IJPPR
INTERNATIONAL JOURNAL OF PHARMACY & PHARMACEUTICAL RESEARCH
An official Publication of Human Journals
ISSN 2349-7203
HUMAN

Human Journals
Research Article
September 2019 Vol.:16, Issue:2

Quality by Design Approach for Bioanalytical Method Development and Validation of Pidotimod in Rat Plasma by RP-HPLC Method

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***Sonali P. Mahaparale, Vrushali B. Londhe**

**Dept. of Pharmaceutical Quality Assurance, Dr.D. Y. Patil College of Pharmacy, Akurdi, Pune 411044, Maharashtra, India*

Dept. of Pharmaceutical Quality Assurance , Dr.D. Y. Patil College of Pharmacy, Akurdi, Pune 411044, Maharashtra, India

Submission: 25 August 2019
Accepted: 30 August 2019
Published: 30 September 2019

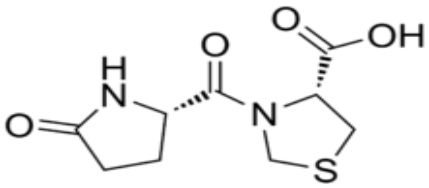
Keywords: Pidotimod, Reverse phases HPLC, Accuracy, Precision

ABSTRACT

A simple, rapid, selective, sensitive, accurate and precise High-Performance Liquid Chromatography (HPLC) with UV detection method has been developed and validated for the determination of Pidotimod in rat plasma. C18 (250x4.6mm) column was used with the mobile phase containing a mixture of Methanol: Water (10:90v/v). The flow rate was 2 ml/min and the drug was monitored at 215 nm. Method development was done by using Quality by Design. QBD provides an optimized method for validation. In analytical method development linearity for Pidotimod was found to be 5-100µg/ml with regression coefficient 0.9950. The accuracy and recovery were found within the limit. The robustness study was performed and the method was found to be fully robust. Plasma samples were processed using acetonitrile as a precipitating agent to extract the drug. The linearity for Pidotimod was found to be 4 to 200 µg/ml with regression coefficient (r2) 0.9992. The recovery was found to be 98.03%. Stability studies for the freeze-thaw cycle, short term stability, and long term stability were done. The % CV for stability study was found within a limit.

INTRODUCTION

Pidotimod is an immunostimulant drug. Chemically, it is described as (R)-3-[(S)-(5-oxo-2-pyrrolidinyl) carbonyl]-thiazolidine-4-carboxylic acid. The molecular formula is $C_9H_{12}N_2O_4S$ which corresponds to molecular weight 244.2. Pidotimod is extremely water soluble. Pidotimod is used for the prevention and treatment of respiratory and urinary tract infections in patients with a compromised immune system. It is used along with other mainstream medications to treat and prevent chronic bronchitis, rhinitis, sinusitis, infections of the urinary tract, etc. This work aimed to develop a simple, accurate, reproducible and sensitive method for determination of Pidotimod in rat plasma using rapid, convenient and simple reverse phase HPLC method.



MATERIALS AND METHODS

Chemicals and reagents

A pure sample of Pidotimod, Methanol (HPLC grade), Water and Drug was obtained from Wockhardt Pvt. Ltd, Aurangabad.

Instruments used

Sr. No.	Instrument	Make/Model
1	HPLC	Agilent 1120 Compact LC
2	UV Spectroscopy	Shimadzu-1700 UV/VIS
3	Balance	LC/GC
4	Ultrasonic bath	Lifecare

Optimization of Chromatographic conditions

Optimization of the mobile phase was performed based on resolution, asymmetric factor and peak area obtained for Pidotimod. The mobile phase Methanol (HPLC grade): water (HPLC grade) (50:50, 60:40, 65:35, 70:30, 80:20) was also tried. Methanol: Water (10:90 v/v) at a flow rate of 2.0 ml/min was found to be satisfactory and gave symmetric and well-resolved peaks for Pidotimod. The chromatogram was recorded at 215.0 nm as the spectrum of

Pidotimod showed maximum response at this wavelength. Chromatogram showed symmetrical peaks with good shapes; tailing factor for Pidotimod was within range & the resolution of the standard drug was satisfactory. The retention time for Pidotimod was found to be 2.67 min. The system suitability parameters observed by using this mobile phase are reported.

Preparation of mobile phase:

HPLC grade Methanol: HPLC grade Water (10: 90v/v) which was filtered through 0.45 µm membrane filter and sonicated on ultrasonic bath for 15 min.

Preparation of standard stock solution:

Pidotimod standard stock solution was prepared by transferring 100 mg of Pidotimod working standard into a 50ml volumetric flask, approximately 10 ml of methanol (HPLC grade) was added and sonicated for 20 min. the volume was made up to 50 ml with HPLC grade water to get the concentration of 2000 µg/ml. This solution was filtered through a 0.45µm pore size nylon66 membrane .the subsequent dilutions were prepared by diluting the stock solution with the methanol.

Separation of plasma from Rat Blood:

The blood was removed from healthy rat by the retro-orbital method of blood collection. Blood will be collected into purple top EDTA tubed and centrifuge (3000 rpm) at 4ºC for 20minutes. After centrifugation uses clean pipette technique place 1.0 ml of plasma into a 1.5ml Eppendorf tube labeled with a tracking number and ‘plasma'.

Preparation of sample solution:

The sample solution was prepared by taking 0.90 ml of rat plasma and 100µl of working standard solution of 40, 200, 500, 1000, 1500, 2000 µg/ml and 1ml of precipitating agent acetonitrile to precipitate plasma protein, were added and mixed. The resulting solution 4, 20, 50, 100, 150, 200 µg/ml was centrifuged at 3000 rpm for 15min. at 2-4ºC.the supernatant, layer was separated and analyzed.

Spiking of Pidotimod in plasma

Table no 1. Spiking of Pidotimod in plasma

Concentration (µg/ml)	Vol. of spiking (ml)	Vol. of plasma (ml)	Final vol. (ml)	Final conc. (µg/ml)
40	0.1	0.9	1	4
200	0.1	0.9	1	20
500	0.1	0.9	1	50
1000	0.1	0.9	1	100
1500	0.1	0.9	1	150
2000	0.1	0.9	1	200

Validation Method Development:

Analysis of tablet formulation:

The marked tablet formulation contained 400 mg of Pidotimod was used. Twenty tablets were accurately weighed and the average weight was determined and transferred top clean and dry glass mortar and ground into a fine powder. The quantity of powder equivalent to 5 mg of Pidotimod was weighed accurately and transferred to 50 ml volumetric flask and 10 ml of methanol (HPLC grade) was added. The contents were sonicated for 20 min and made up the volume up to the mark 50 ml by Water (HPLC grade). This solution was filtered through 0.45 µm pore size nylon 66 membrane the solution was further diluted to get 100 µg/ml and injected into HPLC.

The fundamental parameters for Bioanalytical method validation are accuracy, precision, Selectivity, sensitivity, reproducibility, and stability. The measurements for each analyte in the biological matrix should be validated. Typical method development and establishment for the bioanalytical method includes determination of

(1) Selectivity,

(2) Accuracy, precision, recovery,

(3) Calibration curve and

(4) Stability of analyte in spiked samples.

Selectivity

Analysis of a blank sample of the appropriate biological matrix (plasma) should be obtained from at least six sources were tested for interference, & no interference at reported retention time was found.

Calibration curve

The concentration range over, which the linearity was found to be 4-200 µg/ml. The results are shown in Table 2.

Preparation of quality control standards

The quality control standard solution 20µg/ml, 100µg/ml, 200µg/ml were prepared.

Accuracy & Precision

Accuracy was measured using three determinations of LQC (20 µg/ml.), MQC (100 µg/ml.) and HQC (200 µg/ml.). The precision was carried out by within batch intraday& inter batch precision.

Accuracy & Precision within a batch

The within batch accuracy & precision was performed in a single day by taking three different Concentrations, & each concentration has three determination.

Inter batch Accuracy & Precision

The inter batch accuracy & precision was performed on different days by taking three different concentrations, & each concentration has three determination.

Recovery

Recovery experiment should be performed by comparing the analytical results for extracted samples at three concentrations (low, medium, high) with unextracted standards that Represent 100% recovery.

Stability

a. Freeze & Thaw stability

The freeze-thaw cycle was repeated two more times, and then analyzed on the third cycle.

b. Short term temperature stability

The short term stability was performed by three aliquots of each of the low and high concentrations were tested at room temperature and kept at this temperature from 8 hours and analyzed.

Long term stability

Long-term stability should be determined by storing at least three aliquots of each of the low and high concentrations under the same conditions for 15 days.

RESULTS AND DISCUSSION

Table no 2: Linearity of Pidotimod.

Standard calibration data of Pidotimod Spiked in Plasma

Sr. No	Concentration (µg/ml)	Peak Area* (mAU)	S.D.*
1	4	4432430	0.0215
2	20	11299396	0.0325
3	50	30133354	0.0227
4	100	60275837	0.0743
5	150	93295738	0.0518
6	200	121302138	0.0725

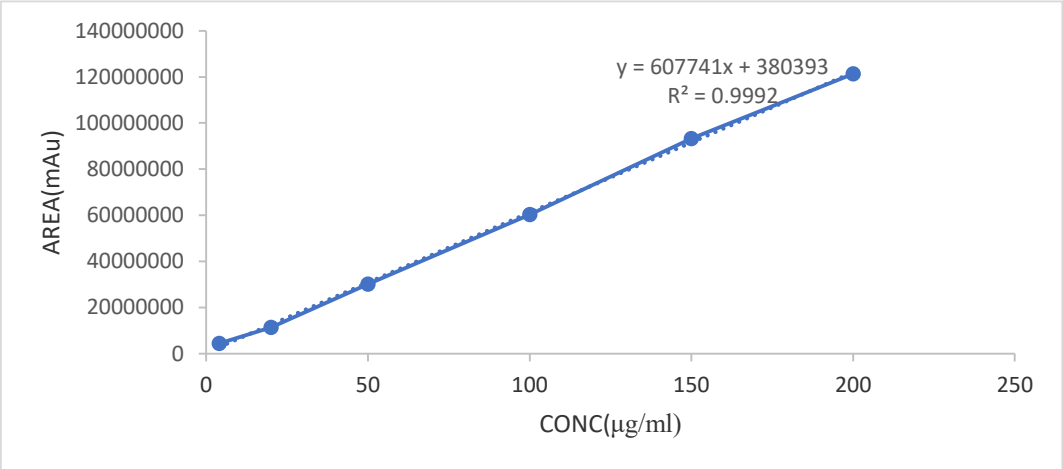


Figure no 1: Calibration curve of Pidotimod spiked in plasma

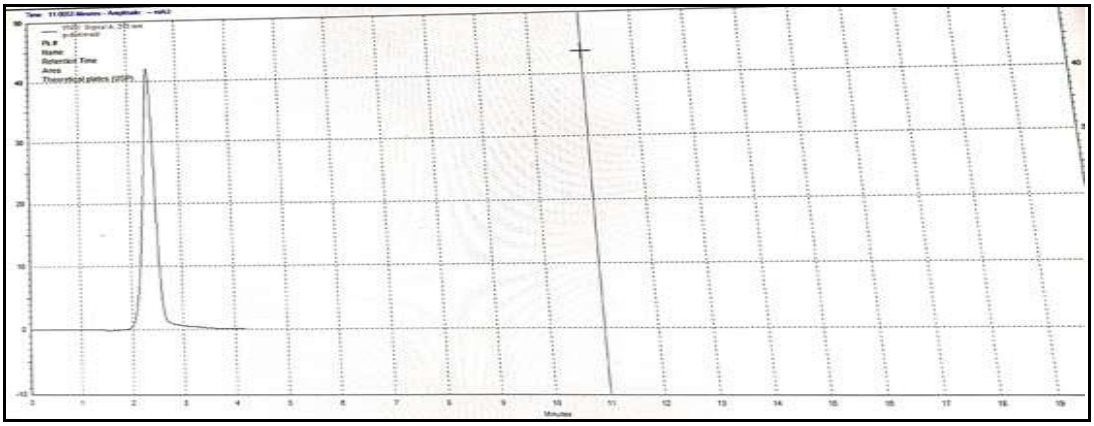


Figure no 2: Final chromatogram of Pidotimod

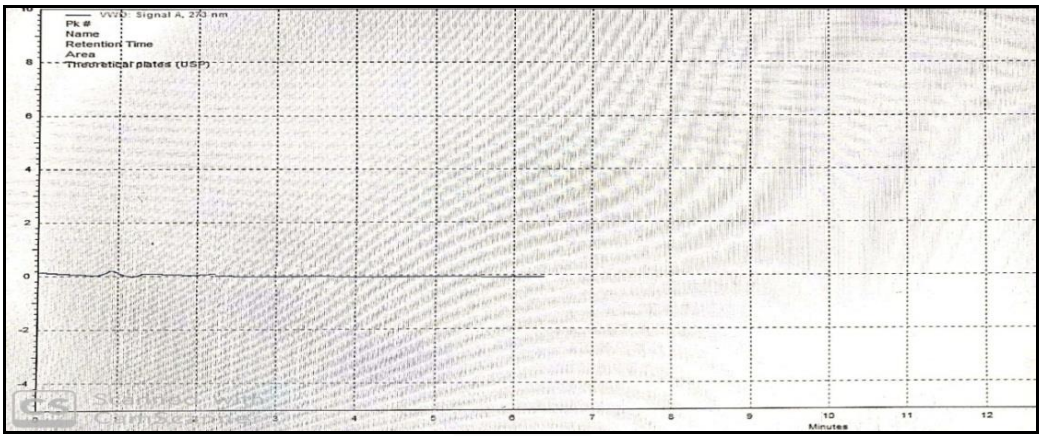


Figure no 3: Chromatogram of plasma.

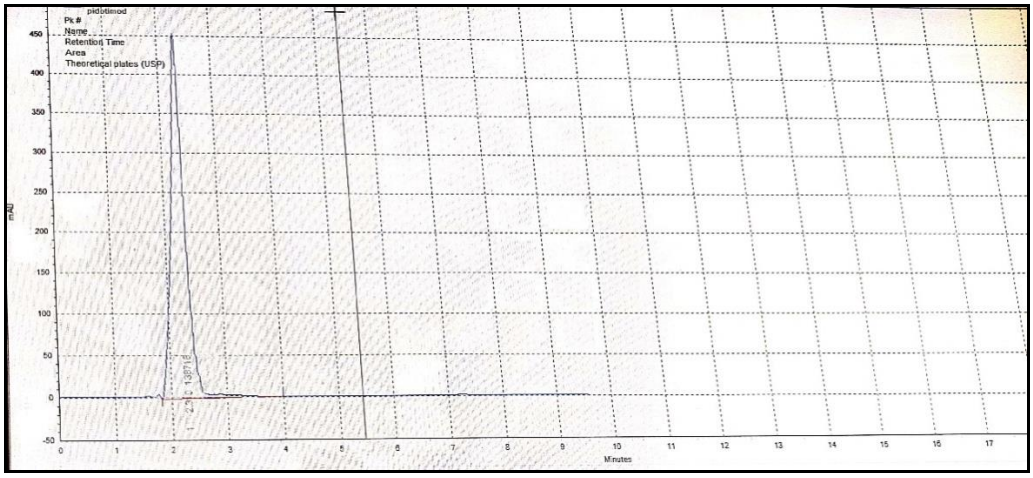


Figure no 4: Chromatogram of LQC Sample spiked in plasma.

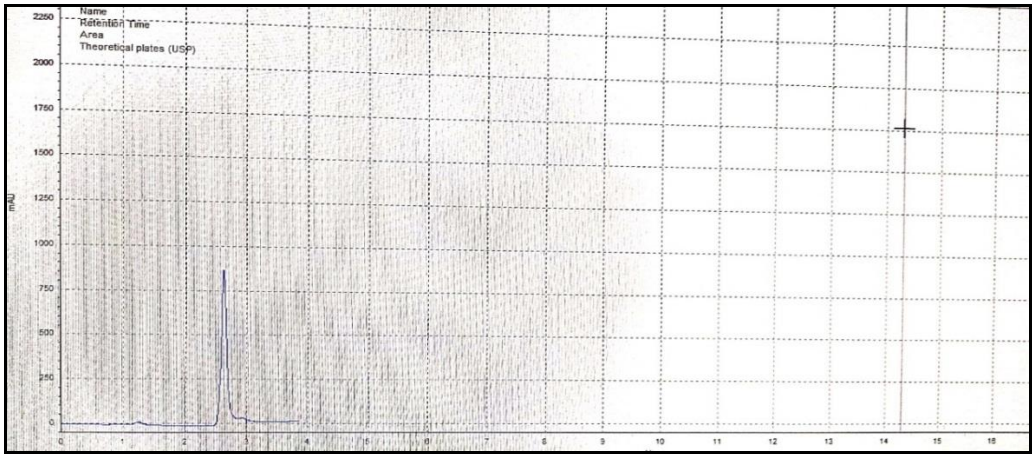


Figure no 5: Chromatogram of MQC sample spiked in plasma.

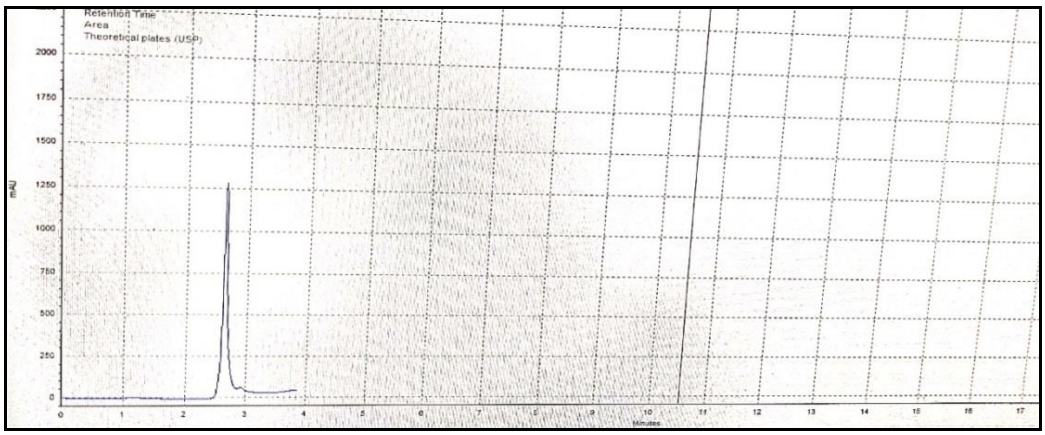


Figure no 6: Chromatogram of HQC sample spiked in plasma.

Table no 3: Accuracy and Precision within a batch

Quality control sample	Amt. Added (µg/ml)	Peak Area*	Amt found (µg/ml)	% Accuracy	%C.V
LQC	20	11299296	19.73	98.65	0.2280
	20	11299385	19.82	99.10	
	20	11299311	19.78	98.90	
MQC	100	60275737	99.83	99.83	0.8407
	100	60275695	98.92	98.92	
	100	60275291	97.85	97.82	
HQC	200	121302138	195.85	97.92	0.3179
	200	121302341	196.95	98.47	
	200	121302245	195.88	97.94	

***Average of three determination**

Table no 4: Inter batch Accuracy and Precision

Quality control sample	Amt. Added (µg/ml)	Peak Area*	Amt. found (µg/ml)	% Accuracy	% C. V
LQC	20	11299344	19.80	99.00	0.2391
	20	11299286	19.71	98.55	
	20	11299311	19.78	98.9	
MQC	100	60275690	98.90	98.90	0.2449
	100	60275720	99.79	99.79	
	100	60275291	97.85	97.85	
HQC	200	12130338	196.93	98.96	0.6052
	200	121302130	195.85	97.92	
	200	121302245	195.88	97.94	

***Average of three determinations**

Table no 5: Recovery study

Conc. (µg/ml)	Peak Area* (Extracted)	Peak Area* (Un-extracted)	% Recovery
20	11298296	11299385	96.96
100	60273695	76275737	98.56
200	121303138	121302341	98.84

***Average of three determinations**

Stability:

Table no 6: Freeze and Thaw stability

Conc. (µg/ml)	Peak Area	Conc. Found	% Purity*	S.D	% C.V
20	11299313	19.75	98.72	0.9295	0.9430
100	60275288	99.33	99.33	0.2468	0.2486

***Average of three determinations**

Short term stability: -

Table no 7: Short term temperature stability

Conc. (µg/ml)	Peak Area	Conc. Found (µg/ml)	% Purity	S.D	% C.V
20	11299212	19.73	98.70	0.5661	0.5761
100	60275233	99.12	99.12	0.2100	0.2114

Table No. 8 Long Term Stability

Conc. (µg/ml)	Peak Area	Conc. Found (µg/ml)	% Purity	S.D	% C.V
20	11299385	19.82	99.1	0.9112	0.9225
100	60275695	98.82	98.82	0.6900	0.6982

CONCLUSION

Bioanalytical method for Pidotimod has been developed and the method was validated as per USFDA guideline. The proposed methods were found to be simple, accurate, precise and reproducible and can be applied for the analysis of drug in rat plasma. The proposed method was also applied for the estimation of bioavailability, bioequivalence, pharmacokinetic & toxicokinetic data of Tablet formulation.

Acknowledgment

The authors are thankful to the Principal, Dr. D. Y. Patil College of Pharmacy, Pune for providing excellent research facilities.

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