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Research Article

Development and Validation of a Stability Indicating RP-HPLC Method for the Determination of Prucalopride succinate in Bulk and Tablet

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ABSTRACT

A very simple, precise, economical, accurate, robust, and reproducible reverse phase-high-performance liquid chromatography method along with stability-indicating attributes has been developed for estimating of prucalopride (PRU) succinate in both bulk and tablet formulation (PRUVICT 2). The estimation of the solutes was performed on a Grace C_{18} column of dimension 150 mm × 4.6 mm, 5 µm. PRU was eluted with acetonitrile: 0.02 M potassium dihydrogen phosphate in the ratio of 20:80 v/v in a 10 min isocratic mode at a flow rate of 1 mL/min at 30°C column temperature and monitored at a wavelength of 277 nm. The retention time of PRU was found to be 5.416 minutes. The Q2B validation of the analytical method revealed good linearity over the concentration range 2–12 µg/mL for PRU with r^2 of 0.999. The mean recovery % over the three tested ranges of 50, 100, and 150% were found to be 100.173, 99.077, and 98.575%, respectively. In intra-day variability study, the % RSDs was detected to be 0.754, 1.032, and 0.482 whereas the inter-day variability study demonstrated % RSDs of 0.797, 0.559, and 0.524, respectively. The acid, alkali, boiled water, hydrogen peroxide, dry heat, and UV radiations based stress studies presented the formation of a variety of characteristic degradation products. The developed analytical method may be employed for the routine analysis of PRU in bulk and tablet formulations.

Introduction

Prucalopride (PRU) (Fig. 1) (IUPAC name: 4-amino-5-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-HT_4 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] This medication is available as tablet products (1 or 2 mg) such as PRUVICT 2®, MOTEGRITY®, and

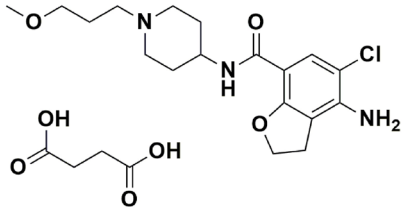

Fig. 1: Structure of prucalopride succinate

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RESOTRANS® for treating chronic idiopathic constipation (CIC) when other popular medications used in constipation do not provide any relief.[6,7]

While searching the literature available in the global pharmaceutical databases, not even a single data were reported regarding the stability-indicating validated RP-HPLC method for the routine analysis of PRU in bulk and pharmaceutical (tablet) formulation (PRUVICT 2). From the review of existing literature, it concluded that an analytical method is essentially required to routinely evaluate the quality attributes of PRU containing tablet formulations as well as in bulk form, so as to meet up the quality-related issues of any pharmaceutical industries that manufacture it. The present work focuses on the rational development of a validated stability-indicating RP-HPLC method, which is very simple, precise, economical, accurate, robust, and reproducible for the determination of PRU in both tablet formulations and in bulk products.

Materials and Methods

Materials

A gift sample of PRU was procured from SL Drugs and Pharmaceuticals Ltd., Hyderabad. Analytical grade chemicals (30% v/v H_2O_2, etc.), reagents (NaOH, HCl, etc.), and solvents (acetonitrile, potassium dihydrogen orthophosphate, etc.) were purchased exclusively from HiMedia Ltd., Mumbai. PRUVICT 2 (Torrent Pharmaceuticals Ltd., India)–each film-coated tablet contains PRU equivalent to 2 mg of PRU was purchased from a local pharmacy shop at Nanded, Maharashtra.

Instruments

VSI® VSI-1B digital pH meter was employed for the estimation of solution pH. Weighing of the chemicals was done using Shimadzu® AUW220D (Kyoto, Japan) balance. Transonic Digital S sonicator was utilized for the sonication. The photostability chamber (Newtronic® Model IC DAC v.1.2) was used for the forced degradation study. Elga Lab (PURELAB UHQ-II) water purification system provided the distilled water for the experiment. The method was developed on a Grace C_{18} column (250 × 4.6 mm i.d., 5 µm particle size) connected to a Jasco HPLC system equipped with JASCO UV 2075 Plus detector having rheodyne sample injection port with 20 µL loop. The chromatographic system was controlled by Empower v.2 software for the elution.

Selection of the Mobile Phase

The appropriate selection of a mobile phase is very crucial for the separation of drugs from a mixture. Based on the peak purity index, theoretical plates, and symmetry of peaks, the mobile phase was chosen. The trial was initiated employing the buffer systems along with acetonitrile (ACN). The elution with ACN:water in the ratio of 50:50 v/v (poor peak shape and tailing; Rt = 9.1 min) (Fig. 2a), 60:40 v/v (poor peak shape with doublet; Rt = 7.12 min) (Fig. 2b), and 70:30 v/v (broad peak; Rt = 3.56 min) (Fig. 2c) showed either very short or too long duration and thus are unsuitable. Further use of ammonium acetate:ACN (50:50 v/v) did not improve the peak, rather peak splitting with broad peak shape was perceived (Rt = 7.1 and 8.05 min) (Fig. 2d). Changing the buffer from ammonium acetate to phosphate buffer (0.02M):ACN (70:30 v/v) resulted

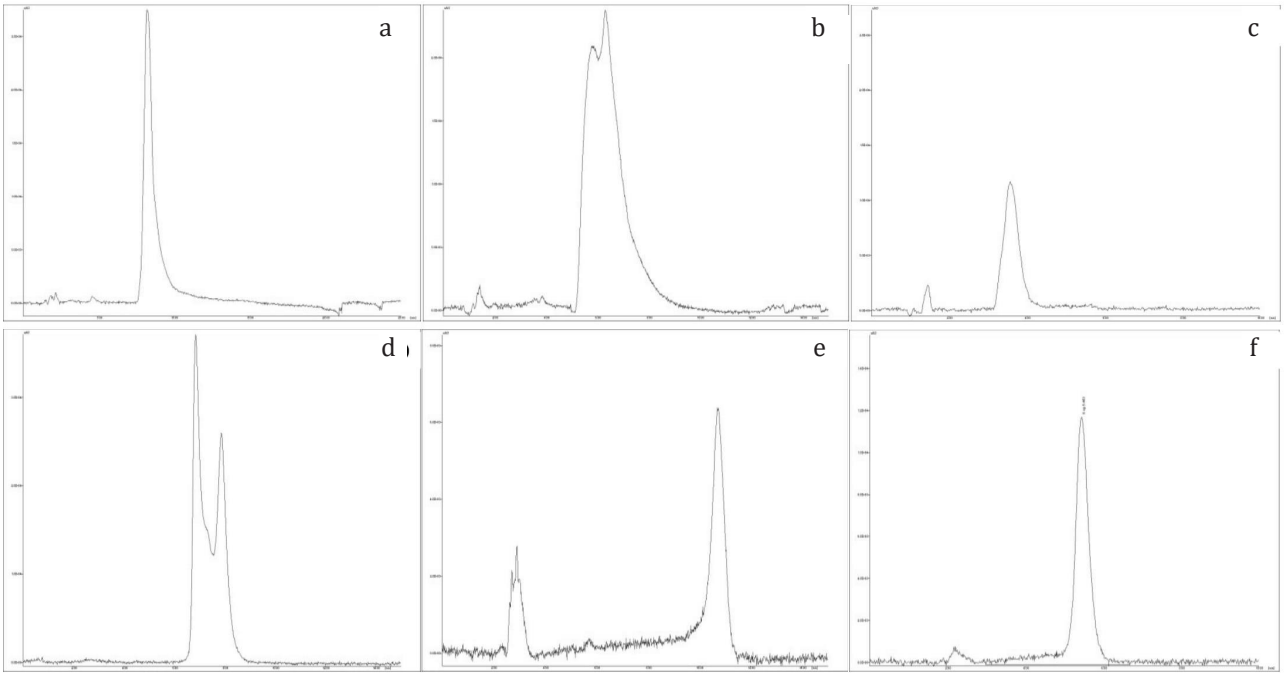

Fig. 2: Selection of mobile phase: (a) ACN:water (50:50 v/v); (b) ACN:water (60:40 v/v); (c) ACN:water (70:30 v/v); (d) Ammonium acetate:ACN (50:50 v/v); (e) Phosphate buffer (0.02 M): ACN (70:30 v/v); and (f) ACN: Phosphate buffer (0.02 M) (20:80 v/v).

in a poor peak with peak tailing (Fig. 2e). Ultimately, the composition ACN: phosphate buffer (0.02 M) (20:80 v/v) was selected for the elution (Fig. 2f). A sharp peak with ideal Gaussian characteristics was predominantly seen. The composition presented the largest number of theoretical plates and the highest peak purity index. The mobile phase content was then degassed under vacuum, filtered through a membrane filter (0.45 µm), and allowed to equilibrate until a steady baseline was achieved.

Determination of Wavelength

From the standard stock solution (B), few dilutions were prepared using the diluent, and the content was scanned over the range of 400–200 nm. The spectrum was obtained with prominent absorbances 227, 254, 277, 296, and 309 nm. It was observed that the drug showed the highest absorbance (λmax) at 277 nm, and thus selected for the analysis (Fig. 3).

Chromatographic Conditions

The PRU was eluted with acetonitrile: 0.02 M potassium dihydrogen phosphate in the ratio of 20:80 v/v in a 10 min isocratic mode at a flow rate of 1 mL/min at 30°C column temperature and monitored at a wavelength of 277 nm.

Preparation of Analytical Solutions

Preparation of 0.02M Potassium Dihydrogen Phosphate

0.02 M buffer was prepared by dissolving 2.72 gm of potassium dihydrogen phosphate in sufficient HPLC grade water to produce 1000 mL.

Preparation of the Mobile Phase

The mobile phase was composed by mixing 0.02 M potassium dihydrogen phosphate buffer and acetonitrile in 80:20 v/v ratios. The content was then filtered through a membrane filter (0.45 µm) using a vacuum filtration assembly and further sonicated on an ultrasonic water bath for the duration of 15 minutes.

Preparation of Standard Stock Solution

The standard stock solution of PRU was prepared by dissolving 10 mg of drug in 10 mL of ACN to get the desired concentration of 1000 µg/mL (A). From the standard stock solution, the standard working solution of PRU was prepared in ACN, which formed a resultant concentration of 100 µg/mL (B). Further dilution was made in ACN from the standard working solution to get the final solution of PRU (10 µg/mL) (C).

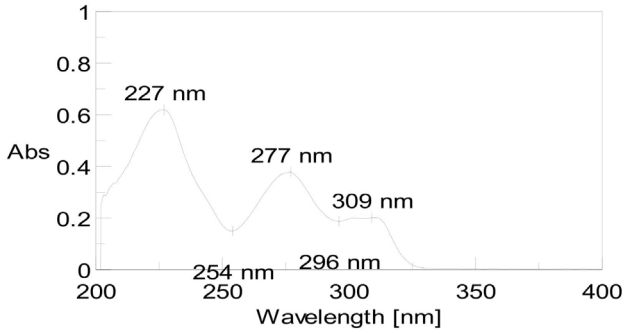

Fig. 3: UV spectrum of prucalopride succinate.

Preparation of Sample Solution (Formulation Analysis)

20 tablets of Pruvict 2 containing 2 mg prucalopride were weighed and powdered uniformly. In a volumetric flask, 10 mg equivalent of PRU was taken, diluent was half-filled, and sonicated further for the duration of 10 min. The resulting sample stock solution was then filtered with Whatman filter paper 41, and the volume was made by the dilute to the required mark to produce 1000 µg/mL strength. Further, 10 µg/mL concentration was prepared by dilution with the diluent.

Method Validation

The validation of the developed analytical method was achieved according to the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines Q2A (Validation of analytical methods: definitions and terminology-presents a discussion of the characteristics for consideration during the validation of the analytical procedures) and Q2B (Validation of analytical procedures: methodology-presents a discussion of the characteristics that should be considered during the validation of analytical procedures) along with the compliance towards USFDA guidance.

Linearity and Range

In the concentration range of 2-12 µg/mL, the linearity of the developed analytical method was determined by spiking six concentrations of PRU (equivalent volume) in the chromatographic HPLC system. The peak area of the method was estimated after preparing the solutions in the presence of the diluent. The linearity graph was formed by plotting the average area against the concentration for each drug solution. The linear regression equation and the regression coefficient value (r^2) were established.[8]

Accuracy

The recovery data (accuracy) was determined at concentration levels of 50, 100, and 150% by spiking the standard drug solutions in the HPLC system. On the basis of definite concentrations, the study was conducted in a triplicate manner and data were reported as % recovery ± % relative error.[9]

Precision

The precision data was determined at a concentration of 6, 8, 10 µg/mL by spiking the standard drug solutions in the HPLC system for intra-day (six times in a single day) and inter-day (three different days). The study was conducted

in a triplicate manner and data were reported as precision ± % relative error.[10]

Robustness

The robustness was determined for the developed chromatographic method by making deliberate changes in the conditions such as alteration in flow rate [at 0.9 mL/min (-1) and 1.1 mL/min (+1)]; wavelength [at 275 nm (-2) and 279 nm (+2)]; and mobile phase composition [18:82 v/v (-2:+2) and 22:78 v/v (+2:-2)]. The other parameters such as column temperature, column length, and its composition, etc., were kept fairly constant.[11]

Systems Suitability Parameters

The systems suitability parameters are determined by injecting the standard solution 5 times in the HPLC system and further determining the imperative components like tailing factor, peak area, retention time, and theoretical plates. This parameter is a measure of the reproducibility attribute of any developed chromatographic method.[12]

Limit of Detection

The limit of detection (LoD) is said to be the lowest possible concentration that can be detected by any sophisticated analytical method; however, it is not always essential to estimate the exact amount of the solute precisely.[13] The given formula determines it:

$$LoD = 3.3 (\sigma / S)$$

Where σ = standard deviation of response; S = slope of the calibration curve. The slope S may be estimated from the calibration curve of the analyte.

Limit of Quantification

The limit of quantification (LoQ) is said to be the lowest possible concentration that can be detected by any sophisticated analytical method, however, with a significant level of precision and accuracy.[14] The given formula determines it:

$$LoQ = 10 (\sigma / S)$$

Where σ = standard deviation of response; S = slope of the calibration curve. The slope S may be estimated from the calibration curve of the analyte.

Degradation Studies

The forced degradation studies under the conditions of acid, alkali, boiled water, hydrogen peroxide, dry heat, and UV radiations were performed according to the procedure given by Kanthale *et al*., 2019.[15]

Acid Degradation Studies

A total of 0.5 mg of equivalent drug was taken in a volumetric flask and diluent was added to half-level. The content was further sonicated (10–15 min) to make the solution clear and to remove the dissolved gases. The content was then filled with the residual diluent quantity to make up the volume up to 100 mL. The above content was stirred for 30 min using a stirrer and afterward centrifuged at 3000 rpm for 5 min duration. From the prepared solution, 5 mL of the sample was taken and mixed with 5 mL of 2 N HCl. The mixture was boiled for an hour under a water bath and further neutralized by an equal volume of 2 N NaOH. The volume was made up to 100 mL and filtered through the membrane filter (0.45 µm). 20 µL of the above content was spiked into the system and data were witnessed.

Alkali Degradation Studies

A 0.5 mg of equivalent drug was taken in a volumetric flask and diluent was added to half-level. The content was further sonicated (10–15 min) to make the solution clear and to remove the dissolved gases. The content was then filled with the residual diluent quantity to make up the volume up to 100 mL. The above content was stirred for 30 minutes using a stirrer and afterward centrifuged at 3000 rpm for 5 minutes duration. From the prepared solution, 5 mL of the sample was taken and mixed with 5 mL of 2 N NaOH. The mixture was boiled for an hour under a water bath and further neutralized by an equal volume of 2 N HCl. The volume was made up to 100 mL and filtered through the membrane filter (0.45 µm). 20 µL of the above content was spiked into the system and the data was witnessed.

Neutral Hydrolysis Studies

0.5 mg of equivalent drug was taken in a volumetric flask and diluent was added to half-level. The content was further sonicated (10-15 min) to make the solution clear and to remove the dissolved gases. The content was then filled with the residual diluent quantity to make up the volume up to 100 mL. The above content was stirred for 30 min using a stirrer and afterward centrifuged at 3000 rpm for 5 min duration. From the prepared solution, 5 mL of the sample was taken, boiled for an hour under a water bath, and the volume was made up to 100 mL. The above content was filtered through the membrane filter (0.45 µm), 20 µL of the above content was spiked into the system, and the data was witnessed.

Oxidation Degradation Studies

0.5 mg of equivalent drug was dissolved in H_2O_2 (5 mL) and boiled for 1 hr duration in a volumetric flask to commence the oxidative stress on the drug molecule. By using the diluent, the above reaction content was diluted and the volume was made up to the mark. Further, the above content was centrifuged at 3,000 rpm for 5 minutes duration, and the supernatant was collected. A 20 µL of the above content was spiked into the system and the data was witnessed.

Dry Heat Degradation Studies

A 0.5 mg of the equivalent drug, taken in a petri dish, was exposed to dry heat in over at a temperature of 90 ± 1°C for 1 hour. The content was added to a volumetric flask, and diluent was added to it. The above content was stirred for half

an hour, sonicated for 10–15 min, and the volume was made up to 100 mL. Further centrifugation was performed at 3,000 rpm for 5 minutes duration, filtered through the membrane filter (0.45 µm), and spiked (20 µL) into the HPLC system.

UV-rays Induced Degradation Studies

A 0.5 mg of equivalent drug was placed in a petri dish and kept under the UV-chamber at 254 nm for the duration of 72 hours. After the UV exposure, the content was added to a volumetric flask and the diluent was added in a half-filled manner. The content in the volumetric flask was stirred uniformly for the duration of 30 minutes, further sonicated for 10–15 minutes, and the volume was made up to 100 mL. The content was centrifuged at 3000 rpm for 5 minutes, filtered through the membrane filter (0.45 µm), and spiked (20 µL) into the HPLC system to determine the degraded product(s).

Results and Discussion

Method Development and Optimization of Chromatographic Conditions

The novel developed analytical method was based on several trials and errors and ultimately the best possible mobile phase (acetonitrile:0.02 M potassium dihydrogen phosphate in the ratio of 20:80 v/v) was selected for the chromatographic 10 min run on a Grace C_{18} column (250 × 4.6 mm i.d., 5 µm particle size) at a flow rate of 1 mL/min under ambient temperature (30°C) and detected wavelength at 277 nm. The selection of this C_{18} column was based on inspiration drawn from previous studies. The elution was achieved after several continuous trials run at 5.416 minutes (averagely) under isocratic mode (Fig. 4). A low pH was preferred in the mobile phase as it enables an acute reduction in the peak tailing, prevents the dissolution of silica-based reversed-phase columns, and remarkably improves the method robustness. The pH was made in a similar constitution with the pKa value to ascertain the solute in the unionized state, essential criteria for achieving high resolution. So, the pH = pKa up to 2 units were chosen rationally. The short-time run will have several advantages in context to solvent and time. The tablet formulation sample assay provided a mean recovery of 99.17% with % RSD value of 1.172 (Table 1). The developed chromatographic method helped in routine analysis of drugs in bulk, very precisely, accurately, and with adequate robustness.

Method Validation

Linearity and Range

A high degree of linearity was found for PRU over the range of 2–12 µg/mL (Table 2). A linear range (regression coefficient value; r^2 = 0.996) was perceived between the concentration and the peak area with linear regression equation y = 31497x + 8257.3 (Fig. 5).

Accuracy

From the formed calibration curve, the % recovery of the drug PRU from the developed chromatographic method was estimated based on the slope of the graph

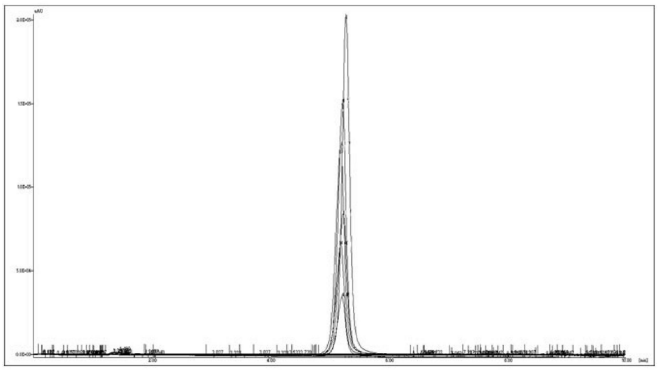

Fig. 4: Chromatogram of prucalopride succinate obtained from multiple sampling

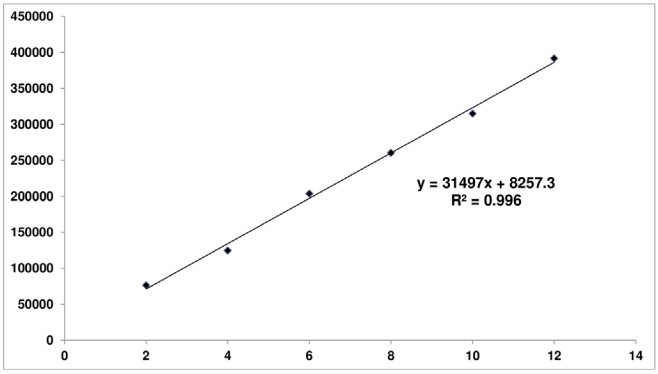

Fig. 5: Linearity plot of prucalopride succinate

Table 1: Assay performed for tablet formulation sample of prucalopride succinate

S. No.	*Peak area (4 µg/mL)*	*Amount recovered (µg/mL)*	*% Recovery*
1	136135	4.060	101.500
2	132914	3.958	98.943
3	133381	3.973	99.314
4	132193	3.935	98.371
5	133565	3.978	99.460
6	135158	4.029	100.724
Mean	133891	3.989	99.719
SD	1472.75	0.047	1.169
% RSD	1.09996	1.172	1.172

Table 2: Linearity study of prucalopride succinate

Conc. (µg/mL)	*Replicates*	Area	*Mean*	*SD*	*% RSD*
2	1	74890	76462	1369.939	1.792
	2	78630			
	3	75895			
	4	76530			
	5	76365			
4	1	123367	124788.6	2346.6	1.880
	2	125193			
	3	122465			
	4	128564			
	5	124354			
6	1	200993	203675	3026.817	1.486
	2	204564			
	3	201468			
	4	202865			
	5	208485			
8	1	265148	260540	4327.437	1.661
	2	262489			
	3	256412			
	4	255460			
	5	263191			
10	1	318986	315075.2	5707.997	1.812
	2	322102			
	3	307409			
	4	312564			
	5	314315			
12	1	398410	391874.2	6438.445	1.643
	2	385272			
	3	391456			
	4	385823			
	5	398410			

and the Y-intercept, which played a prominent role. The % RSD values were found to be 0.755, 0.588, and 0.482, respectively, which corresponded within the acceptance limit (± 2%) given in the USP pharmacopeia (Table 3).

Precision

The study comprising of both intra-day and inter-day variability represented considerably high precision over the tested PRU range of 6-10 µg/mL. The peak area of the sample solution matched exactly with the standard solution, with % RSD meeting the essential requirement of < 2%. In intra-day variability study, the % RSDs were detect to be 0.754, 1.032, and 0.482 (Table 4), respectively, whereas the inter-day variability study demonstrated % RSDs of 0.797, 0.559, and 0.524 (Table 5), respectively. The study highlighted minimum disparity

Table 3: Recovery for accuracy studies for prucalopride succinate

Level	*Conc. of sample solution (µg/mL)*	*Conc. of standard solution spiked (µg/mL)*	*Area*	*Amount recovered (µg/mL)*	*% recovery (mean ± %RSD)*
50%	4	2	195951	5.959	100.173 ± 0.755
			198666	6.045	
			198080	6.027	
100%	4	4	257427	7.911	99.077 ± 0.588
			259554	7.978	
			256740	7.889	
150%	4	6	317558	9.820	98.575 ± 0.482
			320423	9.911	
			318236	9.842	

Table 4: Precision data of intra-day variability.

Concentration (µg/mL)	*Area*	*% recovery*	*SD*	*% RSD*
	198331			
6	201079	101.442	0.765	0.754
	200485			
	257518			
8	262707	99.919	1.031	1.032
	259859			
	321415			
10	324315	99.804	0.481	0.482
	322102			

Table 5: Precision data of inter-day variability.

Concentration (µg/mL)	*Area*	*% recovery*	*SD*	*% RSD*
	198485			
6	198988	100.337	0.800	0.797
	196155			
	260529			
8	263191	100.758	0.563	0.559
	262707			
	318986			
10	322102	99.240	0.520	0.524
	321415			

and very high precision of the developed chromatographic method.

Robustness

After varying the chromatographic parameters (flow rate, wavelength, and mobile phase composition) intentionally, no prominent alteration in the retention time was observed in the chromatogram (Fig. 6). A minor change (2–3%) in the retention time was detected on enhancing or decreasing the parameters. The peak area (high), theoretical plates (> 2000), and the tailing factor (< 2%) were found to meet the minimum requirements of the USP pharmacopeia. The study indicated that the developed chromatographic method possesses robust attributes.

System Suitability Parameters

The monitored system suitability parameters pointed toward a very high degree of reproducibility of the developed chromatographic method. The novel method at 10 µg/mL concentration had average retention time (Rt) of 5.416 minutes, mean peak area (PA) of 263,465, mean theoretical plates (TP) of 2588, and tailing factor (TF) of 1.08 (Table 6). Which reflected towards high column efficacy, noteworthy resolution, good precision, better reproducibility, significant separation, and can be utilized for the routine pharmaceutical analysis. The chromatographic method meets the minimum essential requirements of US Pharmacopoeia monographs regarding theoretical plates (2000), tailing factor (< 2%), and presence of ideal Gaussian peak (symmetric = asymmetric factor = 1).

Limit of Detection and Quantification

The LoD and LoQ of PRU were found to be 0.367 µg/mL and 1.111 µg/mL, respectively, which indicated towards remarkable solute detection attribute of the developed chromatographic method. The method can possibly detect the lowest possible concentration of PRU in bulk or even in the formulation.

Forced Degradation Studies

The influence of photolytic effect, peroxide treatment, neutral condition, thermal environment, acidic treatment,

Table 6: Systems suitability parameters.

Spiking	*Rt (min)*	*Conc. (µg/mL)*	*Area*	*Theoretical plates*	*Asymmetry*
1	5.416	10	263465	2588	1.08
2	5.418	10	263524	2629	1.11
3	5.413	10	263208	2477	1.03
4	5.411	10	262719	2416	1.06
5	5.419	10	263591	2698	1.09
Mean	5.416	10	263302	2562	1.074

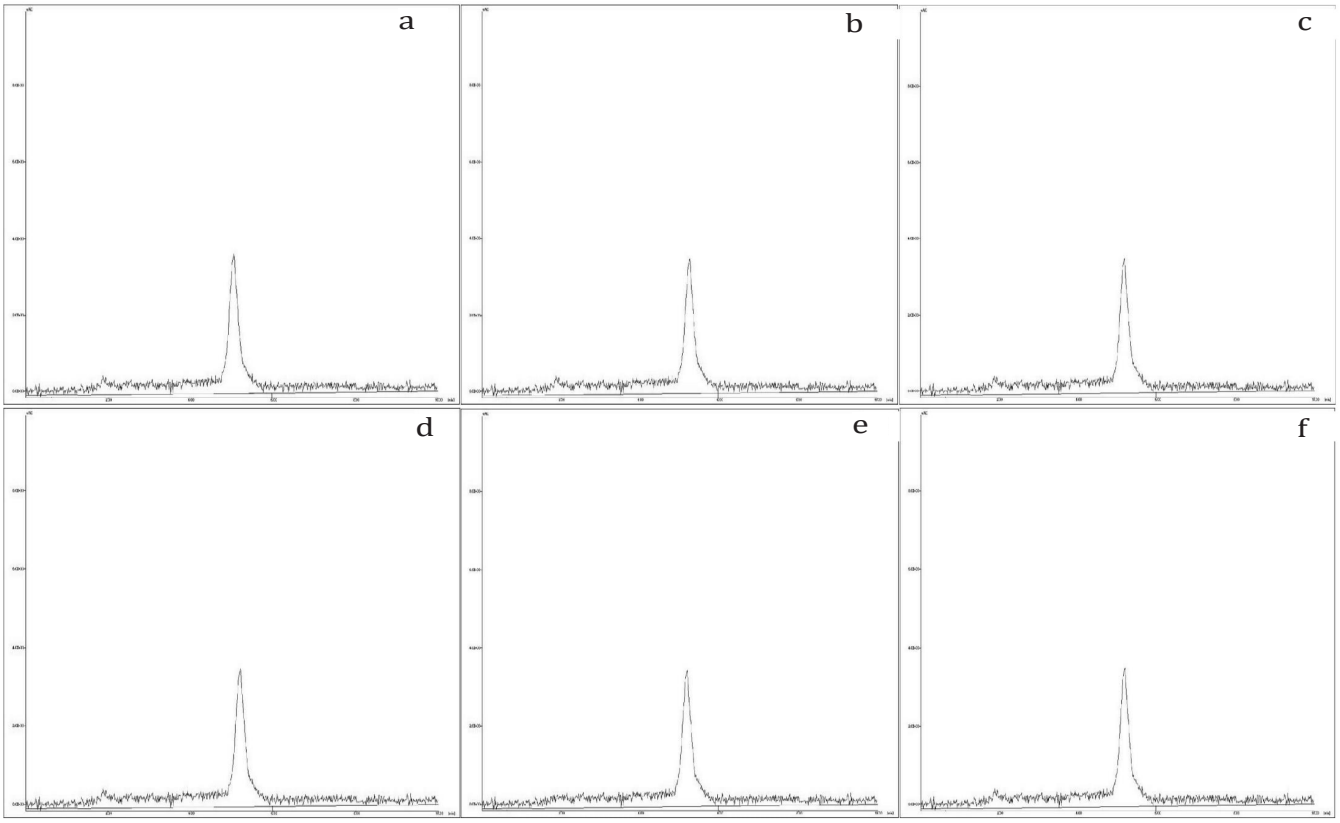

Fig. 6: Robustness studies of prucalopride succinate: (a) Flow rate at 0.9 mL/min; (b) Flow rate at 1.1 mL/min; (c) Wavelength at 275 nm; (d) Wavelength at 279 nm; (e) Mobile phase composition 18:82 v/v; and (f) Mobile phase composition 22:78 v/v

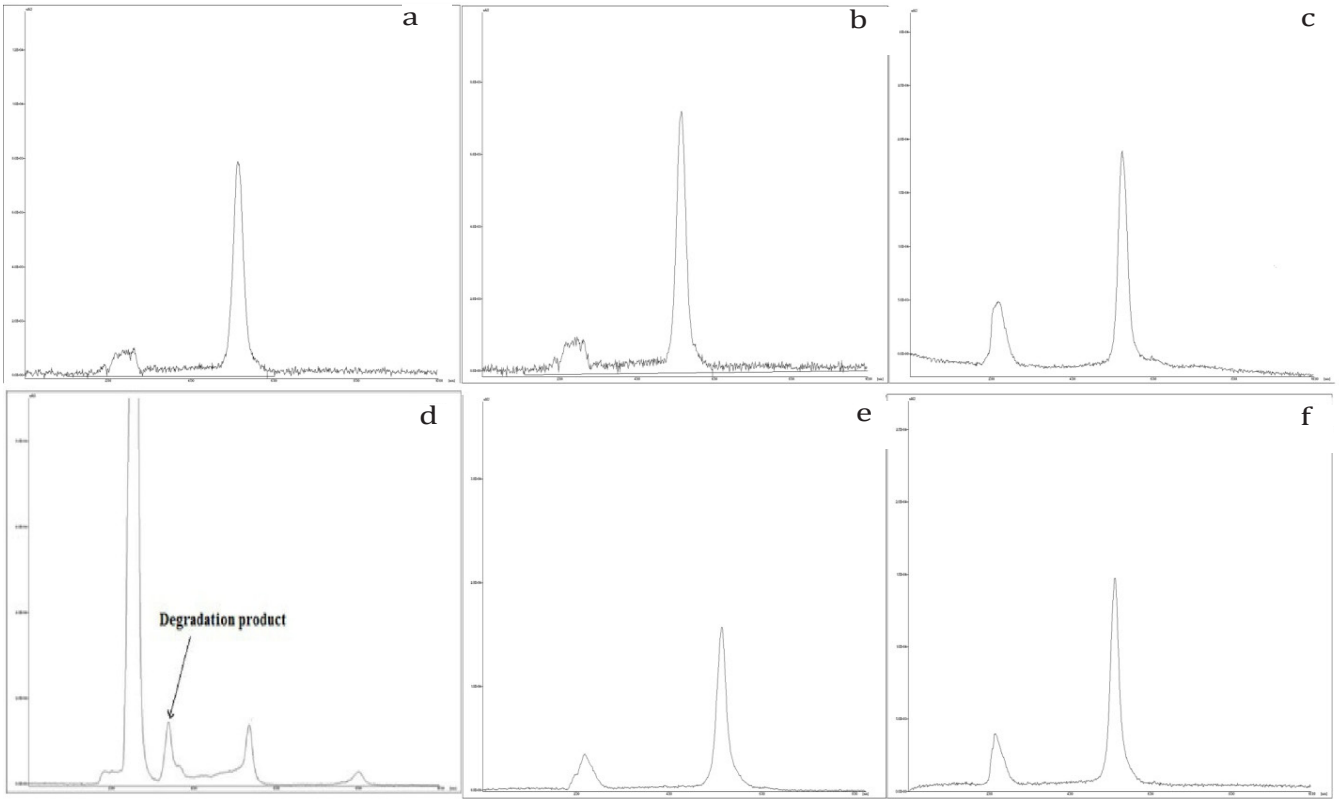

Fig. 7: Force degradation studies of prucalopride succinate: (a) Acidic condition; (b) Alkaline condition; (c) Neutral hydrolysis; (d) Oxidative condition; (e) Dry heat condition; and (f) UV-light

and base treatment produced the formation of a degraded product at the Rt range of 2.1–3.4 minutes. While the acidic treatment and basic treatment did not produce any prominent peak in the forced degraded chromatogram, a distorted peak was observed in the Rt range of 2.1–2.3 minutes. In contrast to it, the chromatograms of neutral, oxidation, heat, and UV exposed samples demonstrated an eye-catching sharp peak at 2.148 minutes. The highest degradation was perceived under oxidative stress, where the degradants were located at Rt of 2.148 and 3.434 minutes (Fig. 7). Although, acidic and basic treatment has a well-established mechanism(s), however, the exact degradation mechanism through oxidative stress, the most prominent way is still unknown. But, it may be predicted that oxidative stress breaks the prevailing weak bonds and rapidly abstracted the protons from the drug substance.

Comparison with Other Methods for Estimation of Prucalopride

As there are no reported reverse-phase stability-indicating HPLC method previously developed or reported in any databases for the estimation of PRU in both bulk and tablet formulation, no comparison can be done in context to attributes such as accuracy, robustness, reproducibility, precision, and linearity. The method developed by us may be regarded as the "index RP-HPLC method" for the estimation of PRU. A few years back, researchers had estimated the content of PRU in rat plasma using ultra-high-performance liquid chromatography with tandem mass spectrometry method.[16] This selective, rapid, accurate, and sensitive method comprising of acetonitrile-water (containing 0.1% formic acid) mobile phase system was found to have immense applications in pharmacokinetics. In comparison with our system, the method developed by our team for estimation of PRU in bulk might also be applied for estimating PRU in biological samples, particularly in rat plasma, maybe with reduced detection ability.

Conclusion

The developed validated chromatographic method can be used by the analytical chemists for quality assurance of the product in daily basis to estimate PRU in both bulk and tablet formulations due to high accuracy, robustness, reproducibility, precision, and linearity (the essential ICH-Q2A and Q2B requirements) as well as compliance with the minimum necessities of the US Pharmacopoeia monographs in terms of tailing values, % RSD, and theoretical plates. The validated stress degradation studies (acid, oxidation, UV-induced, dry heat, neutral, and alkaline) revealed the possible degradants which will be beneficial for the quantification.

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