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Development And Validation Of HPLC-UV Method For Prucalopride Succinate Quantification In Biological Samples

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KEYWORDS
Prucalopride succinate, Bioanalytical method development, HPLC-UV, ICH Guidelines

ABSTRACT:
Prucalopride succinate is a pharmacologically active compound used in the treatment of gastrointestinal disorders. Accurate and reliable quantification of Prucalopride succinate in biological samples is crucial for pharmacokinetic studies, therapeutic monitoring, and drug development. In this study, we developed and rigorously validated a high-performance liquid chromatography with ultraviolet detection (HPLC-UV) method for the quantification of Prucalopride succinate in human plasma. The method was carried out with an Agilent HPLC with a UV detector. BDS Hypersil C8 Column (250 × 4.6 mm, 5 µ) was used at a flow rate of 1.0 mL/min. Detection was carried out at 276 nm. The mobile phase consisting of a mixture of Methanol: 0.1 % Formic Acid (80:20 v/v) respectively. The method involved sample preparation, chromatographic separation, and UV detection. Developed method demonstrated excellent linearity over a wide concentration range (0.05- 0.5µg/ml) and exhibited precision and accuracy within acceptable limits. The lower limit of quantification (0.05µg/ml) was, indicating the method's sensitivity. These bioanalytical validations play a significant role in the evaluation and interpretation of bioavailability, bioequivalence, pharmacokinetic, and toxicokinetic studies of Prucalopride succinate.

Introduction

Validating bioanalytical methods for the precise measurement of drugs and their metabolites in biological fluids is crucial. This process plays a significant role in assessing and interpreting data derived from bioavailability, bioequivalence, pharmacokinetic, and toxicokinetic studies along with sample preparation, storage, transportation, handling, and collection of the sample. The validation of analytical techniques is particularly essential for ensuring the accuracy of quantitative measurements of analytes in specific biological matrices, such as blood, plasma, serum, or urine. The validation of bioanalytical methods is of utmost importance as it contributes to obtaining well-founded and reliable results for drug dosing and monitoring patient safety.

Prucalopride (PRU) (Fig.1) (IUPAC name: 4-amino5-chloro-2,3-dihydro-N-[1-(3-methoxy propyl)-4-piperidinyl]-7-benzofurancarboxamide butanedioate) is a dihydro benzofuran carboxamide derivative belonging to the family of benzofuran that selectively stimulates 5-HT4 receptors and embodies enterokinetic activity.[1] The PRU was first produced by Shire Development LLC Ltd., USA, and endorsed for application in Europe in the year 2009 and by the Food and Drug Administration (FDA) in the year 2018.[2] It selectively performs an activity on the gut muscle wall, thus, helping to reinstate the regular working of the human bowel.[3] In subjects suffering from chronic constipation, there was a lessening in small bowel transit time, an augmentation in the gastric emptying, and further swift colonic filling.[4] There was an enhancement in the bowel motion frequency but no considerable consequence on the transit time of the colon. [5,6,7,8,9]

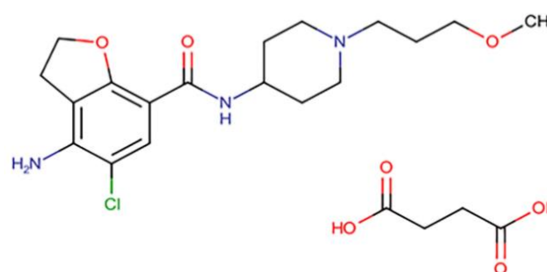

Fig 1*:* Chemical structure of Prucalopride succinate

A thorough literature review identified limited UV spectroscopic methods, [2,9,10,11] a few High-Performance Liquid Chromatography (HPLC)

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methods, [12,13,14,15,16] Spectrofluorimetric method [17] and High-Performance Thin-Layer Chromatography (HPTLC) methods [18] for analyzing the drug Prucalopride succinate. Also two LC-MS/MS methods are reported for stress degradation study of Prucalopride succinate[19,20]. In this context, the current investigation focused on the development of a novel Bioanalytical Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) method, which has not been reported elsewhere. The method established in this study is characterized by its simplicity, reliability and cost-effectiveness.

Materials and Methods

Preparation of standard stock solution of Prucalopride Succinate:

For standard stock solution accurately weighed 10 mg of Prucalopride Succinate transferred to 10 ml volumetric flask and the volume was made up to 10 ml with methanol, to get standard stock solution of Prucalopride Succinate (1000 µg/ml). Further dilutions were made with methanol to produce the stock solutions of 0.5, 1, 2, 3, 4 and 5µg/ml.

Preparation of Plasma Sample (Quality Control Samples)

Sample preparation consisted of the addition of 0.1 ml of plasma sample in 10 ml test tubes, then 0.1 ml of standard stock solution was added, then 0.8 ml of methanol was added as precipitating agent to produce the final conc. of 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5µg/ml, then vortex for 3 minutes followed by centrifugation for 10 minutes at 5000 rpm, then the supernatant was injected into HPLC system. The protein precipitation was the preferred choice of separation because of the minimized steps in extraction of drug from matrix.

Selection of analytical wavelength

A solution of 10 µg/ml was prepared from standard stock solution of Prucalopride succinate (1000µg/ml) and scanned over 200-400 nm in UV Spectrophotometer. The maximum absorbance was shown at 276 nm. Hence it was selected as analytical wavelength; UV spectrum is given in Fig 2.

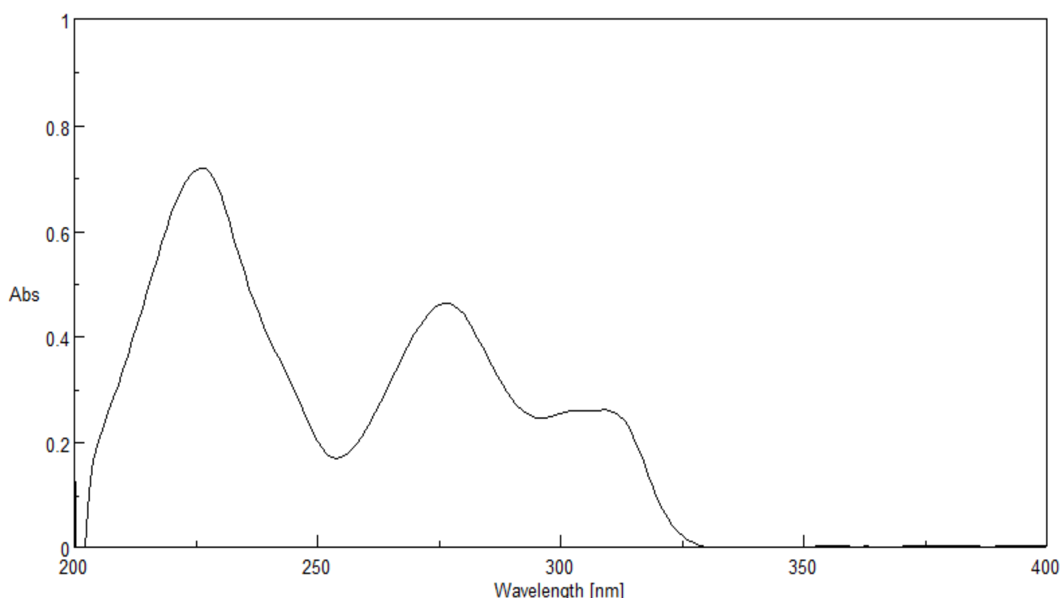

Figure 2: UV-Spectrum of Prucalopride succinate in methanol(10µg/ml)

Mobile Phase Optimization

To achieve optimum chromatographic condition various mobile phases were checked using column BDS Hypersil C8 Column (250 × 4.6 mm, 5 µ). The Methanol: 0.1 % Formic Acid (90:10 v/v) system was initially tried but did not get a considerable number of theoretical plates as well as peak shape. The ratio changed (80:20 v/v) has resulted in considerable improvement of theoretical plates and appropriate peak shape with appropriate system suitability parameters. The system suitability parameters are given in the Table 1.

Table 1: System Suitability Parameters

Parameter	Obtained values
RT (min)	3.692 ± 0.05
Asymmetry	1.14
Plates (N)	2536.58

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Bioanalytical Method Validation [21,22]

1. Selectivity/Specificity

Selectivity of analytical method is ability of method to differentiate and quantify the drug sample in presence of other interfering substance. The specificity of method is demonstrated by analysing blank (mobile phase), blank plasma, API and spiked plasma with API, given in fig 3, 4, 5 and 6 respectively. There was no any interfering peak at the same RT of Prucalopride Succinate.

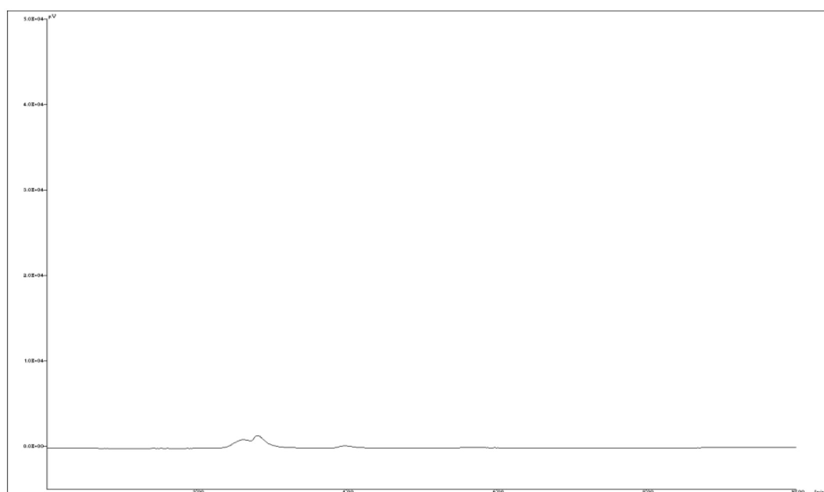

Fig 3: Chromatogram of Blank (MP)

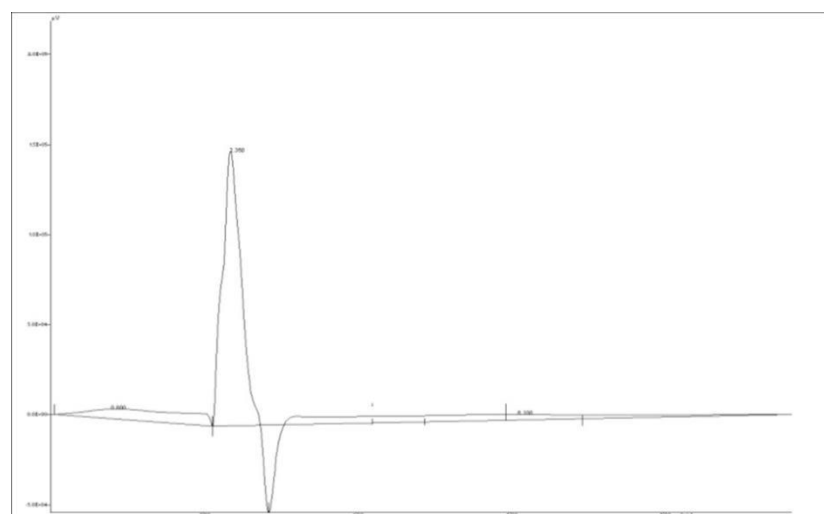

Fig 4: Chromatogram of Blank Plasma

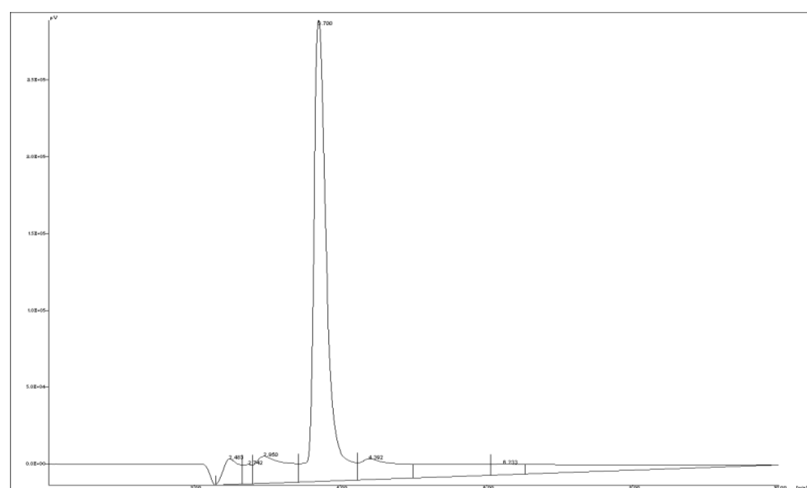

Fig 5: Chromatogram of API - Prucalopride Succinate

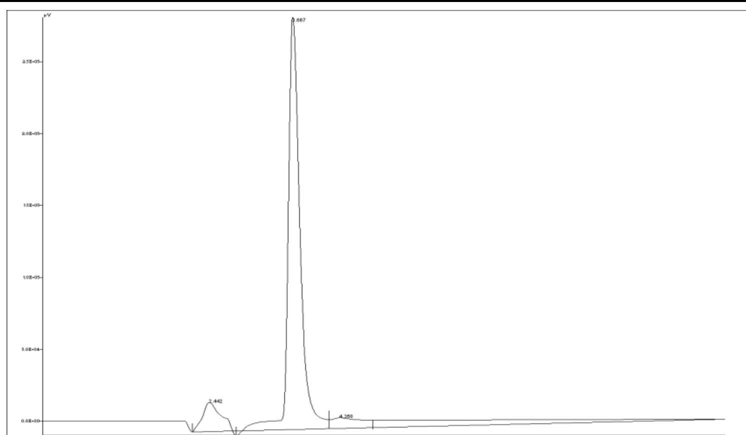

Fig 6: Chromatogram of Spiked Plasma (with API - Prucalopride Succinate)

2. Calibration curve / Linearity

Calibration curve or linearity of method exhibit direct proportionality between detector response and concentration of analyte of interest. Linearity was tested for the range set in concentration of 0.05-0.5µg/ml. 6 replicates of QC samples were analysed and peak areas were recorded (Fig 7). The correlation between the known concentration and response was evaluated through a regression analysis of calibration curve constructed using six-point (0.05, 0.1, 0.2, 0.3, 0.4 and 0.5µg/mL) standard calibration curve. Calibration curve was constructed with drug response on Y-axis and concentration on X-axis. The correlation coefficient (R^2) values were calculated (Fig 8).

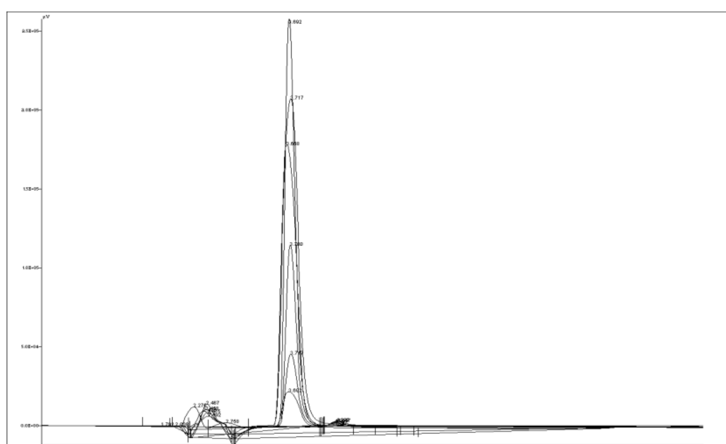

Fig 7: Overlay of Linearity (0.05 - 0.5µg/ml)

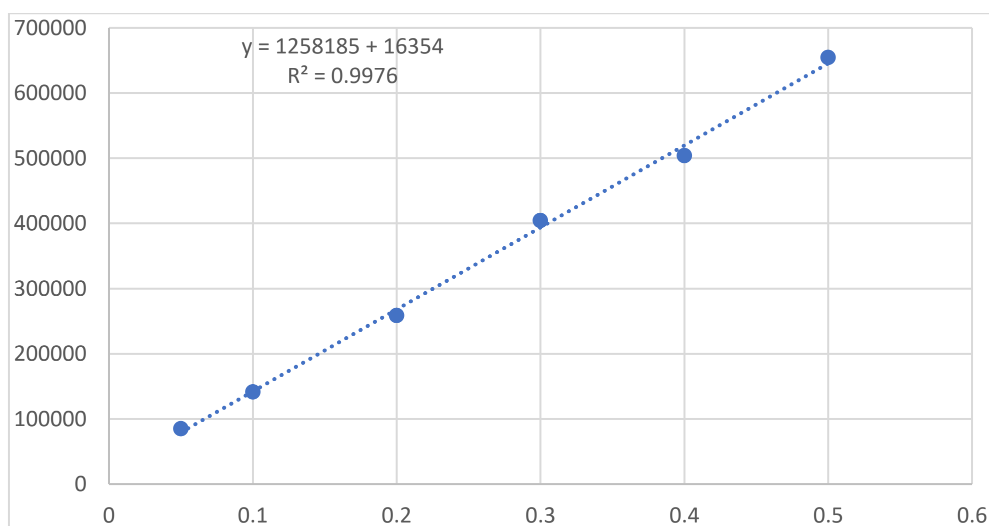

Fig 8: Calibration curve for Prucalopride Succinate in spiked plasma

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Table 2: Linearity of Prucalopride Succinate

Conc. (µg/ml)	Area 1	Area 2	Area 3	Area 4	Area 5	Mean	SD	%CV
0.05	88267	87180	83137	82913	83382	84976	2543	2.99
0.1	133502	139733	155269	147261	130741	141301	10068	7.12
0.2	268090	261955	258708	245718	258783	258651	8175	3.16
0.3	413220	410038	407496	396913	395162	404566	8069	1.99
0.4	514582	500206	483885	515562	505362	503919	12908	2.56
0.5	658173	660797	660410	652039	643079	654900	7479	1.14

3. Accuracy

Accuracy was estimated by using minimum 5 replicates of 3 concentrations i.e., at LQC (0.05 µg/ml, MQC (0.2µg/ml), HQC (0.4µg/ml). The % mean accuracy was determined for all QC samples. Drug area was substituted in regression equation (y=mx+c) to get the concentration of the given sample (Table 3). The deviation of the average from the theoretical value served as the estimation of accuracy. The accuracy at each concentration level should be within ±15% of the nominal concentration.

Table 3: Results of Accuracy Studies

Replicates	LQC (0.05µg/ml)		MQC (0.2µg/ml)		HQC (0.4µg/ml)	
	Area	Calcu. Conc	Area	Calcu. Conc	Area	Calcu. Conc
1	78438	0.049	264944	0.198	532969	0.411
2	79711	0.050	267010	0.199	524028	0.403
3	77560	0.049	266201	0.199	500932	0.385
4	79441	0.050	257610	0.192	514028	0.396
5	80169	0.051	249345	0.185	524168	0.404
Mean Area	79064		261022		519225	
SD	941.9		6723.9		10936.6	
%CV	1.2		2.6		2.1	
%Accuracy	99.68		97.23		99.92	

4. Precision

Closeness of the individual measured value of the drug analyte among all aliquots of same volume of the plasma was assessed by injecting six replicates at, LQC, MQC and HQC levels. The precision of the method performed on HPLC-UV was evaluated by determining the %CV of the repeated injections. Intraday precision was evaluated by determining %CV of the response of the repeated injections injected on the same day (Table 4). On the contrary, Interday precision was calculated after comparison of the measured values of the samples injected on three different days (Table 5). According to the ICH M10 guideline, the precision (%CV) of the concentrations determined at each level should not exceed ±15%.

Table 4: Intraday Precision Studies

Concentration Level	Morning	Afternoon	Evening	Mean	SD	%CV
LQC(0.05µg/ml)	82799	88018	91187	88001	4236	**4.81**
MQC(0.2µg/ml)	265013	281951	318501	311821	27337	**8.77**
HQC(0.4µg/ml)	504543	554408	538265	565739	25444	**4.50**

Table 5: Interday Precision Studies

Concentration Level	Day 1	Day 2	Day 3	Mean	SD	%CV
LQC(0.05µg/ml)	91288	88577	80041	87636	5870	**6.70**
MQC(0.2µg/ml)	315752	293101	290918	309924	13751	**4.44**
HQC(0.4µg/ml)	569292	516639	595059	660330	39971	**6.05**

5. Recovery

Recovery studies were performed by comparing the chromatographic response for samples after extraction at LQC, MQC and HQC with standard samples in three replicates (Table 6). Recovery need not be 100

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percent, but the extent of the recovery of an analyte should be consistent and reproducible.

Table 6: Results of Recovery Studies

Conc level	Area		%Recovery	%Mean Recovery
	Standard	Spiked plasma		
LQC (0.05µg/ml)	86473	81585	94.35	
	86147	78605	91.25	**92.46**
	87118	79960	91.78	
MQC (0.2µg/ml)	247691	231720	93.55	
	255459	235189	92.07	**91.87**
	249137	224228	90.00	
HQC (0.4µg/ml)	638053	588227	92.19	
	614827	587831	95.61	**94.20**
	626698	594200	94.81	

6. Carry Over:

Carryover is the impact of the previous injection to the next injection of the analyte. It was determined by injecting blank samples after HQC injection of 0.4µg/ml. According to the guidelines, response of samples should be below the LLOQ. Chromatograms obtained are shown in Fig. 9.

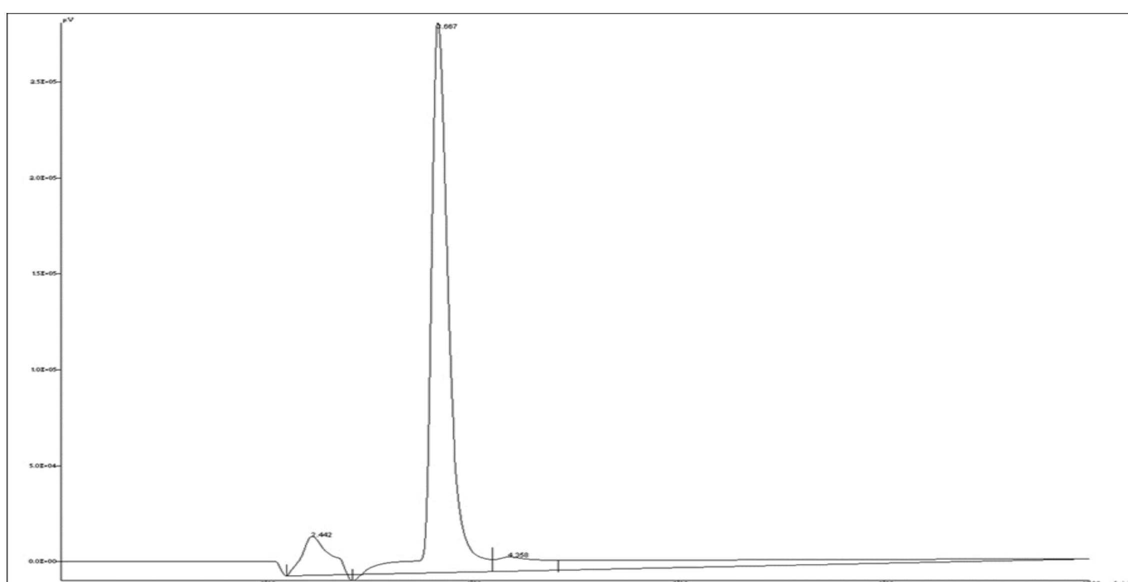

Fig.9: Chromatogram at HQC (0.4 µg/ml)

7. Stability

The purpose of determining stability is to detect any degradation of analyte occurred during entire process of sample collection, storage, extraction, and analysis. It is recommended to determine stability during short term storage, long term storage as well as during freeze thaw cycles. Stability samples should be compared with freshly prepared QC samples. The acceptance criteria for % mean stability is 85-115%.

Prucalopride Succinate stability was evaluated using two concentration levels i.e., at LQC, HQC. For each sample to be tested mean of 3 samples was taken that were stressed, stored, and analyzed. Following types of stability studies were performed:

i. Short term (Bench Top) stability: LQCs and HQCs were kept at room temperature for 4 hours and checked for its stability.

ii. Long term stability: LQCs and HQCs were kept in deep freezer at -20^{0}C for 7 days, brought to room temperature and then checked for its stability.

iii. Freeze thaw stability: The stability of low- and high-quality concentration samples was determined after three freeze thaw cycles stored at -20^{0}C till it freezes, brought to room temperature, and then checked for its stability.

iv. Stock solution stability: Stock solution stability of the drug was determined for 2 hrs at room temperature.

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Comparing them against the freshly weighed stock solution assessed for stability.

Table 7: Results of Stability Studies

Stability	Conc. (µg/ml)	Area	Avg. Area	SD	%CV	%Mean Stability
Freeze thaw stability (three cycles)	**LQC**	76718	78625	1680.09	2.14	91.45
		79885				
		79272				
	HQC	474301	481936	8013.70	1.66	93.78
		481226				
		490281				
Short term stability (for 4h at RT)	**LQC**	81398	80486	797.43	0.99	93.61
		80138				
		79921				
	HQC	472402	472931	7619.76	1.61	92.02
		480801				
		465589				
Long term stability (for 7 days at -20^{0}C)	**LQC**	79971	80074	1730.64	2.16	93.13
		81853				
		78397				
	HQC	489069	484004	7345.46	1.52	94.18
		487364				
		475580				
Stock solution stability (for 2 hrs)	**LQC**	83910	83317	515.50	0.62	96.91
		83068				
		82973				
	HQC	503948	495915	7468.05	1.51	96.50
		489183				
		494612				

8. Matrix Effect

A matrix effect is defined as an alteration of the analyte response due to interfering and often unidentified component(s) in the sample matrix. During method validation the matrix effect between different independent sources/lots should be evaluated. No matrix interference was observed.

Summary of Validation Parameters is shown in Table 8.

Table 8: Summary of Bioanalytical Validation Parameters

Sr. No.	Validation Parameter	Results	
1.	**Linearity**	y = 1258185x + 16354 $R^2 = 0.9976$	
2.	**Range**	0.05-0.5 µg/ml	
3.	**Precision**	**Conc**	**% CV**
	A) Intraday precision	LQC	**4.81**
		MQC	**8.77**
		HQC	**4.50**
	B)Interday precision	LQC	**6.70**
		MQC	**4.44**
		HQC	**6.05**
4.	**Accuracy**	**% Mean ± % CV**	
	LQC	99.68 ±1.19	
	MQC	97.23 ± 2.58	
	HQC	99.92 8± 2.11	
5	**Recovery**	**% Mean**	
	LQC	92.46	
	MQC	91.87	
	HQC	94.20	
6.	**Stability**	**% Stability**	
	Freeze thaw stability	LQC	91.45
		MQC	93.78
	Shortterm (Bench Top)stability	LQC	93.61
		MQC	92.02
	Long term stability	LQC	93.13
		MQC	94.18
	Stock solution stability	LQC	96.91
		MQC	96.50

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5.	**Specificity**	Specific
9.	**Robustness**	Robust

Conclusion:

In this study, we successfully developed and rigorously validated a high-performance liquid chromatography with ultraviolet detection (HPLC-UV) method for the quantification of Prucalopride succinate in biological samples. The method demonstrated robustness, precision, accuracy, and linearity, making it a reliable analytical tool for pharmaceutical and clinical research. Our method allowed for the accurate quantification of Prucalopride succinate within a wide concentration range, with a lower limit of quantification (LLOQ) as low as (0.05 µg/ml). The validation results, including intra-day and inter-day precision and accuracy, demonstrated the method's reliability and reproducibility. The developed HPLC-UV method offers several advantages, including simplicity, cost-effectiveness, and rapidity, making it an attractive choice for routine analysis in pharmaceutical laboratories and clinical settings. The successful validation of this method opens up opportunities for pharmacokinetic studies, bioequivalence assessments, and therapeutic monitoring of Prucalopride succinate in diverse biological matrices. Further studies and applications of this method are encouraged to harness its full potential in drug development and clinical practice.

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