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RP-HPLC Method Development and Validation of Rilpivirine Hydrochloride Quantification of Saliva Samples

Alekhya CH* **and Vijay Shree K**

Department of Pharmaceutics, Malla Reddy College of Pharmacy, India

***Corresponding author:** Alekhya CH, Department of Pharmaceutics, Malla Reddy College of Pharmacy, India; E-mail: alekhyachintanippu [at] gmail [dot] com

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Abstract

Determination of Rilpivirine hydrochloride in saliva samples by High Performance Liquid Chromatography with ultraviolet detection. Samples of saliva was extracted with methanol and spiked with Rilpivirine hydrochloride. The chromatographic separation was performed on Agilent Eclipse C18 (4.6x100mm) 3.5µm column, with a mobile phase comprising of a mixture of methanol : acetate buffer of pH 4.0 in the ratio 70:30 v/v. The flow rate was 1.0 mL/min with detection at 300 nm. Retention time of Rilpivirine hydrochloride was found to be 2.707 min. Linearity was found to be in the range of 0.25-25 µg/ml with regression equation y =1000000x+34542 and correlation coefficient 0.999. The low % RSD values are indicates the method is accurate and precise. The limit of detection (LOD) and limit of quantification (LOQ) were found to be 0.076 and 0.232 µg/ml, respectively.

Keywords: Rilpivirine; HPLC; Saliva; Method validation; Extraction

Introduction

Rilpivirine hydrochloride is a di-amino pyrimidine derivative. Chemically, it is 4-[[4-[[4-[(E)-2-cyanoethenyl]-2,6-dimethylphenyl]amino]-2-pyrimidinyl]amino]benzonitrile mono hydrochloride structure are shown in (Figure 1).

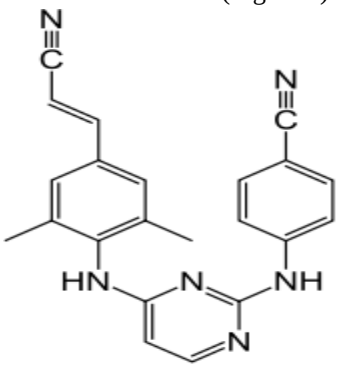

Figure 1: *Structure of Rilpivirine hydrochloride.*

Rilpivirine (TMC278) is Non –nucleoside reverse transcriptase, which was approved by the FDA in May 2011 [1-3]. It is a basic, white, amorphous powder which is readily soluble in methanol, dichloromethane and insoluble in water. Rilpivirine hydrochloride is not official in Indian Pharmacopoeia and British Pharmacopoeia. A thorough literature survey has revealed that UV spectroscopy [4-6], HPLC [7-9] method for Rilpivirine hydrochloride with combination of other drugs, UPLC [10], LC-MS [11,12] for its estimation in bulk, pharmaceutical dosage forms and biological samples.

Rilpivirine is a poorly soluble drug with intermediate permeability in vitro studies and its biological half-life 34-50 hrs [13,14]. Thus, bioavailability of poorly water-soluble drugs will be affected positively when their dissolution rate is increased. These drugs show serious adverse clinical effects like non-steady absorption due to variability among patients and individual patient dosing.

Till date there is no report to estimation of Rilpivirine in saliva samples. The aim of present research work for the development and validation of a HPLC method. The present work describes the development and validation of HPLC method, an attempt was

made to develop a simple, accurate, precise and rapid method for the Rilpivirine hydrochloride in human saliva.

Experimental

Materials and reagents

Rilpivirine was gift sample from Hetero drugs. Methanol Grade was purchased from Merck Chemical Company. HPLC Grade water was purchased from India mart. The 0.45 µm pump Nylon filter was obtained from Advanced Micro Devices (Ambala Cantt, India) & whatman no 5 filter paper was obtained from Modern Science lab, (Nashik, India). Glasswares used were Class A grade.

Sample preparation

In order to investigate the effects of medium on calibration curve linearity and equation parameter, working standard solutions of Rilpivirine are prepared in acetonitrile and saliva matrices. Human saliva are obtained from healthy volunteers and stored at -20 ˚C until analysis. Stock standard solution of Rilpivirine was prepared by dissolving 10mg in 10ml acetonitrile and stored at -20 ˚C for 1 month and protected from light. Further dilutions were prepared, by diluting stock solution with mobile phase to achieve calibration concentration (25-1000 ng/mL^{-1}).

Extraction process

Trail 1: To 0.2ml of saliva samples, 50µL of Ortho phosphoric acid and 3ml of n-hexane was added. The sample were mixed in a mechanical shaker for 20 min and centrifuged at 1000 rpm for 10 min. After centrifuged, supernant layer was separated and make up to 10ml with mobile phase [acetonitrile:water (80:20) v/v].

Trail 2: To 0.2ml of saliva samples, 50µL of ortho phosphoric acid and 3ml of methanol was added. The sample were mixed in a mechanical shaker for 20 min and centrifuged at 1000 rpm for 10 min. After centrifuged, supernant layer was separated and make up to 10ml with mobile phase [acetonitrile:water (80:20) v/v].

Out of 2nd trial performed, the 2nd trial was selected for further studies because 2nd trial was found good separation of saliva, with good peak symmetry.

Standard preparation

Weigh accurately about 10 mg of Rilpivirine hydrochloride working standard to a 10 ml volumetric flask. Add about 5 ml diluent to dissolve it completely (sonicate if necessary), make up the volume with diluents it gives 1000 µg/ml. Further dilute, 1ml of this solution to 10ml with diluents it gives 100µg/ml.

Standard stock solution preparation

Further dilutions were prepared, by diluting stock solution with mobile phase to achieve calibration concentration (0.25-25 µg/ml).

Preparation of sample solution

To the extract solution, different concentration of Rilpivirine hydrochloride was spiked to get the concentration of 0.25-25 µg/ml.

Validation of RP-HPLC method

As per the International Conference on Harmonization (ICH) guidelines, the method validation parameters like linearity, precision, accuracy, limit of detection, limit of quantitation, specificity and robustness were experimentally determined and the method was validated.

System suitability parameters

System suitability tests are an integral part of chromatographic method. To ascertain its effectiveness, system suitability tests were carried out by injecting freshly prepared standard stock solution of 10µg/ml Rilpivirne hydrochloride in six replications and the parameters like retention time, peak area, plate number (N), and peak asymmetry of samples were calculated.

Specificity

Specificity for an assay ensures that the signal measured comes from the substance of interest and there is no interference from excipient and/or degradation products and/or impurities. Specificity of the method was done by comparing the chromatogram of drug with the chromatogram of blank (mobile phase).

Linearity

Calibration and quality control samples were prepared by adding Rilpivirine hydrochloride solution in blank saliva. The amount corresponded to the saliva concentration of Rilpivirine hydrochloride ranged from 0.25 to 25 µg/ml. The calibration curves for the saliva spiked by Rilpivirine hydrochloride were obtained by platting Rilpivirine peak areas for the concentration range 0.25,0.5,1,5,10,15,20 and 25 µg/ml.

Precision

Precision studies were carried out to ascertain the reproducibility of the proposed method.

Repeatability (Method precision)

Repeatability was determined by preparing six replicates of 10 µg/ml Rilpivirine hydrochloride spiked with saliva separately inject equal volumes (20 µl) of each solution. Record the

chromatograms and measure the peak response of drug. The results were reported as %RSD.

Intermediate Precision

Interday precision

Intraday precision study was carried out by preparing drug solution spiked with saliva of concentration (10 µg/ml) and analysing it at three different times in a day. Record the chromatograms and measure the peak response of Rilpivirne hydrochloride. The results were reported as %RSD. The precision result showed a good reproducibility with percentage relative standard deviation less than 2.

Accuracy

The accuracy of methods was evaluated by performing triplicate analyses of concentrations of 125ng/mL, 250ng/mL and 500ng/mL in saliva spiked drug samples.

Limit of detection and limit of quantitation

The limit of detection (LOD) and the limit of quantitation (LOQ) of the drug were derived by calculating the signal-to-noise ratio (S/N, i.e., 3.3 for LOD and 10 for LOQ) using the following equations designated by International Conference on Harmonization (ICH) guidelines.

$LOD = 3.3 \times \sigma/S$

$LOQ = 10 \times \sigma/S$

Where,

σ = the standard deviation of the response and

S = slope of the calibration curve

Was performed and chromatographed. The sample solution 20µl was injected.

Robustness

Robustness studies were carried by changing the flow rate of mobile phase from 0.9 to 1.1 mL/min, and wavelength from 298 to 302. Rilpivirne hydrochlorides made in triplicates and were analysed.

Ruggedness

Ruggedness studies were performed by preparing three replicates of 10 µg/ml of Rilpivirine hydrochloride , analysing by two different analyst and the results are reported as %RSD. Structure of Rilpivirine hydrochloride is shown in (Figure 1).

Results

The Eclipse C_{18} (100 mm x4.6mm, 3.5µm) column was used and the mode of elution was isocratic. The flow rate 1.0m/min, injection volume was 20µL and run time of sample was 5min. Initially various mobile phase compositions were tired, to separate the ingredients. Out of 5 trials performed, the 5th trail was selected because when compared to other trails, the 5th trial had the least in retention time, with good peak symmetry as mention in and (Figures 2 and 3).

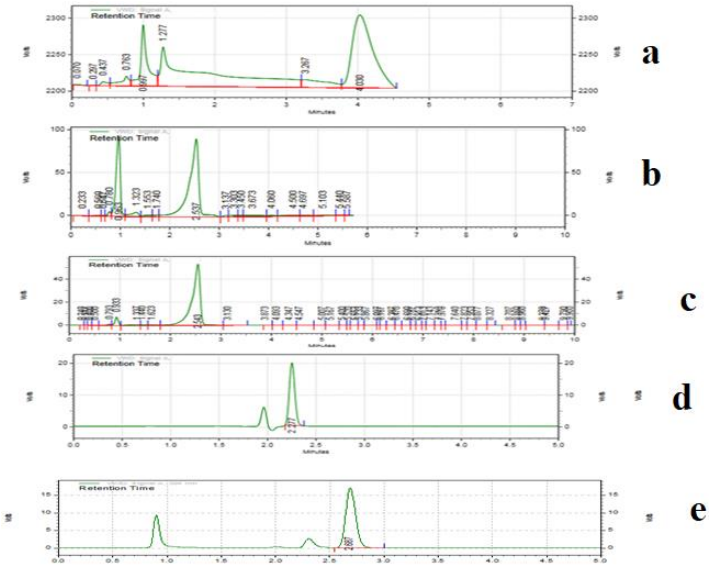

Figure 2: *Trails of mobile phases a. Methanol:water (50:50) b. Acetonitrile:water (50:50) c. Acetonitrile:methanol (50:50) d. Acetate buffer (pH 4):methanol (50:50) e. Acetate buffer (pH 4): methanol (40:60).*

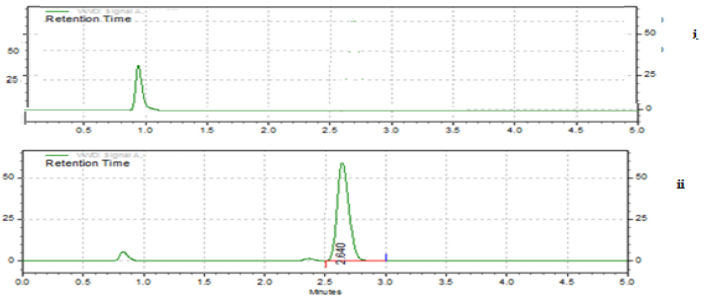

Figure 3: *Typical Chromatogram of i. blank saliva ii. Rilpivirine spiked with saliva.*

Extraction process

To 0.2ml of saliva samples, 50µL of ortho phosphoric acid and 3ml of methanol was added. The sample were mixed in a mechanical shaker for 20 min and centrifuged at 1000 rpm for 10 min. After centrifuged, supernant layer was separated and make up to 10ml with mobile phase [methanol:acetate buffer (70:30) v/v]. To the above solution, different concentration of drug solution was spiked and solution was injected. (Table 1) shows mobile phase section.

HPLC Method Validation

As per the International Conference on Harmonization (ICH) guidelines, the method validation parameters like linearity, precision, accuracy, limit of detection, limit of quantitation,

specificity and robustness were experimentally determined and the method validated.

Table 1: *Selection of mobile phase.*

S.No	Mobile phase conditions	Observation
1	Water : Methanol (50:50% v/v)	Broad peaks appear
2	Water : Acetonitrile(50:50 v/v)	Extra peak with tailing
3	Acetonitrile : Methanol (50:50 v/v)	
4	0.05M Acetate buffer of pH 4.0 : Acetonitrile (50 : 50 % v/v)	Sharp peak with fronting
5	0.05M Acetate buffer of pH 4.0 : Acetonitrile (60 : 40 % v/v)	Sharp peak with extra peak

Specificity

Specificity of the method was done by comparing the chromatogram of drug (drug spiked with saliva) with the chromatogram of blank (saliva). The chromatogram of the blank was recorded and it did not show any peaks. The chromatogram of the drug is given in (Figure 3) respectively.

System suitability parameters

System suitability test are an integral part of chromatographic method. They were used to verify that the reproducibility of the chromatographic system is adequate for the analysis. To ascertain its effectiveness, system suitability tests were carried out by injecting freshly prepared standard stock solution of Rilpivirine hydrochloride. Results are shown in (Table 2).

Table 2: *System Suitability Parameters.*

Parameters	Results
Retention time (min)	2.640
Theoretical plates	5962
HETP	0.016
Asymmetry	1.2

Linearity

Standard solutions of Rilpivirine hydrochloride in the concentration range of 0.25-25µg/ml were injected into chromatographic system and peak areas were measured. A graph of peak areas (on Y-axis) versus concentration (on X-axis) was plotted and calibration graph was shown in (Figure 4). The corresponding values are given in (Table 3). The regression equation was found to be y = 1000000x+34542. Coefficient of determination was found to be 0.999.

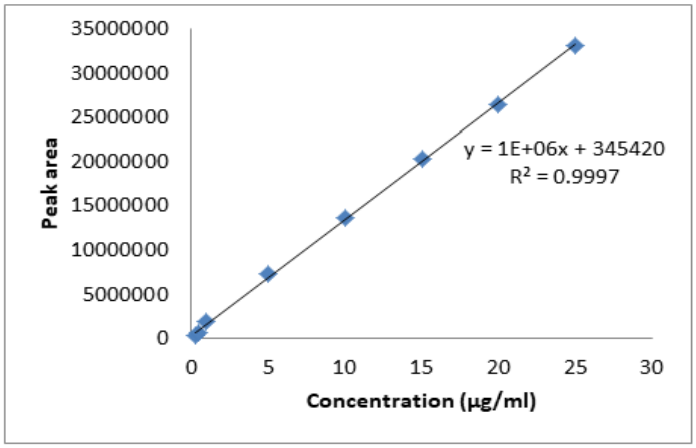

Figure 4: *Calibration curve of Rilpivirine hydrochloride.*

Table 3: *Repeatability studies of Rilpivirine hydrochloride.*

Concentration (µg/ml)	Peak area	
10	13593009	Mean =13556868 S.D = 73291.8 %RSD =0.540
10	13526588	
10	13485698	
10	13685472	
10	13547892	
10	13502548	

Precision

The results were reported as %RSD. The precision result showed a good reproducibility shown with percent relative standard deviation less than 2. Corresponding results were mentioned in (Table 4).

Accuracy

The accuracy of HPLC analysis tested by the recovery of Rilpivirine hydrochloride in saliva is summarized in (Table 5).

Robustness

Robustness studies were carried out by changing the flow rate of the mobile phase from 0.8 to 1.2 mL/min and by changing the wavelength from 238 to 242 nm. 125ng/mL Rilpivirine hydrochloride was analysed.

Ruggedness

Robustness studies were carried by changing the flow rate of mobile phase from 0.9 to 1.1 mL/min and by changing in wavelength from 298 to 300 nm. 10 µg/ml Rilpivirine hydrochloride was analysed and the %RSD is determined. Referred in (Table 6).

Table 4: *Precision of Rilpivirine hydrochloride.*

	Intraday precision		Interday precision	
Concentration (µg/ml)	**Peak area**		**Peak area**	
10	13526584	Mean 13565961 ± 92006.3 %RSD = 0.678	13524587	Mean = 13572845 ± 78610.4 %RSD=0.579
10	13565845		13658742	
10	13542584		13458751	
10	13658742		13548752	
10	13698521		13587498	
10	13569852		13658742	

Table 5: *Accuracy studies of Rilpivirine hydrochloride.*

Saliva Concentration (µg/ml)	Amount recovered (µg/ml)	% Recovery	% Mean recovery± SD	%RSD
5	4.92	98.4	97.7 ± 0.989949	1.01
5	4.85	97.0		
5	4.81	96.2		
10	9.70	97.0	97.85 ± 1.20	1.22
10	9.87	98.7		
10	9.61	96.1		
15	14.72	98.1	98.73 ± 1.66	1.64

Table 6: *Ruggedness studies of Rilpivirine hydrochloride.*

Concentration (µg/ml)	Change in flow rate (ml/min)		Retention time (min)		Change in wavelength (nm)		Retention time (min)	
	0.9	**1.1**	**0.9**	**1.1**	**298**	**302**	**298**	**302**
10	13526589	13548695	2.907	2.273	13569852	13587456	2.640	2.640
	13654857	13658745			13658745	13594526		
	13458752	13459854			13487536	13655421		
	13558745	13548752			13569852	13518742		
	13548754	13554874			13542658	13487521		
	13569854	13569854			13652158	13598521		
Mean area ± SD	13552925 ± 63702.64	13556796 ± 63396.98			13580134 ± 65661.9	13573698 ± 60604.5		
%RSD	0.470	0.467			0.483	0.446		

Limit of detection and limit of quantitation

The parameters LOD and LOQ were determined on the basis of response and slope of the regression equation. The limit of detection (LOD) and the limit of quantitation (LOQ) of the drug were derived by calculating the signal-to-noise ratio (S/N, i.e., 3.3 for LOD and 10 for LOQ) using the following equations

designated by International Conference on Harmonization (ICH) guidelines.

$LOD = 3.3 \times \sigma/S$ $LOQ = 10 \times \sigma/S$

Where, σ = the standard deviation of the response and; S= slope of the calibration curve.

- LOD = 0.076
- LOQ = 0.232

Conclusion

The proposed RP-HPLC method is uncomplicated, quick, accurate, precise, robust, and sensitive. This process has been observed to be improved over previously reported analytical methods of Rilpivirine hydrochloride in terms of validation parameters, use of a cost-effective as well as a mobile phase methanol:acetate buffer (70:30) v/v], low Rt (speedy analysis), no internal standard and UV detection. A simple, rapid precise, accurate and robust RP-HPLC-UV method was developed, validated for the determination of Rilpivirine hydrochloride in saliva and optimization parameters. The simplicity of the method allows for application in laboratories that lack sophisticated analytical instruments such as LC-MS/MS or GC-MS/MS that are complicated, costly and time consuming rather than a simple HPLC method. The contribution of each of the above mentioned factors indicate the superiority of the developed method above other described analytical methods for the regular investigation of Rilpivirine hydrochloride as an active pharmaceutical ingredient and biological samples like saliva.

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Declaration of Interest

The author has no relevant affiliations or financial involvement with a financial interest in or financial with the subject matter or materials discussed in the manuscript.

Conflicts of Interest

There is no conflict of interest.

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