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SYSTEMATIC DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR THE ESTIMATION OF PRUCALOPRIDE IN BULK DRUG AND DOSAGE FORM

Running title: RP-HPLC Method Validation for Estimation ofPrucalopride

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ABSTRACT: Several spectrophotometric and HPLC methods have been reported for determination of Prucalopride in drugs and in pharmaceutical dosage forms. Hence, in the present study, a new, sensitive, suitable and robust reversed-phase high performance liquid chromatography method was developed and validated for the determination of Prucalopride in bulk drug and in tablet formulation.
In RP-HPLC method, Acetonitrile and 0.1% OPA in Water (25:75 % v/v) was used as mobile phase, at a flow rate of 1.0 ml/min, on HPLC system containing UV- detector with OpenlabEZchrome software and Kromasil C18, 250 mm X 4.6 mm, 5 µm. The detection was carried out at 226 nm. The method gave suitable retention time i.e. 3.88 min for Prucalopride. The results of analysis in the method were validated in terms of Filter study, Solution stability, specificity, Linearity, accuracy, precision (Repeatability and intermediate precision), limit of detection, limit of quantification and robustness. A simple and precise method was developed for the assay of Prucalopride in bulk drug and in tablet formulation.
The method needs regular reagents for doing analysis and also less time consuming, it can be performed routinely in industry for routine analysis of bulk drug and marketed product of Prucalopride.

Keywords: RP-HPLC, Prucalopride, Acetonitrile, Validation.

Introduction:

Prucalopride acts as a selective stimulator of the 5-HT4 receptors while having no interaction with hERG channel or 5-HT1 receptors which reduces significantly the cardiovascular risk found in other similar drugs. 15-HT4 receptors can be found throughout the gastrointestinal tract primarily in smooth muscle cells, enterochromaffin cells, and myenteric plexus. Its activation produces the release of acetylcholine which is the major excitatory neurotransmitter in the GI tract. Hence, prucalopride stimulates motility by interacting specifically with 5-HT4 receptors in the GI tract which causes a release of acetylcholine and further contraction of the muscle layer of the colon and relaxation of the circular muscle layer leading to the propulsion of luminal content. [19]

Material and methods:

Chemical and reagents:

Vidisha Aalytical laboratories provided gift sample of Prucalopride. The HPLC-grade solvents used in this study were obtained from Merck Ltd. in Bangalore, India, including acetonitrile, methanol and water from Siddhi Lab, Ortho-Phosphoric Acid from Qualigens. All of the chemicals used were of the highest quality for HPLC.

Instruments and chromatography condition:
The HPLC system consisted of Agilent 1100 series HPLC instrument with Quaternary 1260 Infinity II with DEACX16446 detector for the detection and separation of components from the column at specific RT with flow rate 1.0 ml/min. The column used dimensions Kromasil, C18, 250 mm X 4.6 mm, 5 µm with an Injection volume of 20µl at 226nm. Standard and samples was filtered with through a membrane filter of 0.45 µm (Sigma-Aldrich). The HPLC system was operated at 40°C. Before performing the analysis, degas the mobile phase using sonication for up to 5-10 minutes.

Determination of solubility:
The solubility was determined in Water at a concentration of 2 mg/mL as follows and are given in results**.**
Water: Weighed approx 26.42 mg of Prucalopride Succinate (Equivalent to 20 mg of Prucalopride)and sonicated for 5-10 minutes to dissolve in 10 ml of Water.

Selection of analytical wavelength:

Selection of solvent
Water was selected as the solvent for dissolving Prucalopride Succinate.
Preparation of standard solution for UV scan to find Absorption maxima:
In order to prepare stock solution, weighed accurately 13.21 mg Prucalopride Succinate (Equivalent to 10 mg of Prucalopride) and transferred into 20 ml volumetric flask, added 15 ml of water and sonicated to dissolve the standard completely and diluted up to the mark with water (500 PPM). Further diluted 0.8 mL to 20 mL with water.(20 PPM)
Selection of analytical wavelength
Water as a blank and Prucalopride standard solution (20 PPM) was scanned from 400 nm to 200 nm. Absorption maxima was determined for drug. Prucalopride showed maximum absorbance at 226 nm shown in results.

Method Development by RP – HPLC:
Preparation of standard stock solution for Chromatographic development:
Prucalopride Standard stock solution was prepared by dissolving 13.21 mg Prucalopride Succinate (Equivalent to 10 mg of Prucalopride) into a 20 mL clean and dried volumetric flask, added about 15 mL of water to dissolve it completely and made volume up to the mark with water (500 PPM). Further diluted 2 ml of stock solution to 10 mL with water (100 PPM).
Selection of analytical wavelength for HPLC method development: Analytical wavelength for the examination was selected from the wavelength of maximum absorption from the spectrophotometric analysis and it was 226 nm**.**
Method of analysis:
The chromatographic conditions were maintained as previously mentioned, and the baseline stabilization procedure was carried out for 30 minutes total. Following stabilization, the repeatability of the Blank and the prepared concentration solution of the standard drug was tested in the respective peak regions of the Blank and the prepared concentration solution of the standard drug. For the purpose of quantification, the solution of the sample was injected. We estimated the response factor based on the standard peak ratio and the sample peak ratio. The same technique was done nine times to ensure that the created method was adhering to the established standard of repeatability.
Validation of RP-HPLC method:
The developed method for estimation of Prucalopride was validated as per ICH guidelines for following parameters.
Filtration Study:
Filtration study of an analytical procedure checks the interference of extraneous components from filter, deposition on filter bed and compatibility of filter with sample.This study was conducted with

Prucalopride Test sample (Tablet solution).Filtration study carried out with unfiltered and filtered test solution. During filtration activity 0.45 µm PVDF and 0.45 µm Nylon syringe filters used by discarding 5 mL of aliquot sample.

Stability Of Analytical Solution:

Stability study was conducted for standard and test sample solution. Stability study was performed at normal laboratory conditions.The solution was stored at normal illuminated laboratory conditions and analyzed after 12 hours and 24 hours.Standard and Test solution stability study was performed by calculating the difference between results of test solution at each stability time point to that of initial.

Specificity:

Specificity is the ability to access unequivocally the analyte in the presence of components which may be expected to be present.

Linearity and Range:

Preparation of linearity solution

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. 5 levels of Linearity was performed from 10% to 150% of working concentration

Limit of Detection (LOD) And Limit of Quantitation (LOQ):

Detection limit:

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

Quantitation limit:

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy.

As per ICH Q2R1 guidelines LOD and LOQ was determined by using the approach Based on the Calibration Curve in which residual standard deviation of a regression line was calculated and determined the LOD and LOQ by using following formula:

$LOD = 3.3\ \sigma / S$

$LOQ = 10\ \sigma / S$

Where,

σ = residual standard deviation of a regression line

S = Slope of regression line

Accuracy (% Recovery):

The accuracy of the analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value of the value found, Accuracy will be conducted in the range from 50 % to 150 % of working concentration. Solution of each accuracy level was prepared in triplicate. Calculated % Recovery for each sample, Mean % recovery for each level and overall recovery and also calculated % RSD for each level and % RSD for overall recovery.

Precision:

Precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous test under the prescribed conditions. Precision is of two types, Repeatability and Intermediate precision. It is performed on tablet test sample.

Robustness:

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

Results and Discussion:

Development of RP-HPLC method:

The development of an RP-HPLC technique for the measurement of Prucalopridein dosage form was completed. Mixture of Acetonitrile: 0.1% OPA (25:75 %V/V) in a isocratic mode used as mobile phase. A variety of chromatographic conditions, including flow rate, column temperature, and the components ratio in the mobile phase used, were tested in order to generate a crisp and symmetric peak with an appropriate retention period. In order to get a superior peak, the column Kromasil C18**,** 250 mm X 4.6mm ID, 5 µm was employed. Because of the mobile phase employed in the procedure, the characteristics of the chromatographic settings such as retention duration, theoretical plate number (N), retention factor, and selectivity could be tailored to meet the needs of the researchers. The determination of wavelength was carried out using an Agilent 1100 system equipped with a photodiode array detector in order to ensure adequate sensitivity of Prucaloprideusing the RP-HPLC technique (PDA). In order to discover the wavelength maxima, the standard solution of Prucalopridewas scanned throughout a range of 200–400 nmwith a better peak being identified at 226 nm.

Selection of analytical wavelength:

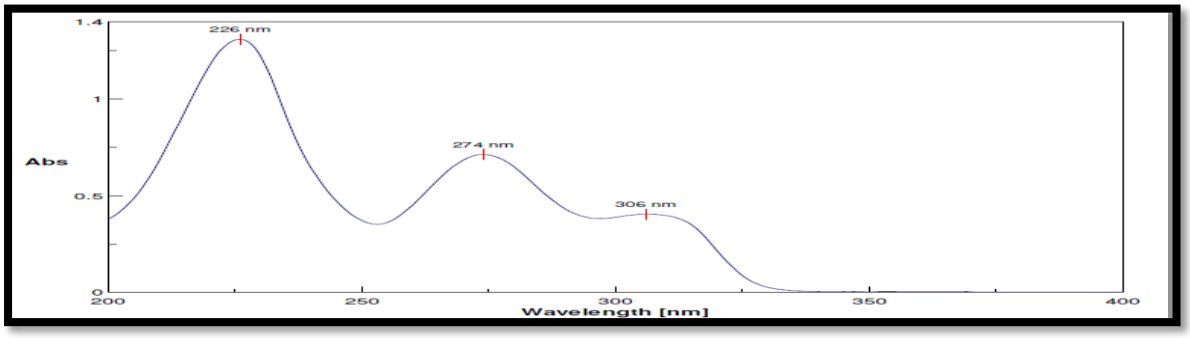

Fig. No. 1 UV spectrum of Prucalopride

Optimization of HPLC method:

Observation:

Prucalopride eluted with good chromatography. (Theoretical plate: 3648 & Tailing Factor: 1.67)

Conclusion: From the observations of trials first to Nine, it was concluded that chromatographic conditions in trial Nine gives better peak, good retention time , Theoretical plate & tailing factor therefore chromatographic conditions in trial Nine was used for Method Validation.

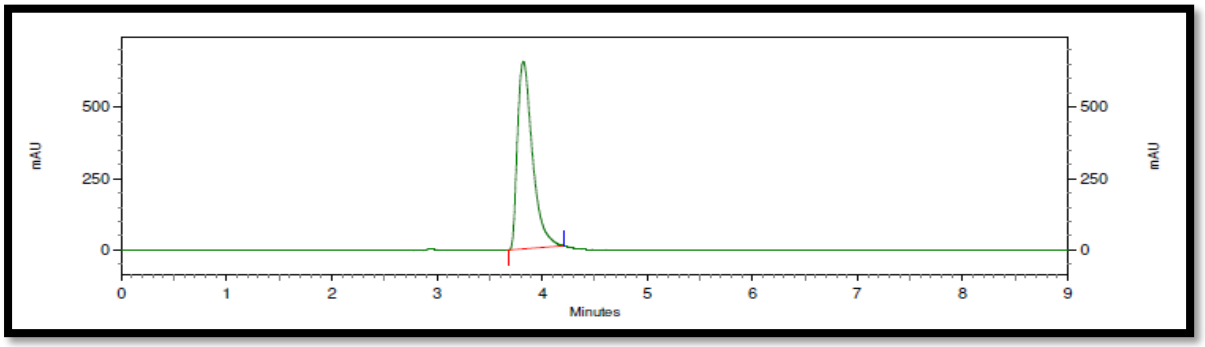

Fig. No. 2 Typical chromatogram of Trial 9

System suitability test:

It was observed from the data tabulated above; the method complies with system suitability parameters. Hence, it can be concluded that the chromatographic method is adequate for intended analysis.

System Suitability Acceptance Criteria:
1. Relative standard deviation of the area of analyte peaks in standard chromatograms should not be more than 2.0 %.
2. Theoretical plates of analyte peak in standard chromatograms should not be less than 2000.
3. Tailing Factor (Asymmetry) of analyte peaks in Standard Chromatograms should be less than 2.0

Table No. 1 Results for System Suitability Test of Prucalopride:

Sr No.	Standard solution	Area	Asymmetry	Theoretical plates
1	Standard_1	10926316	1.52	6169
2	Standard_2	10924161	1.52	6179
3	Standard_3	10931529	1.52	6182
4	Standard_4	10914288	1.52	6146
5	Standard_5	10935137	1.53	6135
Mean		**10926286**	**1.52**	**6162**
STD Dev		**7973.358**		
% RSD		**0.07**		

VALIDATION OF RP-HPLC METHOD

- **FILTRATION STUDY:**
  Both filters PVDF and Nylon passes the criteria for filter study, hence both filters can be used. We used Nylon filter because it showed less absolute difference as compare to PVDF filter.

Table No. 2 Results of Filter study:

Sample description	Area	% Absolute difference
Unfiltered	10895206	NA
0.45 µ PVDF filter	10835149	0.55
0.45 µ Nylon filter	10783087	1.03

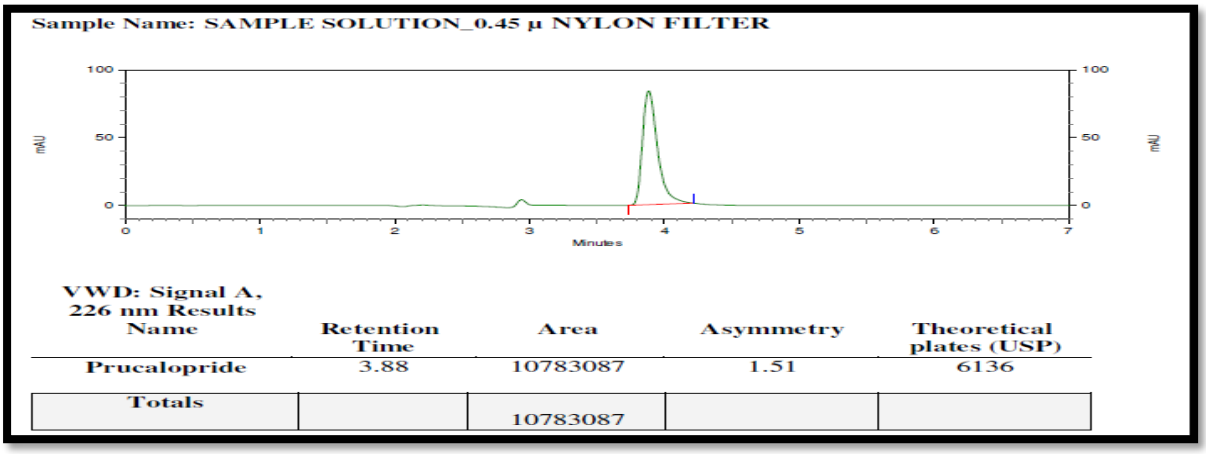

Fig. No. 3 Typical chromatogram of sample filtered through 0.45µ Nylon filter.

➢ **SOLUTION STABILITY:**

Standard and Test solution was found stable up to 24 Hrs. Hence both solutions can be used up to 24 Hrs.

Table No. 3 Results of Solution stability:

Sample solution			Standard solution		
Time point	**Area**	**% Absolute difference**	**Time point**	**Area**	**% Absolute difference**
Initial	10836109	NA	**Initial**	10925360	NA
12 Hours	10736297	0.92	**12 Hours**	10855397	0.64
24 Hours	10685098	1.39	**24 Hours**	10806305	1.09

SPECIFICITY:

Blank and placebo was not having interference at R.T. of Prucalopride. Peak purity for Standard as well as test solution was well within limits. Hence developed chromatographic method passed the criteria for specificity.

Table No. 4 Results of Specificity:

Description	**Observation**
Blank	No interference at R.T. of Prucalopride due to blank
Placebo	No interference at R.T. of Prucalopride due to placebo
Standard solution	Peak purity was 0.995
Test Solution	Peak purity was 0.989

➢ **Linearity and Range :**

From the calibration curve it was concluded that the Prucalopride shows linear response in the range of 1.0 -15.0 µg/ml. The Regression value was found well within the limit.

Table No. 05 Linearity Data for Prucalopride

Level	**Conc (µg/mL)**	**Area**	**Mean**	**% RSD**
10%	1.0	1065938	1065287	0.155

		1063412		
		1066510		
50%	5.0	5423415	5425148	0.029
		5425638		
		5426391		
100%	10.0	10935067	10925741	0.089
		10926518		
		10915637		
125%	12.5	13872936	13888717	0.123
		13886395		
		13906819		
150%	15.0	16550587	16565958	0.103
		16563095		
		16584193		

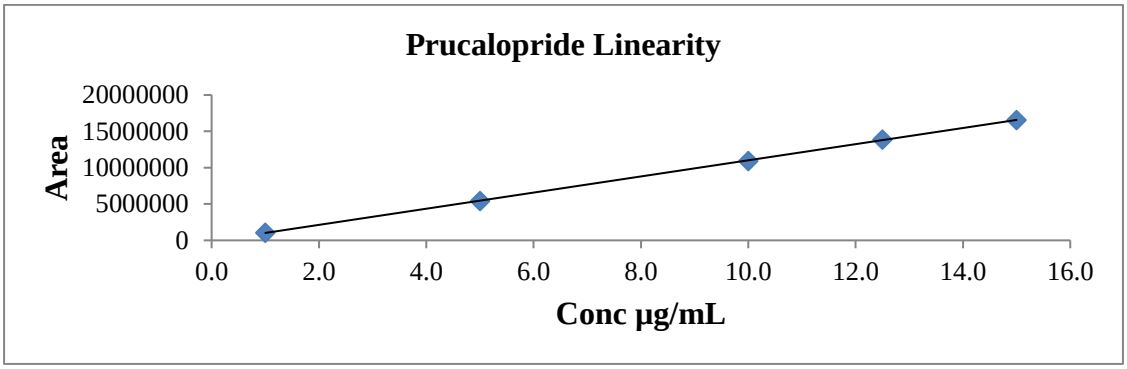

Fig. No. 4 Calibration curve of Prucalopride

- **ACCURACY (RECOVERY):**

Recovery of analytical procedure was found well within acceptance criteria at all 3 levels. % Recovery not get hampered by changed in analyte concentration.

Table No. 6Result and statistical data of Accuracy of Prucalopride

Level (%)	Area	Recovered conc (µg/mL)	Added conc (µg/mL)	% Recovery	Mean Recovery	% RSD
50	5486391	5.02	5.00	100.40	100.07	1.331
	5389518	4.93	5.00	98.60		
	5535193	5.06	5.00	101.20		
100	10942039	10.00	10.00	100.00	99.73	0.932
	10793617	9.87	10.00	98.70		
	10993508	10.05	10.00	100.50		

150	16573067	15.15	15.00	101.00	100.36	0.842
	16513069	15.10	15.00	100.67		
	16312361	14.91	15.00	99.40		

Overall Recovery: 100.05 **% RSD for Overall Recovery:** 0.954

- **PRECISION:**

% Assay and % RSD was found well within acceptance limit and hence method is precise (Reproducible).

Table No. 7 Result of Intra- day and Inter- Day Precision for Prucalopride:

Repeatability	**Sample**	**Test Sample (mg)**	**Area**	**% Assay**
	Sample 1	1030.9	10923164	99.84
	Sample 2	1032.6	10762917	98.35
	Sample 3	1031.8	10692869	97.87
	Sample 4	1030.9	10925398	99.88
	Sample 5	1032.4	10953186	100.08
	Sample 6	1032.8	10936221	99.96
	Mean			**99.33**
	STD DEV			**0.96062**
	% RSD			**0.967**
Intermediate precision (Inter-Day)	Sample 1	1030.9	10856318	99.39
	Sample 2	1032.6	10962913	100.20
	Sample 3	1031.8	10861631	99.35
	Sample 4	1030.9	10762519	98.53
	Sample 5	1032.4	10896315	99.61
	Sample 6	1032.8	10816069	98.84
	Mean			**99.32**
	STD DEV			**0.586310**
	% RSD			**0.590**
Repeatability Plus Inter-day	**Mean**			**99.325**
	STD DEV			**0.75877**
	% RSD			**0.764**

- **ROBUSTNESS:**

The robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

Following changes made under Robustness:

- Change in Wavelength
- Change in flow rate
- Change in column oven temperature

Table No. 8 Result of Robustness study of Prucalopride:

Change in Parameter	R.T.	Standard area	Asymmetry	Theoretical plates
Wavelength by +3 NM (229 NM)	3.88	11027346	1.68	5765
Wavelength by -3 NM (223 NM)	3.88	10564414	1.64	5779
Flow rate by +10% (1.1mL/min)	3.53	10137981	1.65	5405
Flow rate by -10% (0.9mL/min)	4.34	12398976	1.68	5755
Column oven temp by +2ºC (42 ºC)	3.86	11151318	1.50	6284
Column oven temp by -2ºC (38 ºC)	3.89	10352639	1.54	6039

SUMMARY AND CONCLUSION:

The present work involved the development of simple, accurate, precise and suitable RP-HPLC method. Literature survey revealed that several methods have been reported for determination of Prucalopride in bulk drug or in pharmaceutical dosage forms. Hence, in the present study, a new, sensitive and suitable reversed-phase high performance liquid chromatography method was developed and validated for the determination of prucalopride in bulk drug and pharmaceutical dosage form. In developed RP-HPLC method, the analyte were resolved by using isocratic program and mobile phase was used Acetonitrile : 0.1% OPA in water (25 : 75 % V/V) at a flow rate of 1.0 ml/min, on HPLC system containing UV- visible detector with Openlab EZ-Chrome Software and Kromasil C18, 250 mm X 4.6 mm, 5 µm. The detection was carried out at 226 nm. The results of analysis in the developed method were validated in terms of linearity, accuracy, precision, robustness, limit of detection and limit of quantification. The developed method has several advantages, including reproducibility of results, rapid analysis, simple sample preparation and improved selectivity as well as sensitivity. The regression coefficient (r2) for each analyte is not less than 0.999 which shows good linearity. The % recovery was in the acceptable range in tablet dosage form. The %RSD was also less than 2% showing high degree of precision of the proposed method. Since the developed method is robust and reproducible and also less time consuming, it can be performed for routine analysis in pharmaceutical industry for bulk drug of Prucalopride and also in pharmaceutical dosage form.

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