript review.

**Dr. Purnima Ashok:** Research guidance and final manuscript approval.

### ETHICAL APPROVAL

This research work involves analytical method development and formulation studies only. No experiments involving human participants or animals were conducted.

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### REFERENCES

1. Bunten S, Happe S. 2006. Rotigotine transdermal system: a short review. *Neuropsychiatr Dis Treat.* 2(4):421–426.
2. de Carvalho Mendes T, et al. 2021. Rotigotine: a review of analytical methods for the raw material, pharmaceutical formulations, and its impurities. *J AOAC Int.* 104(3):592–604.
3. de Carvalho Mendes T, et al. 2024. Identification and characterization of rotigotine degradation products by HPLC coupled DAD and CAD detectors and HRMS through Q-Orbitrap and electrospray ionization. *J Pharm Sci.* 113(8):2542–2551.
4. Elshoff JP, et al. 2015. An update on pharmacological, pharmacokinetic properties and drug–drug interactions of rotigotine transdermal system in Parkinson's disease and restless legs syndrome. *Drugs.* 75(5):487–501.
5. Grimes D, et al. 2012. Canadian guidelines on Parkinson's disease. *Can J Neurol Sci.* 39(Suppl 4):S1–S30.
6. International Council for Harmonisation. 2005. ICH Q2(R1): validation of analytical procedures: text and methodology. Geneva: ICH.
7. Li W, et al. 2022. High drug-loaded microspheres enabled by controlled in-droplet precipitation promote functional recovery after spinal cord injury. *Nat Commun.* 13:1262.
8. National Center for Biotechnology Information. 2023. Rotigotine [Internet]. PubChem [cited 2023 Mar 13]. Available from: <https://pubchem.ncbi.nlm.nih.gov/compound/Rotigotine>
9. Nyarko S, et al. 2024. Development of the RP-HPLC method for simultaneous determination and quantification of artemether and lumefantrine in fixed-dose combination pharmaceutical dosage forms. *Adv Pharmacol Pharm Sci.* 2024:3212298.
10. Pahwa R, et al. 2017. Rotigotine for the treatment of Parkinson's disease: a review of safety and efficacy. *Expert Opin Pharmacother.* 18(10):1009–1015.
11. Poewe W, LeWitt PA. 2010. Rotigotine transdermal system: developing continuous dopaminergic

- delivery in Parkinson's disease. *Ann N Y Acad Sci.* 1184(1):113–124.
- Raeder V, et al. 2021. Rotigotine transdermal patch for motor and non-motor Parkinson's disease: a review of 12 years' clinical experience. *CNS Drugs.* 35(2):215–231.
  - Ramireddy AR, Behara DK. 2022. Analytical method validation on simultaneous estimation of ozenoxacin and benzoic acid in pharmaceutical formulation. *Acta Chromatogr.* 35(3):278–285.
  - Saha P, Pandey MM. 2022. DoE-based validation of an HPLC-UV method for quantification of rotigotine nanocrystals: application to in vitro dissolution and ex vivo nasal permeation studies. *Electrophoresis.* 43(4):590–600.
  - U.S. Food and Drug Administration. 2007. NEUPRO™ (rotigotine transdermal system) prescribing information. Silver Spring (MD): FDA.
  - U.S. Food and Drug Administration. 2022. Reviewer guidance: validation of chromatographic methods. Silver Spring (MD): FDA.
  - van der Palen JCM, et al. 2018. Rotigotine transdermal patch in Parkinson's disease: a systematic review and meta-analysis. *Eur J Neurol.* 25(2):247–256.
  - Yawalkar AN, Pawar MA, Vavia PR. 2022. Microspheres for targeted drug delivery: a review on recent applications. *J Drug Deliv Sci Technol.* 75:103659.
  - Yi D, et al. 2023. Analysis of the dissolution behavior and solubility of rotigotine form II in different mono-solvents. *J Mol Liq.* 377:121532.
  - Yabré M, et al. 2018. Greening reversed-phase liquid chromatography methods using alternative solvents for pharmaceutical analysis. *Molecules.* 23(5):1065.

#### LIST OF FIGURES

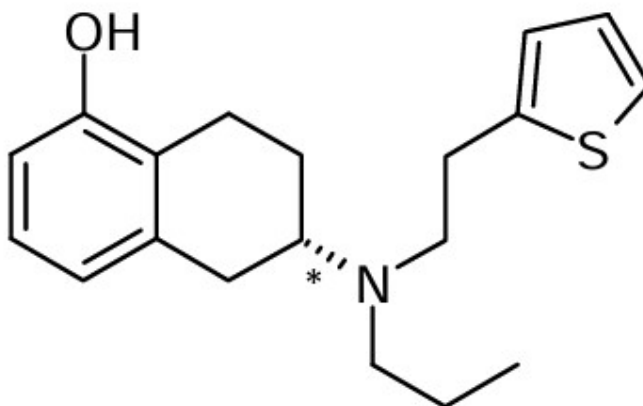


Figure. 1 Structure of Rotigotine

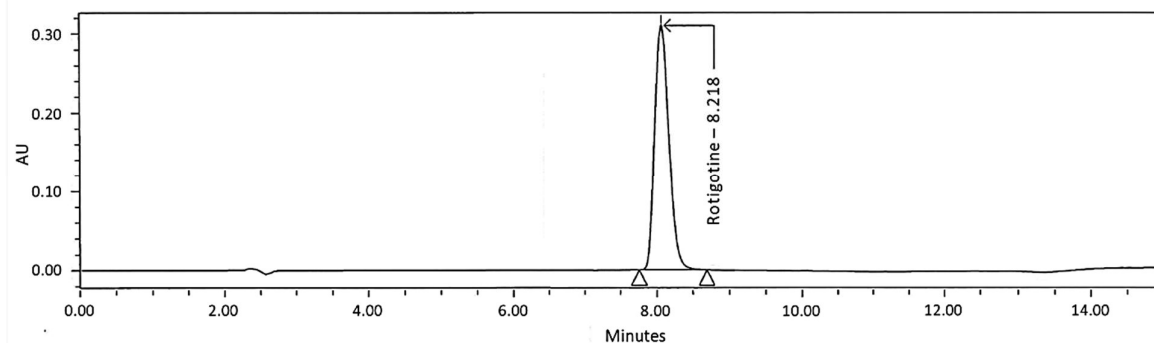


Figure. 2 Typical Chromatogram of ROG Standard Solution

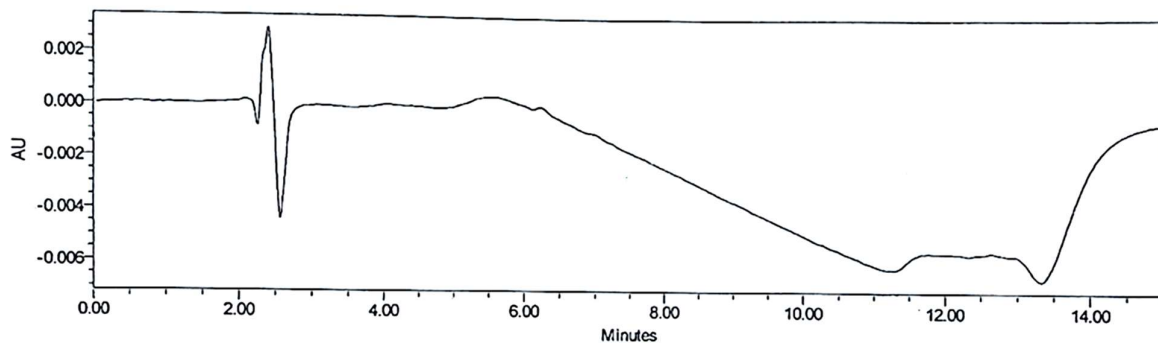


Figure. 3 Typical Chromatogram of Blank sample

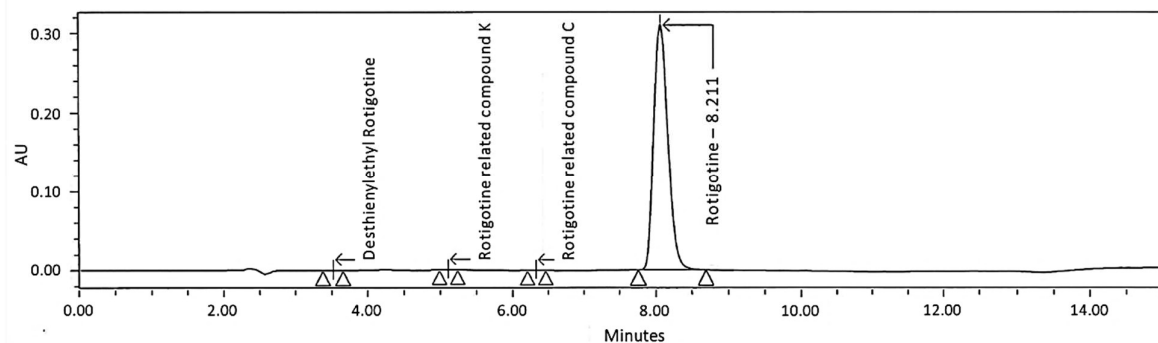


Figure. 4 Typical Chromatogram of Spiked sample

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Table 1: Gradient Elution Program

Time (Minutes)	Mobile Phase A	Mobile Phase B
0.00	70	30
2.00	70	30
10.00	35	65
12.00	35	65
13.50	70	30
15.00	70	30

Table 2: Specificity Results

Compound Name	Retention Time (Minutes)	Purity Angle	Purity Threshold	Peak Purity
Rotigotine	8.211	0.142	0.275	Pass
Desthienylethyl rotigotine	3.534	Not Applicable		
Rotigotine related compound K	5.184			
Rotigotine related compound C	6.361			
Blank	No interfering peaks were detected at the retention time of Rotigotine.			
Placebo Solution				

**Table 3: Linearity data of Rotigotine**

Linearity Level	Linearity of Rotigotine	
	Concentration (µg/mL)	Average Area (mAU)
80%	0.803	1725650
90%	0.903	1938754
100%	1.003	2140539
110%	1.104	2359212
120%	1.204	2574457
Correlation coefficient	0.9999	
Coefficient of determination	0.9998	
Intercept	29694	
Slope	2111273	
Regression equation (y = mx + c)	y = 2111273 x + 29694	

**Table 4: Accuracy or Recovery data of Rotigotine**

Spike Level	Amount added in µg/mL	Amount found in µg/mL	% Recovery	Mean	STDEV	%RSD
80% Level	0.803	0.801	99.837	100.10	0.23	0.23
	0.805	0.807	100.213			
	0.804	0.806	100.251			
100% Level	1.003	1.004	100.129	100.11	0.18	0.18
	1.005	1.004	99.923			
	1.006	1.009	100.291			
120% Level	1.204	1.207	100.237	100.13	0.17	0.17
	1.208	1.207	99.933			
	1.207	1.210	100.213			

**Table 5: Robustness data for Rotigotine**

S. No.	Condition	Retention Time (Minutes)	Assay (%w/w)
1	Actual	8.21	99.78
2	Low Column Temp.	8.46	99.92
3	High Column Temp.	8.11	100.10
4	Low Flow Rate	8.56	100.13
5	High Flow Rate	8.00	99.64

**Table 6: Rotigotine Parameters observed during Forced degradation Study**

S. No.	Condition	Assay (% w/w)	Purity Angle	Purity Threshold	Peak Purity
1	Control Sample	100.53	0.142	0.275	Pass
2	Acid Sample (1N HCl, 24 Hr at 60 °C)	100.35	0.156	0.303	Pass
3	Base Sample (1N NaOH, 24 Hr at 60 °C)	100.65	0.155	0.308	Pass
4	Water Sample (24 Hr at 60 °C)	99.50	0.153	0.293	Pass
5	Peroxide Sample (1% H <sub>2</sub> O <sub>2</sub> , 15 minutes at 60 °C)	99.49	0.156	0.291	Pass
6	Thermal Degradation Sample (60 °C for 48 hours)	100.55	0.152	0.313	Pass
7	White Fluorescent Exposure Sample (1.2 Million Lux Hours)	98.50	0.157	0.297	Pass
8	UV light Exposure Sample (200-Watt hours / square meter)	100.51	0.150	0.289	Pass
9	90% RH Humidity Exposure Sample	98.34	0.160	0.299	Pass

**Supplementary file:**

**System suitability:**

**Table S7: System Suitability Parameters**

S. No.	System suitability parameter	Result
1	Theoretical Plates	3604
2	Tailing Factor	1.18
3	% RSD	0.10

**Table S8: System Suitability Areas**

Injection No.	Area (mAU)
1	2142870
2	2142653
3	2142869
4	2144817
5	2147595
<b>Average</b>	2144160.68
<b>STDEV</b>	2111.42
<b>% RSD</b>	0.10

**System Precision:**

*Table S9: System Precision Areas*

Injection No.	Area (mAU)
1	2142870
2	2142653
3	2142869
4	2144817
5	2147595
6	2145009
<b>Average</b>	2144302.14
<b>STDEV</b>	1920.03
<b>% RSD</b>	0.09

**Specificity:**

*Table S10: Absolute difference between % Assay during specificity*

Name of the sample	Assay (% w/w) of Rotigotine
As such sample	100.53
Spiked sample	99.86
Difference	0.67

The absolute difference between the % Assay in such sample and the spiked sample is less than  $\pm 2.0\%$ .

**Ruggedness (Intermediate Precision):**

*Table S11: Details of Variations during Ruggedness*

S. No.	Changes	Method Precision	Ruggedness
1	Triethylamine	Merck (B. No. TF1Q786019)	Merck (B. No. TF1Q786019)
2	Orthophosphoric Acid	Merck (B. No. CF0C700316)	Merck (B. No. CBOC700056)
3	Methanol	Rankem (B. No. S176F76)	Merck (B. No. TM1ER24623)
4	Acetonitrile	Rankem (B. No. TO66G20)	Rankem (B. No. T038F20)
5	Date	11 MAR 2024	08 APR 2024
6	Column Details	LCC017/23	LCC010/23
7	Instrument Details	HPLC007	HPLC019

*Table S12: System Suitability Parameters during Ruggedness*

S. No.	System suitability parameter	Result
1	Theoretical Plates	3812
2	Tailing Factor	1.21
3	% RSD	0.12

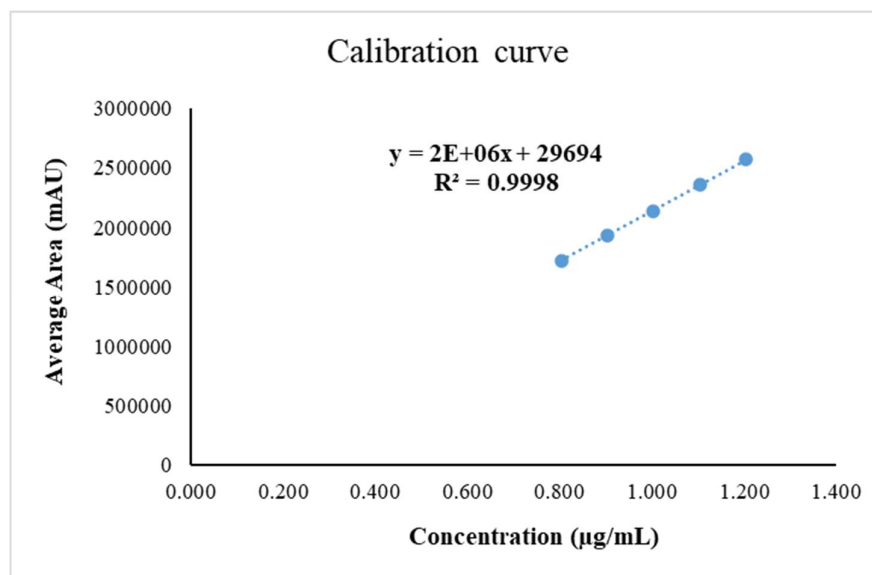
**Table S13: System Suitability Areas for Ruggedness**

Injection No.	Area (mAU)
1	2132857
2	2134772
3	2130727
4	2134796
5	2137561
Average	2134142.51
STDEV	2540.94
% RSD	0.12

**Table S14: Results of Method Precision & Intermediate Precision**

Injection No.	Assay (%w/w) in Method Precision	Assay (%w/w) in Intermediate Precision
1	99.86	99.54
2	100.27	99.34
3	99.67	99.66
4	100.07	99.75
5	100.20	99.35
6	99.72	100.02
Average	99.97	99.61
STDEV	0.25	0.26
%RSD	0.25	0.26
Overall Average	99.79	
Overall SD	0.31	
Overall RSD	0.31	

**Linearity:**



**Figure S5: Calibration curve of Rotigotine**

**Robustness:**

**Table S15: Parameters varied during Robustness**

Parameter	Actual	Low	High
Column Temperature	40°C	38°C	42°C
Flow rate	1.0 mL/min	0.9 mL/min	1.1 mL/min

**Table S16: System Suitability Parameters observed during variation in column oven temp.**

S. No	System Suitability Parameters	Actual Column Temperature (40°C)	Low Column Temperature (38°C)	High Column Temperature (42°C)
1	Theoretical Plates	3812	4529	2984
2	Tailing Factor	1.21	1.26	1.23
3	%RSD	0.12	0.11	0.19

**Table S17: System Suitability Parameters observed during variation in Flow Rate**

S. No	System Suitability Parameters	Actual Flow Rate (0.8 mL/min)	Low Flow Rate (0.7 mL/min)	High Flow Rate (0.9 mL/min)
1	Theoretical Plates	3812	4312	2874
2	Tailing Factor	1.21	1.30	1.24
3	%RSD	0.12	0.13	0.16

**Bench Top Solution Stability of Standard and Sample Solutions:**

**Table S18: Summary Results for solution stability study of standard solution and sample solution for Rotigotine**

Time Interval	Similarity Factor for Standard	Sample Assay (%w/w)	Difference in % Assay of Sample
Initial	Not applicable	99.50	Not applicable
48 hours at RT	1.00	98.99	0.51

**Bench Top Stability of Mobile Phase:**

**Table S19: Summary Results for Mobile phase Stability Study**

System suitability parameter	Result
Theoretical plates	3931
Tailing factor	1.14
% Relative standard deviation (RSD)	0.12

**Filter Validation:**

**Table S20: Filter Details**

Filter description	Filter	
	Nylon	PVDF
Manufacturer Name	mdi Membrane Technologies	mdi Membrane Technologies
Lot Number	SN7353G	SV2462L
Size in µm	0.45 µm	0.45 µm

***Table S21: Assay Results for Filter Validation***

<b>Filter details</b>	<b>% Assay</b>	<b>Absolute difference</b>
Centrifuged	99.50	Not Applicable
Nylon filter	99.72	0.22
PVDF filter	99.62	0.12