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Original Article

Stability Indicating Bioanalytical Method Development and Validation for Estimation of Remogliflozin Etabonate by RP-HPLC in Human Plasma

Poonam Dada Waditake, Mahesh Hari Kolhe*, Mayuri Kailas Mate, Rohit Jaysing Bhor, Pratibha Sudhakar Bhalerao, Manjusha Pandharinath Mhaske

Department of Pharmaceutical Quality Assurance, Pravara Rural College of Pharmacy, Pravaranagar, Loni, Maharashtra, INDIA.

ABSTRACT

Background: Remogliflozin etabonate belongs to the gliflozin class used for the treatment of type 2 diabetes mellitus and non-alcoholic steatohepatitis. **Objectives:** The objective of the present study is to develop an accurate, precise and validated bioanalytical reverse phase high-performance liquid chromatographic method for the estimation of Remogliflozin etabonate in human plasma and to conduct stability studies by forced degradation. **Materials and Methods:** The chromatographic analysis was performed at 224 nm on THERMO C_{18} (250×4.6 mm, 5 µm) column with a flow rate of 1 mL/min and a mixture of methanol-0.1 % acetic acid (80:20 v/v) used as a mobile phase. The method was validated with respect to linearity, accuracy, precision and robustness. In compliance with ICH guidelines, forced degradation studies were conducted in acidic/alkaline hydrolytic, peroxide and photolytic conditions. **Results:** The proposed method was used for the determination of drugs in plasma. Retention time was found to be 4.4 min for REM. The method was found to be linear over the concentration range 5-13 µg/mL. The correlation coefficient was determined to be 0.9992. The recovery of REM was more than 98% and % RSD was found to be less than 2%. LOD and LOQ were determined to be 0.13 and 0.42, respectively. **Conclusion:** The developed RP-HPLC method was found to be linear, accurate, precise and robust, hence it could be successfully used for routine quantification and stability monitoring of drugs in biological samples.

Keywords: Remogliflozin etabonate, Bioanalytical, Validation, Forced degradation, RP-HPLC.

Correspondence:
Mr. Mahesh Hari Kolhe
Department of Pharmaceutical Quality Assurance, Pravara Rural College of Pharmacy, Pravaranagar, Loni-413736, Maharashtra, INDIA.
Email: kolhe.mh@gmail.com
ORCID: 0000-0002-7591-4988

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INTRODUCTION

Diabetes mellitus is a collection of metabolic disorders distinguished by high blood glucose levels that arise from deficiencies in the secretion of insulin, its action, or both. The primary cause of Type 1 diabetes is an autoimmune response that destroys the pancreatic β cells through humoral (B cell) and T-cell-mediated inflammation (insulitis).[1] 90-95% of cases of diabetes are Diabetes Mellitus Type 2 (T2DM), often called "non-insulin-dependent diabetes" or "adult-onset diabetes." This category includes those with peripheral insulin resistance and relative insulin insufficiency. The actual etiologies remain unknown; however, patients do not have an autoimmune breakdown of β cells.[2] T2DM is marked by elevated blood sugar levels (hyperglycemia) and impaired glucose tolerance. The condition arises from the body's insufficient responsiveness to insulin, resulting in an upsurge in insulin secretion and, subsequently, an insulin deficit.[3] Glycemic control gradually deteriorates in people with T2DM. Sodium-dependent glucose co-transporter inhibitors are anti-diabetic drugs.[4] The glucose reuptake in the proximal tubule of the kidneys is facilitated by the Sodium-Glucose co-transporter-2 (SGLT2), which exhibits a low-affinity mechanism.[5] SGLT2 is specifically expressed in the renal system.[4,6]

Remogliflozin Etabonate (REM) has the chemical composition represented by the formula $C_{26}H_{38}N_2O_9$.[6] This prodrug is inactive, but when it is administered and absorbed, it transforms into its active state, remogliflozin.[7] This active compound specifically targets SGLT2 in the renal proximal tubule and is employed in the management of type 2 diabetes.[6] It Top of Formstops the kidneys from reabsorbing glucose, which lowers the blood glucose level in people with T2DM.[8] Remogliflozin etabonate is not included in any pharmacopoeia.

A literature review reveals different analytical methodologies documented for the quantification of remogliflozin, either independently[6,7] or in conjunction with other medications.[8-10]

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The determination of remogliflozin in rat plasma has been documented using the LC-MS method.[11] Additionally, methods involving RP-HPLC and HPTLC have been documented for assessing REM levels in both bulk and tablet formulations.[12,13] However, at present, there is no method available for assessing REM in human plasma using RP-HPLC. The goal of this current study is to establish a new, precise and robust RP-HPLC method for quantifying REM in human plasma.

MATERIALS AND METHODS

Reagents and Chemicals

A gift sample of Pure REM was received from Swapnroop Drugs and Pharmaceuticals, Chha. Sambhajinagar, India. Methanol and acetic acid of HPLC grade were obtained from Omkar Traders Pvt. Ltd., Mumbai. The drug-free EDTA human plasma was obtained from the PMT Blood Bank, Pravaranagar.

Instrumentation

The chromatographic analysis was performed using an HPLC binary gradient system (AGILENT 1100 series) with a reciprocating pump (1100). In the chromatographic study, the stationary phase was THERMO C_{18} (250×4.6 mm, 5 µm) and a combination of methanol and 0.1% acetic acid was used as the mobile phase. The data was processed using CHEMSTATION software. A diode array detector was used for the detection of wavelength, which occurred at 224 nm.

Chromatographic Conditions

The C_{18} column was employed for chromatographic separation. Different compositions of the mobile phase were studied to obtain the best REM detection. The components of the mobile phase were degassed for duration of 15 min and were filtered prior to application. The standard and sample solutions were diluted using a diluent (mobile phase) in an 80:20%v/v ratio. The column was kept at a temperature of 25°C and 20 µL was the volume of injection with a flow rate of 1 mL/min.

Standard Stock Solution Preparation

Into a volumetric flask (10 mL), 10 mg REM was accurately weighed and transferred. Methanol (5 mL) was added to it and sonication was performed for 10 min to ensure the REM dissolves completely. The volume was adjusted with methanol to achieve a stock solution of 1000 µg/mL. 1 mL was drawn from the solution and placed into a volumetric flask (10 mL). The volume was adjusted with methanol to achieve the 100 µg/mL working standard solution.

Preparation of Blank Plasma

To prepare a blank plasma, acetonitrile (4 mL) was introduced to 1 mL of collected plasma as an extracting solvent. The mixture was thoroughly mixed and left at room temperature for 5 min. Then, centrifugation was performed at 10,000 rpm for 12 min. Finally, a clear fluid from the upper layer was separated, filtered and used further for the HPLC analysis.

Preparation of Calibration Standards

To create calibration standards of REM, an appropriate amount of diluted standard solution was added to the blank plasma to achieve drug concentrations ranging from 5-13 µg/mL.

Validation of Method

The validation of the developed method followed ICH guidelines for various parameters such as linearity, accuracy, precision, LOD, LOQ and robustness.

Linearity

The developed method's linearity was assessed by plotting a calibration curve by spiking plasma with a known quantity of REM ranging from 5-13 µg/mL. The regression equation was obtained from the calibration curve of peak area versus REM concentration.

Precision and Accuracy

The precision and accuracy of the developed method were assessed by spiking REM in human plasma. To assess intra-day precision, sample solutions of REM (7, 9, 11 µg/mL) were examined within the same day at low, medium and high concentration levels. The same concentrations were used to analyze inter-day precision.

The method's accuracy was assessed through recovery studies conducted at three different levels of concentration (80%, 100% and 120%). To perform the recovery studies, a specific concentration of the standard drug (80%, 100% and 120%) was added to previously analyzed sample solutions. %RSD and %recovery were calculated.

LOD and LOQ

The minimum detectable quantity in a sample from background noise but not quantitated is the LOD (Limit of Detection). The minimum quantity of substance in a sample that can be precisely and accurately quantified is the LOQ (Limit of Quantitation). Three times the noise level was used to determine LOD and ten times the noise level was used to calculate LOQ.

Robustness

Robustness was examined by implementing slight modifications to the chromatographic conditions. Minor adjustments were made to the mobile phase ratio (79:21 and 81:19), flow rate (1 mL/min±0.1 mL/min) and detection wavelength (224 nm±1 nm). The %RSD was calculated.

Assay in Formulations

Twenty tablets were weighed and then ground into a powdered form. An amount of powder equal to 10 mg of REM was mixed with methanol in a volumetric flask (100 mL). An appropriate quantity of plasma was introduced to a flask, the mixture was thoroughly mixed and centrifugation was performed for 15 min. After filtration, the volume was adjusted with methanol. A volume of 1mL from the solution was added to the volumetric flask (10 mL) and diluted with the mobile phase to achieve a 10 µg/mL REM solution. After sonication, the solutions were introduced into the system. The determination of peak areas was conducted and quantification was performed using a regression equation.

Stability Study

The studies of forced degradation were conducted to assess the intrinsic stability of REM and to find out the degradation pathways. During forced degradation studies, REM was subjected to diverse stress conditions such as acid (0.1 N HCl) and base hydrolysis (0.1N NaOH), peroxide degradation (3% H_2O_2) and photolytic degradation.

RESULTS

Optimized Chromatographic Conditions

The different chromatography parameters such as column temperature, mobile phase concentration, flow rate and other conditions were optimized during the method development.

Temperatures of the column oven were examined in the 25-30°C range. The optimum column temperature was found to be 25°C. Different mobile phase ratios were tried in order to achieve better separation. A mixture of methanol and 0.1% acetic acid at 80:20%v/v ratios was used for the HPLC analysis, as it gives effective resolution and better separation. Various flow rates (ranging from 0.8-1 mL/min) were tested and it was discovered that 1 mL/min was the best for effective separation. The UV spectrophotometer was used to scan the solution in order to select the wavelength and the maximum absorbance was confirmed at 224 nm (Figure 1). Results for optimized conditions are given in Table 1.

System Suitability Test

The test for system suitability was conducted by injecting a standard solution of REM six times into the system. Different parameters, including resolution, tailing factor, retention time and theoretical plates were assessed to ensure the reproducibility of the chromatographic system. All the results were found to be within specifications proving the suitability of the method. Table 2 shows results for system suitability.

Linearity

The response of the calibration curve of REM was observed to be linear across 5-13 µg/mL concentration. The linearity equation was obtained as y=37.0414x-7.695 and the correlation coefficient was found to be 0.9992 (Figure 2). As a result, good linearity was shown by the developed method. Table 3 represents the linearity results.

Precision and Accuracy

The precision and accuracy of the developed method were calculated by using %RSD and percentage recovery. Three different concentrations were used to calculate precision. The method's accuracy was assessed by adding specific concentrations of the standard drug (80%, 100% and 120%).

The %RSD values were found to be in the range of 0.08-0.73 for the intra-day precision and 0.04-0.30 for the inter-day precision. The mean recovery values for REM ranged from 98.63-100.81%, showing the method's accuracy. %RSD for both precision and accuracy was within the limits which shows good precision and repeatability of the method. Results for precision and accuracy are given in Table 4A and Table 4B.

LOD and LOQ

The detection limit for REM was determined to be 0.1390 µg/mL, while the quantification limit was 0.4213 µg/mL.

Robustness

The study showed that after introducing minor changes in the ratio of mobile phase, flow rate and detection wavelength, no significant variations were found. Thus, the method demonstrated robustness, with a %RSD below 2.0%. Table 5 shows the results of robustness.

Assay

The analysis was conducted on a tablet formulation containing 100 mg of REM. The % recovery of REM was found to be 101.85±0.31 %w/w in plasma.

Stability Study

Stability investigation was carried out under several stress conditions, including the hydrolysis with acids (Figure 3A) and alkalis (Figure 3B), peroxide degradation (Figure 3C), photolytic degradation (Figure 3D) and neutral degradation (Figure 3E). The study on degradation revealed that REM exhibited high susceptibility to acid and base hydrolysis as well as peroxide degradation. The drug remained stable under neutral and photolytic degradation conditions. Table 6 represents the results of degradation studies.

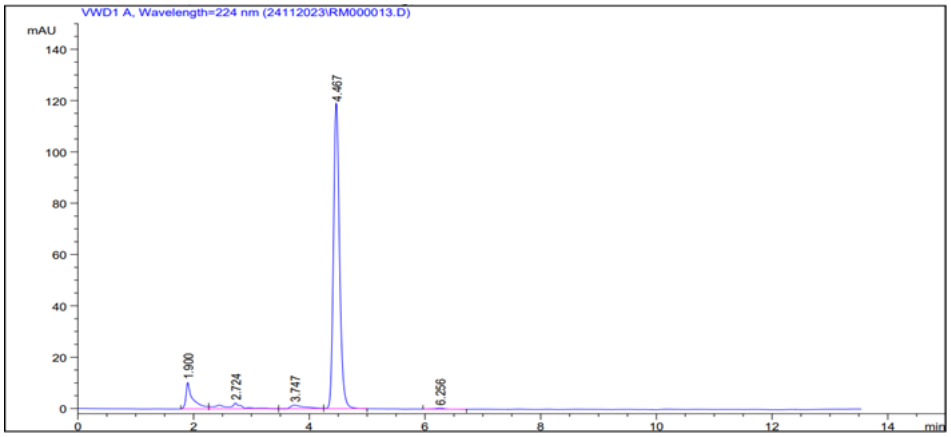

Figure 1: Representative chromatogram of Remogliflozin in human plasma.

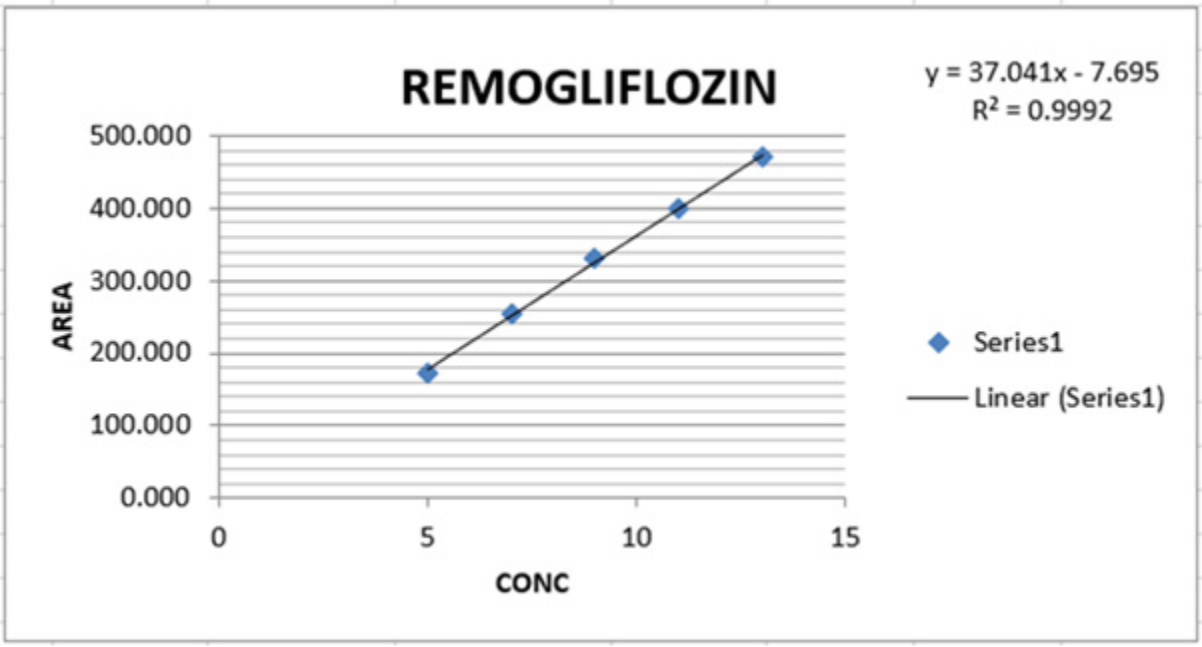

Figure 2: Calibration curve of REM spiked in human plasma.

Acidic Degradation

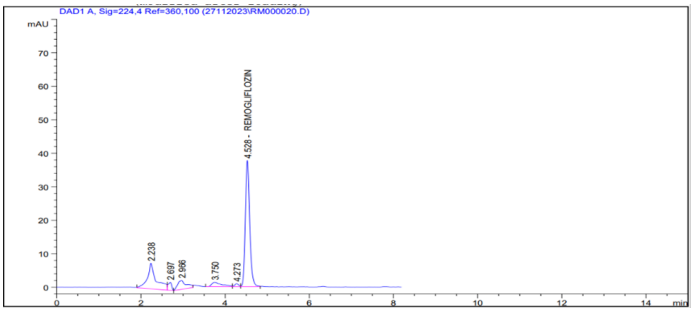

Figure 3A: Chromatogram of acidic degradation after 1 hr.

Basic Degradation

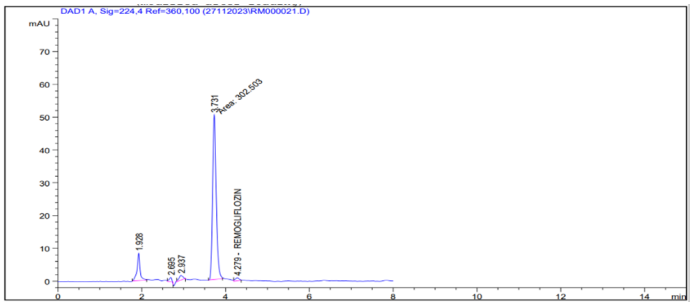

Figure 3B: Chromatogram of basic degradation after 1 hr.

Peroxide Degradation

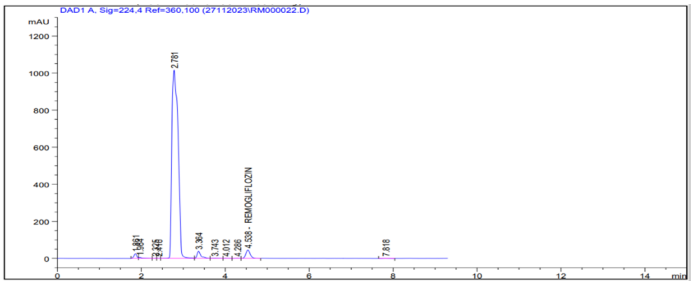

Figure 3C: Chromatogram of peroxide degradation after 1 hr.

Photolytic Degradation

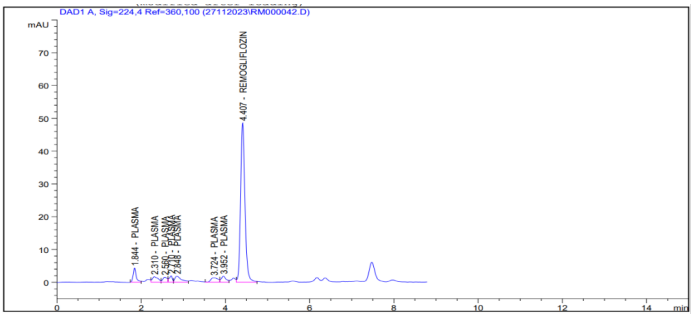

Figure 3D: Chromatogram of photolytic degradation after 24 hr.

Neutral Degradation

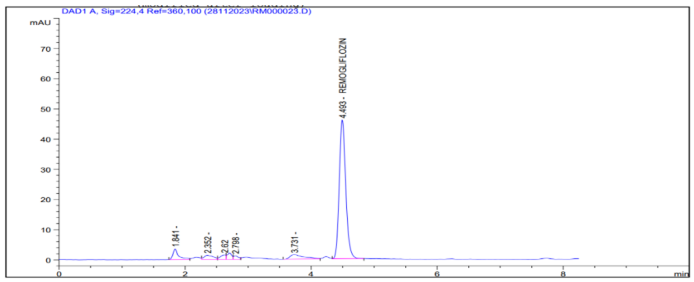

Figure 3E: Chromatogram of neutral degradation after 1 hr.

Table 1: Results of optimized conditions.

Parameters	Optimized conditions
Stationary phase	THERMO C_{18} (250×4.6 mm, 5 µm).
Mobile phase	Methanol: 0.1% acetic acid (80:20 v/v).
Flow rate	1 mL/min
Temperature of column	25°C
Injection volume	20 µL
Detection wavelength	224 nm

Table 2: System suitability parameters.

Parameters	Results
No. of theoretical plates	9408
Tailing factor	0.83
Retention time	4.4
Resolution	2.40

Table 3: Results for linearity studies of REM.

Sl. No.	Concentration (µg/mL)	Area±SD
1	5	173.78±1.41
2	7	254.20±2.68
3	9	329.70±0.66
4	11	398.79±0.63
5	13	471.90±2.42
Correlation coefficient	0.9992	
Slope	37.04	
Y - intercept	7.695	

Table 4A: Results of precision (*n*=3).

Concentration (µg/mL)	Intra-day		Inter-day	
	Area±SD	%RSD	Area±SD	%RSD
7	255.64±1.88	0.73	252.01±0.74	0.30
9	328.46±0.78	0.24	331.88±0.39	0.12
11	398.79±0.33	0.08	396.62±0.16	0.04

Table 4B: Results of Accuracy studies of REM (*n*=3).

Recovery level	Amount taken (µg/mL)	Amount recovered (µg/mL)	Mean % recovery	%RSD
80%	4	4.03	100.81	0.67
100%	5	4.95	98.91	0.94
120%	6	5.92	98.63	0.40

Table 5: Results of Robustness of method (*n*=3).

Chromatographic changes		Area±SD	%RSD
Mobile phase ratio	79:21	399.10±0.80	0.20
	81:19	423.07±0.95	0.22
Flow rate (mL/min)	0.9	459.00±0.61	0.13
	1.1	383.78±0.78	0.20
Wavelength (nm)	223	387.30±1.06	0.27
	225	388.16±1.04	0.27

Table 6: Results for degradation studies.

Stress type	Time	Degradation area	% Degradation
Acid degradation	1 hr.	271.52	17.65
	24 hr.	262.50	20.30
Base degradation	1 hr.	6.34	98.08
Peroxide degradation	1 hr.	314.31	4.67
	24 hr.	294.9	10.56
Neutral degradation	1 hr.	319.2	3.18
	24 hr.	311.04	5.66

DISCUSSION

An RP-HPLC method was developed and validated to analyze Remogliflozin etabonate, a pure drug, using a mobile phase consisting of methanol and 0.1% acetic acid (80:20 v/v). Validation encompassed several parameters including specificity, linearity, accuracy, precision, LOD, LOQ, recovery and stability. When applied to a marketed formulation, the method effectively assessed pharmacokinetic parameters. The method demonstrated linearity from 5-13 µg/mL, indicating a strong linear relationship within the tested concentration range. Accuracy studies revealed results close to the expected value (100%), confirming the method's accuracy, while precision assessment showed % RSD for intermediate precision below 2%, indicating its precision. A low LOQ emphasized the method's sensitivity, enabling precise estimation of nanogram drug quantities. Robustness testing confirmed the method's reliability against deliberate alterations.

Forced degradation studies exposed Remogliflozin etabonate's susceptibility to acid and base hydrolysis, resulting in significant ester hydrolysis and conversion into the active drug, Remogliflozin. The method was found to be stable in the case of neutral conditions. Hence the suggested method can be successfully used for identification and quantification of Remogliflozin in various biological matrices.

CONCLUSION

A simple, accurate and precise RP-HPLC bioanalytical method was developed and validated for REM quantification in human plasma, following the ICH guidelines. The % RSD for validation parameters was found to be less than 2, which suggests that the method is appropriate for the identification and quantitative determination of REM. The developed method has better separation, and linearity and is highly accurate for the determination of drugs in human plasma. Forced degradation investigations were conducted, indicating the drug's susceptibility to acid-base hydrolysis and peroxide degradation, which resulted in its conversion to the active form, Remogliflozin. The method is indicative of stability and is appropriate for the routine analysis of REM in biological fluids.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

%: Percentage; **ºC:** Degree celcius; **µg:** Microgram; **n:** No. of determinations; **mL:** Milliliter; **mg:** Milligram; **nm:** Nanometer; **T2DM:** Type 2 Diabetes Mellitus; **SGLT2:** Sodium glucose co-transporter 2; **HPTLC:** High performance thin layer chromatography; **min:** Minute; **RSD:** Relative standard deviation; **SD:** Standard deviation; **REM:** Remogliflozin etabonate; **hr:** Hour; **UV:** Ultraviolet; **RP-HPLC:** Reverse Phase High Performance Liquid Chromatography; **ICH:** International Council for Hormonization; **LC-MS:** Liquid chromatography-tandem mass spectroscopy; **LOD:** Limit of detection; **LOQ:** Limit of quantification.

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