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Research Article

Development and Validation of a Stability-Indicating Assay (HPLC) Method for quantitative analysis of Prulifloxacin in Bulk Drug

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Abstract

A novel stability-indicating reversed-phase (RP) HPLC method has been developed and validated for quantitative analysis of prulifloxacin in the bulk drug. Use of a 250 mm × 4.6 mm, 5-µm particle size, C18 column with 50:50 (v/v) formic acid in water (pH 3.5)–acetonitrile as isocratic mobile phase enabled separation of the drug from its degradation products. The flow rate and detection wavelength were 1 mL min^{-1} and 277 nm respectively. The method was validated for linearity, limits of detection and quantification, accuracy, precision, selectivity, ruggedness and system suitability. The linearity of the method was excellent over the range 0.030–10.000 µg mL^{-1}. The mean values of slope, intercept, and correlation coefficient were 85169, 9332 and 0.9992 respectively. The limits of detection and quantification were 0.010 and 0.030 µg mL^{-1}, respectively. RSD in intra-day and inter-day precision studies was < 2 %. Recovery of prulifloxacin from bulk drug ranged from 100.08 and 102.00 %. Prulifloxacin was subjected to stress conditions (hydrolysis (acid, base), oxidation, photolysis, and thermal degradation) and the stressed samples were analysed by use of the method. Maximum degradation was observed in acid and base hydrolysis and oxidation. The drug was also susceptible to degradation under photolytic and thermal conditions. The degradation products were well resolved from main peak thus proving the stability indicating nature of the method.

Key words: Prulifloxacin; Stability indicating; Degradation products

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1. Introduction

Prulifloxacin, (±)-7-{4-[(Z)-2,3-Dihydroxy-2-butenyl]-1-piperazinyl}-6-fluoro-1-methyl-4-oxo-1H,4H-[1,3]thiazeto[3,2 a]quinoline-3-carboxylic acid cyclic carbonate, is a new thiazeto-quinolone antibacterial agent prodrug of the quinolone carboxylic acid Ulifloxacin, [1H,4H-[1,3]-thiazeto-[3,2-a]quinoline-3-carboxylic acid, 6-

fluoro-1-methyl-4-oxo-7-(1-piperazinyl), [Figure 1], characterized by a potent and broad-spectrum antibacterial activity. Prulifloxacin structure contains the skeletal quinolone with a four-member ring in the 1, 2-position including a sulfur atom to increase antibacterial activity and an oxodioxolenylmethyl group in the 7-piperazine ring to improve its oral absorption; it is immediately and quantitatively transformed into the active metabolite Ulifloxacin. It exhibits good penetration in target tissues and fluids, and possesses a long half-life, thus allowing for once-daily administration. Ulifloxacin is generally more active in vitro than other fluoroquinolones against a variety of clinical isolates of Gram-negative bacteria, including community and nosocomial isolates of Escherichia coli, Klebsiella spp., Proteus, Providencia and Morganella spp., Moraxella catarrhalis and Haemophilus spp. Prulifloxacin has been successfully tested in Phase III randomized, controlled trials including patients with acute exacerbations of chronic bronchitis, uncomplicated and complicated urinary tract infections, and chronic bacterial prostatitis. Like other quinolones, prulifloxacin showed a broad-spectrum antibacterial activity against both Gram-positive and Gram-negative bacteria, and several anaerobic and atypical bacteria associated to chronic bronchitis and urinary infections. [1-4] Ulifloxacin, the active metabolite of Prulifloxacin inhibits the bacterial DNA gyrase and topoisomerase IV functions [5].

Stability-indicating assay methods (SIAM's) can be specific one, which evaluate the drug in the presence of its degradation products, excipients and additives, or selective one which is able to measure the drug and all the degradation products in the presence of excipients and additives. The International Conference on Harmonization (ICH) guidelines requires performing stress testing of the drug substance, which can help identify the likely degradation products. Moreover, validated stability-indicating method should be applied in the stability study. Stability is considered as one of the most important criteria in pharmaceutical quality control. Only stable preparation would promise precise delivery of the drug to the patients. Expiration dating on any drug product is based upon scientific studies at normal and stressed conditions. [6].

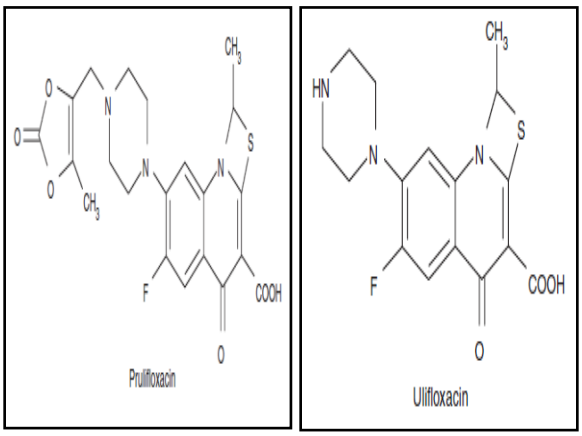

Figure 1. Chemical structure of Prulifloxacin and its active metabolite Ulifloxacin

Literature survey reveals that several methods have been reported for the determination of the active metabolite ulifloxacin in various matrices based on HPLC determinations with UV, fluorescence and mass spectrometric detection [7-10]. Some of these methods suffered from inadequate sensitivity, long analysis time and the use of toxic halogenated solvents. There are few stability indicating analytical methods available for the estimation of prulifloxacin from bulk and formulation. However these methods suffer from drawbacks such as higher linearity range, higher limit of detection and

quantification. [11-12] Hence an attempt has been made to develop and validate a sensitive stability indicating high performance liquid chromatographic method for determination of prulifloxacin from bulk which is specific, precise and accurate.The manuscript describes the development and validation, in accordance with ICH guidelines, of a simple, precise and accurate stability-indicating reversed-phase HPLC method for analysis of prulifloxacin in the presence of its degradation products. This article mainly deals with the forced degradation of prulifloxacin under the stress conditions like acid and base hydrolysis, oxidation, heat, and light, and validation of the method for accurate quantification of prulifloxacin in the bulk drug.

2. Experimental

Reagents and Materials

The working standard of prulifloxacin (98% pure) was procured from Hetero Drugs (Mumbai, India). HPLC grade acetonitrile was purchased from Merck (Darmstadt, Germany). Deionised and ultra pure water used in all experiments was obtained from Milli-Q System (Millipore). The 0.45-µm Nylon pump filter was obtained from Advanced Microdevices (Mumbai, India). Formic acid used for adjusting the pH of buffer solution was of AR grade (Merck, Darmstadt, Germany). Sodium hydroxide (NaOH), hydrochloric acid (HCl), and hydrogen peroxide (H_2O_2) were purchased from Qualigens Fine Chemicals (Mumbai, India).

Preparation of Mobile Phase

500 mL of Milli Q water, adjusted to pH 3.5 with Formic Acid was mixed with 500 mL of acetonitrile.The mobile phase was then filtered through a 0.45-µm Nylon filter and sonicated in an ultrasonic bath for 15 min.

Preparation of Standard Solution

10 mg of working standard of Prulifloxacin was accurately weighed and dissolved in 10 mL acetonitrile to give a stock solution of 1 mg mL^{-1}. Further dilutions were made in mobile phase.

Chromatographic conditions

A Jasco Liquid Chromatographic system with Jasco PU 1580 Intelligent HPLC Pump and a Jasco FP 1575 Intelligent UV-Vis Detector were used for the method development and forced degradation studies. The output signal was monitored and processed using Borwin Chromatography Software on Pentium computer (HCL-Mumbai).

The chromatographic separation was achieved on Qualisil BDS RP-C_{18} column (4.6 mm × 250 mm i.d. 5-µm particle size), with 50:50 (v/v) formic acid in water (pH 3.5): acetonitrile as mobile phase pumped at a flow rate of 1.0 mL min^{-1}. Before use it was filtered through a 0.45-µm Nylon filter and degassed in an ultrasonic bath. Analysis was carried out at room temperature. The injection volume was 20 µL and ultraviolet (UV) detection was at 277 nm.

Forced Degradation Studies

Acid hydrolysis

Forced degradation by acid hydrolysis was performed by adding 10 mL of stock solution (1 mg mL^{-1}) of prulifloxacin to 10 mL each of acetonitrile and 0.1 M HCl and refluxing the mixture at 60°C for approximately 2h. The solution was then left to reach room temperature, neutralized to pH 7 by addition of 0.1 M NaOH, then diluted to 100 mL with mobile phase so as to get concentration 100 µg mL^{-1}. Further 1mL of this solution was

diluted to 10 mL with mobile phase to get final concentration 10 µg mL^{-1}

Alkaline hydrolysis

Forced degradation by alkaline hydrolysis was performed by adding 10 mL of stock solution (1 mg mL^{-1}) of prulifloxacin to 10 mL each of acetonitrile and 0.1 M NaOH and refluxing the mixture at 60°C for approximately 2h. The solution was then left to reach room temperature, neutralized to pH 7 by addition of 0.1 M HCl, then diluted to 100 ml with mobile phase so as to get concentration 100 µg mL^{-1}. Further 1 mL of this solution was diluted to 10 mL with mobile phase to get final concentration 10 µg mL^{-1}.

Neutral hydrolysis

Forced degradation in alkaline media was performed by adding 10 mL of stock solution (1 mg mL^{-1}) of prulifloxacin to 10 mL each of acetonitrile and water and refluxing the mixture at 60°C for approximately 2h. The solution was then diluted to 100 mL with mobile phase so as to get concentration 100 µg mL^{-1}. Further 1 mL of this solution was diluted to 10 mL with mobile phase to get final concentration 10 µg mL^{-1}.

Oxidative degradation

To study the effect of oxidising conditions, 10 mL of stock solution (1 mg mL^{-1}) of prulifloxacin was added to 10 mL 3 % H_2O_2 (v/v) solution and the mixture was kept for 24 h. The solution was then diluted to 100 mL with mobile phase so as to get concentration 100 µg mL^{-1}. Further 1 mL of this solution was diluted to 10 mL with mobile phase to get final concentration 10 µg mL^{-1}

Temperature stress

To study the effect of temperature, approximately 50 mg prulifloxacin was exposed to dry heat (100°C) in a convection oven for 8 h. The drug was then removed from the oven, dissolved in 10 mL acetonitrile and volume was adjusted upto 50 mL with mobile phase.10 mL of this solution was diluted with mobile phase to get concentration 100 µg mL^{-1}. 1 mL of the above solution was further diluted with mobile phase to 10 mL so as to give a solution of final concentration equivalent to 10 µg mL^{-1} of prulifloxacin.

Photolytic degradation

To study the effect of UV light, approximately 50 mg prulifloxacin was exposed to short and long wavelength UV light (254 and 366 nm, respectively) for 24 h, then dissolved in 10 mL of acetonitrile and made up to the volume by mobile phase in 50 mL volumetric flask. Then 1 mL of stock solution was further diluted with mobile phase so as to give a solution of final concentration equivalent to 10 µg mL^{-1} of prulifloxacin. 20 µL of resulting solution was injected into HPLC and chromatograms were recorded.

Method Validation

The method was validated for linearity, limits of detection and quantification, accuracy, precision, selectivity, robustness and ruggedness and system suitability in accordance with ICH guidelines.

Linearity

Linearity was studied by preparing standard solutions at concentrations from 0.03 to 10 µg mL^{-1} and plotting peak area against concentration. Linearity was evaluated by least-squares regression.

Detection Limits

The limits of detection and quantitation were calculated by the method based on the standard deviation (σ) of the responses for blank injection in triplicate and the slope (S) of the calibration plot, by

use of the formulae LOD = $3.3\sigma/S$ and LOQ = $10\ \sigma/S$.

Precision

Precision was studied to determine intra-day (repeatability) and inter-day (injection of samples over three consecutive days) variation in the HPLC method for three different concentrations of prulifloxacin (0.1, 1, and 5 µg mL^{-1}) each analysis being performed in triplicate.

Accuracy

The accuracy of an analytical method is defined as the similarity of the results obtained to the true value and precision is defined as the degree of that similarity. Accuracy was evaluated in triplicate by addition of three different amounts of prulifloxacin to a previously analyzed sample and comparing the amount of prulifloxacin recovered with the amount added. The amounts added were (0.1, 1 and 5 µg mL^{-1}). Recovery, RSD (%) was calculated for amount added.

Robustness and Ruggedness

Robustness of method was investigated by varying the chromatographic conditions such as change of flow rate (±10%), organic content in mobile phase (±2%), wavelength of detection (±5%) and pH of buffer in mobile phase (±0.2%). The ruggedness of the method was assessed by comparison of the intra-day and inter-day assay results for prulifloxacin obtained by two analysts in the same laboratory.

Stability

The solution stability of prulifloxacin was carried out by leaving the test solutions of working standard in tightly capped volumetric flasks at 24, 48, and 72 h. The results were compared with those obtained from freshly prepared standard solutions and % RSD was calculated.

3. Results and Discussion

HPLC Method Development and Optimization

Prulifloxacin is hydrophobic drug; it is freely soluble in organic solvents like acetonitrile and methanol. Initially during method development, acetonitrile: sodium dihydrogen phosphate in water 70:30 % (v/v) was used as mobile phase. With this combination resolution could not be achieved between solvent and drug peaks and also resulted in uneven baseline and tremendous noise. In order to remove noise the buffer was removed and a combination of water and acetonitrile was tried. Then combination comprising of water: acetonitrile 70:30 % (v/v) was used. The mobile phase gave good response and good peak shape but there was tailing for which mobile phase was modified and it was the fine tuned to give good sharp peak and good response without noise, tailing and splitting.

Effect of mobile phase composition

Mobile phase (acetonitrile: water) in different ratio i.e. 70:30, 60:40, 50:50 with flow rate of 1mL min^{-1}. Minimum retention for Prulifloxacin was obtained at 70:30 acetonitrile: water which resulted into inadequate separation of drug peak from coeluting solvent peak, therefore it appeared as split peak. In order to solve this problem the composition of acetonitrile was reduced to 60% from 70% and water proportion was increased from 30% to 40%, however the peak were well resolved and it was not appearing as split peak as earlier. But due to inadequate separation there was fronting which spoiled the peak shape. Therefore in order to improve retention time and peak shape proportion of methanol and water was fixed to 50:50. This composition gave

good sharp peak without tailing and fronting along with acceptable retention time of 6.42 min, which overall reduces the analysis time. In mobile phase composition 70:30 % (v/v), the elution power of the mobile phase was high as result of which the drug was unable to retain on to the column, leading to coeluting of drug peak along with solvent peak. This resulted into split peak which inturn hampered overall resolution. In contrast to this at 60:40 v/v compositions, the drug affinity to stationary phase increased due to relative lower proportion of acetonitrile present in the mobile phase, but this resulted in fronting and asymmetry. Thus at the end acceptable resolution and good peak symmetry was achieved with acetonitrile: water 50:50 (%v/v) and so it was considered to be optimum and it was further fine tuned to give better peak shape.

Effect of mobile phase pH

The peak shape of drug was affected by the pH values of the system. pKa value of Prulifloxacin is approximately 5 so it is an acidic drug which can also be interpreted from its structure. In acidic pH drug remains in unionized form which in turn has an effect on peak shape and retention time (Rt). Therefore considering the pKa value 5, pH of water was adjusted to 3.5 with formic acid. Prior fixing this pH different trial at different pH was carried out, but either drug eluted at lower Rt or there was peak tailing and asymmetry. Thus mobile phase acetonitrile: water 50: 50 % (v/v) pH 3.5 adjusted with formic acid was optimized, as it resulted in improved sensitivity, selectivity good resolution, better peak shape and reproducible results. With the aim of the optimization of mobile phase pH (3.0, 3.5, 4.0, and 5.0), the remaining two factors were kept constant, i.e. mobile phase composition acetonitrile: water 50: 50 % (v/v) and flow rate of 1 ml min-1. According to the Henderson Hasselbalch equation, the drug having a pKa less than 7 is considered to be an acidic, which remain almost 90% in unionized state in acidic pH in contrast with basic pH where it is 90% ionized. The co-relation between pKa and peak shape and retention time can be justified by, the more the drug is unionized, and more it is nonpolar, which can effectively interact with nonpolar group present on column. This property results in to better separation between nonpolar and polar impurities as well as selective separation between two nonpolar molecules depending on pKa and structure. Thus, for best chromatographic separation pH 3.5 was fixed, as it gave improved peak shape with no tailing and reproducible response.

Validation of the Method

A method developed and optimized cannot be applied for analysis unless completely validated. ICH recommended validation parameters including precision (RSD, %), accuracy (recovery, % and RSD, %), linear range (correlation coefficient), and sensitivity (LOD and LOQ), ruggedness and robustness (RSD %), specificity and system suitability were evaluated.

Linearity

The calibration curve was linear over the concentration range studied (0.03–10 μg mL^{-1}; n = 6). The correlation coefficient was 0.9999 with RSD 0.27 %. The mean values of the slope and intercept were 85281 ± 233.69 and 9332 ± 12.69 respectively.

Precision and Accuracy by recovery

The intra-day and interday variability or precision data are summarized in Table 1. The low value (≤ 2%) of RSD indicates the repeatability of the method. The recovery

of the method was 100.12–101.88 % after spiking a previously analyzed test solution with additional drug standard. The values of recovery, %RSD, and SE are shown in the Table 2. RSD was always less than 2%, which indicates the proposed method is accurate.

Limits of Detection (LOD) and Quantification (LOQ), determined by the standard deviation method as described in the experimental section, were 0.010 µg mL^{-1} and 0.030 µg mL^{-1}, respectively, indicating the method is sensitive and can be used for detection and quantification of prulifloxacin in a very wide concentration range. Robustness and Ruggedness was tested using the so-called 'one factor at a time' method. Influences of small changes in chromatographic conditions, such as change in flow rate (±10%), organic content in mobile phase (±2%), wavelength of detection (±5%), and pH of buffer in mobile phase (±0.2%), were studied to determine the robustness of the method. There was no significant change in the RT and area of Prulifloxacin when these conditions were varied as described in the experimental section. The low value of the RSD indicates the method is robust (Table 3). Results from system-suitability data are listed in Table 4. The number of theoretical plates (N) was higher than 2000 (the value regarded as minimum for an acceptable method). The tailing factor (T), another property which the ICH guidelines stipulate should be monitored, was within the limits established by these guidelines. These system-suitability tests aid checking of the method to ensure the HPLC system and procedure are capable of providing data of acceptable quality. It can therefore be concluded that the method gives consistent results if mobile phase pH, composition, and flow rate vary slightly. The peak purity results are listed in Table 5. The resolution data for all the stress conditions is listed in Table 6. This indicates that the drug was well resolved from its degradation products which indicate specificity of the method. The % RSD for Prulifloxacin in stability studies was (≤ 2%).

Stress Degradation behaviour

The results from stress-testing studies were indicative of the high specificity of the method (Table 7). The degradation products were completely resolved from the parent compound. The drug was susceptible to maximum degradation (87.70%) when subjected to oxidation followed by acid hydrolysis (71.67%) and base hydrolysis (64.30%). The drug was also subject to degradation under photolytic (58.95%) and thermal conditions (52.44%).

Table 1. Precision data

Intra-day Precision			**Inter-day Precision**		
Actual Concentration (µg/ml)	**Measured Concentration (µg/ml) ± S.D.**	**% R.S.D**	**Actual Concentration (µg/ml)**	**Measured Concentration (µg/ml) ± S.D.**	**% R.S.D**
0.1	0.102± 0.0008	0.82	0.1	0.102± 0.0007	0.71
1.0	1.008± 0.0145	1.44	1.0	1.011± 0.0152	1.53
5.0	5.017± 0.0596	1.19	5.0	5.027± 0.0267	0.53

Table 2. Accuracy by percent recovery data

Accuracy by percent recovery			
Actual Concentration (µg/ml)	**Measured Concentration (µg/ml) ± S.D.**	**% R.S.D**	**% Recovery**
0.1	0.101 ± 0.0013	1.35	101.01
1.0	1.008 ± 0.0158	1.56	101.88
5.0	5.003 ± 0.0655	1.30	100.12

Table 3. Robustness studies for Prulifloxacin

Conditions	Assay %	RT	Theoretical plates	Tailing	RSD (%) (n=5)
Acetonitrile: buffer (50:50)	99.09	6.60	5969	1.23	0.29
Acetonitrile: buffer (52:48)	100.88	6.56	6345	1.27	0.23
Acetonitrile: buffer (48:52)	99.64	6.65	6215	1.22	0.24
Flow rate (1.1 mL/min)	98.42	6.54	6176	1.25	0.15
Flow rate (0.9 mL/min)	99.05	6.68	6479	1.27	0.25
Mobile phase pH (pH 3.3)	100.22	6.67	5993	1.25	0.30
Mobile Phase pH (pH 3.7)	100.89	6.69	6412	1.24	0.27

Table 4. System suitability studies for Prulifloxacin

Sr. No.	Area (6.000 µg/ml)	Tailing factor	Theoretical plates
1	2799432.45	1.21	5925
2	2754930.33	1.25	6053
3	2685948.47	1.19	6114
4	2697327.60	1.16	6136
5	2732937.40	1.25	6079
6	2789236.47	1.35	5961
Mean	2734115.25	1.212	6061
±S.D.	45755.7685	0.0390	82.4495
%CV	1.67	3.21	1.36

Table 5. Peak Purity results for Prulifloxacin in presence of degradation products

Stress Conditions	Peak Purity of Doxofylline
Acidic hydrolysis	999.956
Basic hydrolysis	999.572
Thermal stress	999.587
Oxidative stress	999.683
Photolysis	999.893

Table 6A. Resolution data for the degradation product peaks observed in Acid Hydrolysis

Resolution factor between	A*	B*	Prulifloxacin
A & B	1.47	-	-
A & Prulifloxacin	2.77	-	-
B & Prulifloxacin	-	1.55	-

A & B are the suspected degradation products

Table 6B. Resolution data for the degradation product peaks observed in Base Hydrolysis

Resolution factor between	C*	B*	Prulifloxacin
C & B	0.82	-	-
C & Prulifloxacin	2.79	-	-
B & Prulifloxacin	-	2.34	-

C* & B* are the degradation products

Table 6C. Resolution data for the degradation product peaks observed in Oxidation

Resolution factor between	A*	Prulifloxacin
A & Prulifloxacin	3.08	-

A* is the degradation product

Table 6D: Resolution data for the degradation product peaks observed in Photolysis

Resolution factor between	C*	B*	Prulifloxacin
C & B	0.45	-	-
C & Prulifloxacin	2.5	-	-
B & Prulifloxacin	-	1.55	-

C* & B* are the degradation products

Table 7. Percent Degradation of Prulifloxacin and Retention time of Degradation products

Sr. No.	Condition	Retention time of drug/ degradation products (Min)	Peak Area (µV. sec)	Percent Degradation (%)
1	Untreated Stock	6.43	1082585.65	--
2	Acid Hydrolysis	2.88 4.21 6.62	186063.55 144904.12 306663.34	71.67
3	Base Hydrolysis	3.61 4.10 6.68	119988.38 31221.91 386406.25	64.30
4	Oxidation	2.92 6.62	141257.37 133073.24	87.70
4	Photolysis	3.42 4.19 6.67	27764.24 28506.57 444371.48	58.95
5	Thermal degradation	6.60	514873.50	52.44

Figure 2. Representative Chromatogram of stress studies of Prulifloxacin

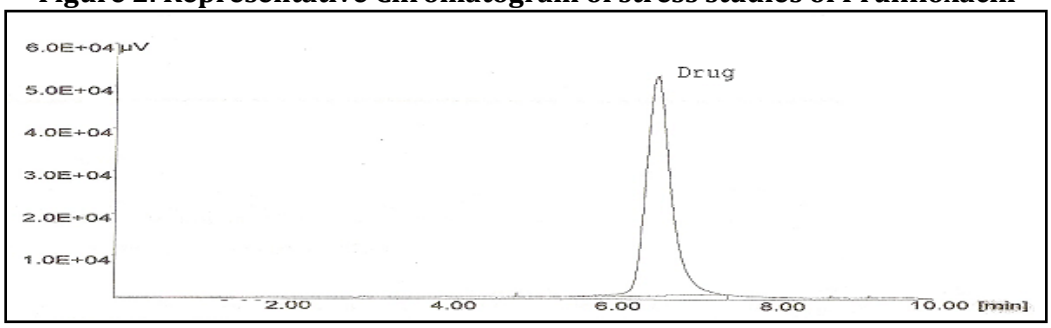

Figure 2A. Untreated stock solution (10 µg/ml)

#	Name	Rt	Area
1	Drug	6.63	1082585.65

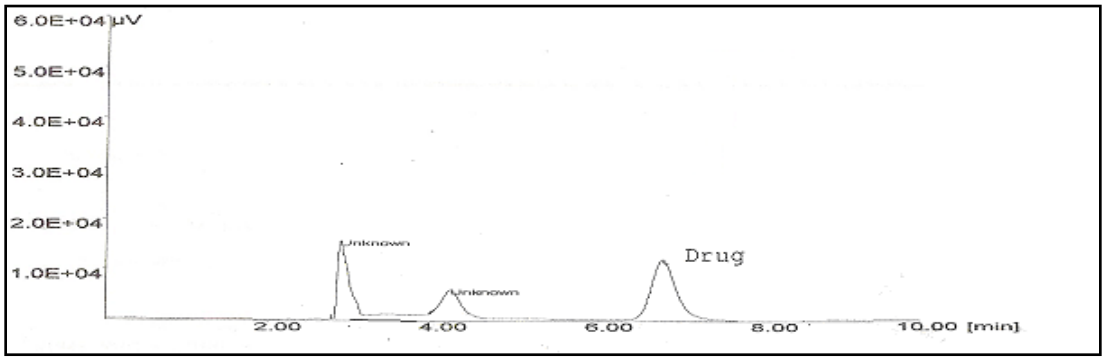

Figure 2B. Acid Hydrolysis

#	Name	Rt	Area
1	Unknown	2.88	186063.52
2	Unknown	4.21	144904.39
3	Drug	6.62	306663.50

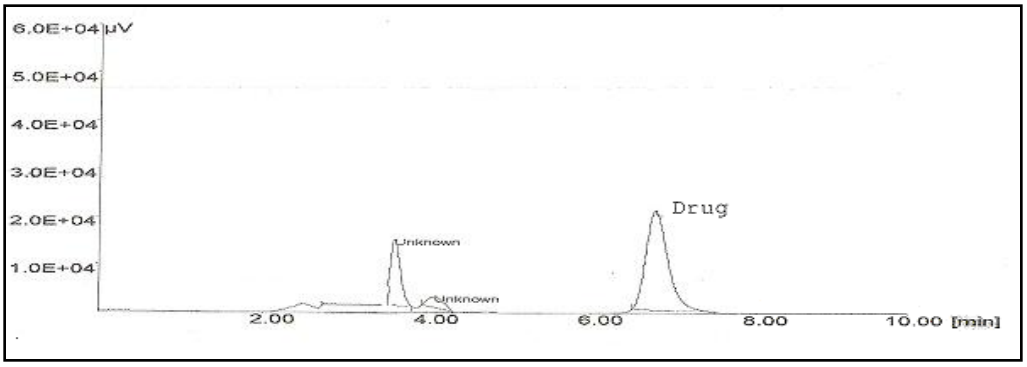

Figure 2C. Base Hydrolysis

#	Name	Rt	Area
1	Unknown	3.61	119988.38
2	Unknown	4.10	31221.91
3	Drug	6.68	386406.25

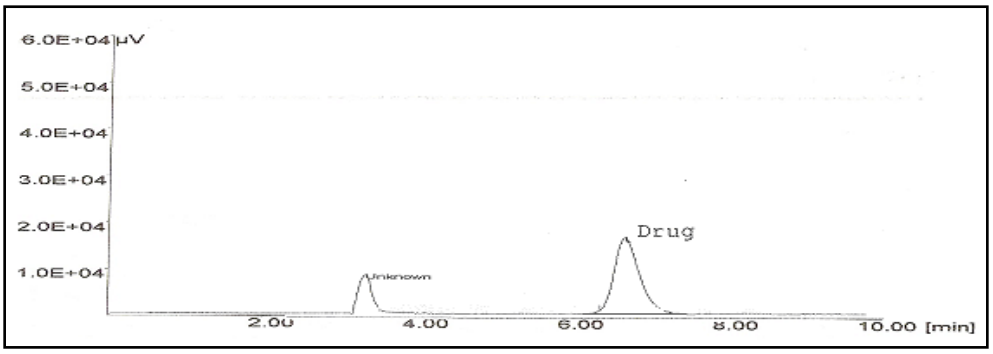

Figure 2D. Oxidation

#	Name	Rt	Area
1	Unknown(A)	2.92	141257.37
2	Drug	6.62	133073.24

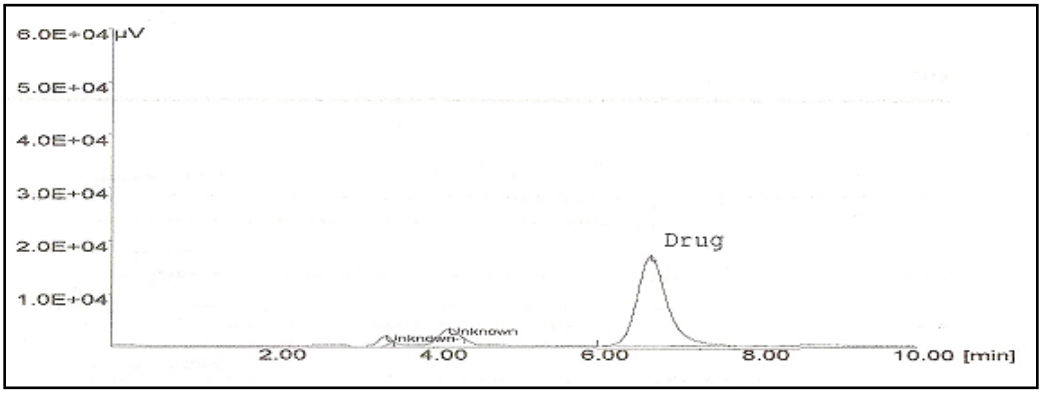

Figure 2E. Photolysis

#	Name	Rt	Area
1	Unknown	3.42(C)	27764.24
2	Unknown	4.19(B)	28506.57
3	Drug	6.67	444371.48

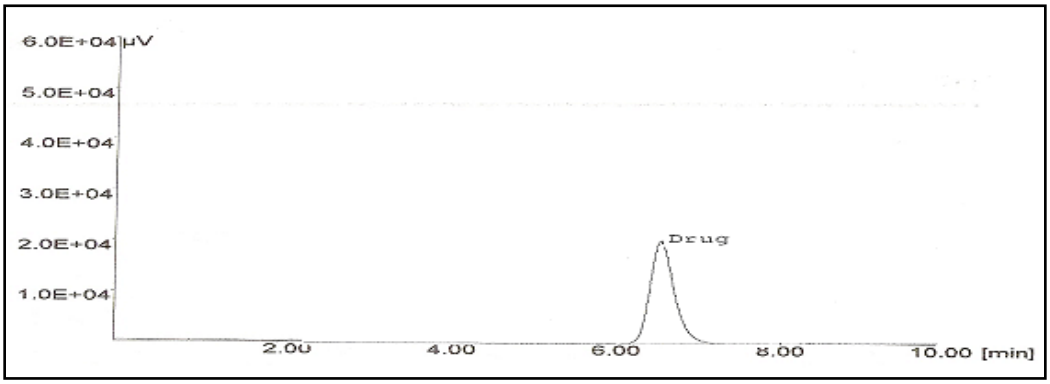

Figure 2F Thermal Degradation

#	Name	Rt	Area
1	Drug	6.60	514873.50

Conclusion

Thus the method developed for determination of prulifloxacin is simple, precise, accurate and selective. The method was successfully validated for all the validation parameters as per ICH guidelines. The method is stability-indicating and can be used to assess the stability of prulifloxacin in the bulk drug. The method can be conveniently used for assay of prulifloxacin in the bulk drug and in pharmaceutical dosage forms in pharmaceutical industry.

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