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Abstract: Stability is considered one of the most important criteria in pharmaceutical quality control. With this objective a stability indicating high performance liquid chromatographic method has been established for analysis of Pazufloxacin mesylate in the presence of degradation products. The drug was subjected to stress condition of hydrolysis, oxidation, photolysis, thermal degradation. Extensive degradation was found in Oxidative medium. Minimum degradation was found in acid degradation while there was no degradation found in Basic, thermal and photolytic condition. Successful separation of a drug from degradation product formed under stress condition was achieved on C18 column using methanol and 50mM Potassium dihydrogen Orthophosphate (40:60, v/v), pH4.5 adjusted with Acetic acid as a mobile phase. Flow rate was 1ml min⁻¹ and the detector was set at wavelength of 249nm. The method was validated for linearity, range, precision, and accuracy, limit of quantification and limit of detection. Because method effectively separates the drug from their degradation products, it can be used as stability indicating method.

Keywords: Pazufloxacin mesylate; stress degradation; stability indicating method; RP-HPLC.

1. Introduction

Pazufloxacin mesylate, (3R)-10-(1-aminocyclopropyl)-9-fluoro-3-methyl-7-oxo-1*H*, 7*H*-[1, 3] oxazino [5, 4, 3-*ij*] quinoline-carboxylic acid (Figure 1) [1-2]. Pazufloxacin is a fused tricyclic quinolone derivative with a 1-aminocyclopropyl substituent at C-10 position. The presence of aminoacyl group at C-10 is a unique feature of the molecule imparting potent broad spectrum activity against gram-positive and gram-negative bacteria including variety of resistant strains and anaerobic bacteria [3-7].

Stability is considered one of the most important criteria in pharmaceutical quality control. Only stable preparation would promises precise delivery of drug to the patient. Expiration dating on any drug product is based upon scientific studies at normal and stress conditions [8-9].

Literature survey reveals that there are analytical methods available for determination of Pazufloxacin mesylate from biological matrix, bulk drug and dosage form, and analytical method for determination of Pazufloxacin mesylate [10-14].

Literature survey further reveals that the drug Pazufloxacin mesylate is not official in USP or

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BP and there is no official method available for the studying the impurities and related substances in Pazufloxacin mesylate. There is validated stability indicating analytical method for the determination of Pazufloxacin in the presence of degraded product But this method suffer from drawbacks like use of high conc. Of organic solvents, and also time consuming [15-16]. Hence attempt was made to develop a stability indicating HPLC method for the degraded substances determination.

Keeping in view of susceptibility of Pazufloxacin mesylate under variety of condition, it was felt that a HPLC method of analysis that separates the drug from the degradation products which are formed under ICH suggested condition such as hydrolysis, oxidation, photolysis, and thermal degradation would be remarkable interest. These studies serve to give information on drugs inherent stability and help in the validation of analytical method to be used in the stability studies. Therefore, the objective of current study was to study the degradation of Pazufloxacin mesylate under different ICH stress condition and to establish accurate, specific, reproducible stability indicating HPLC method.

This paper deals with the forced degradation of Pazufloxacin mesylate under stress condition like acid hydrolysis, alkaline hydrolysis, oxidation, photolysis, and thermal. It also deals with the validation of the developed method for the accurate quantification of degradation product.



Figure 1. Structure of Pazufloxacin Mesylate

2. Materials and methods

2.1. Chemical and reagent

The working standard of Pazufloxacin mesylate was procured from Macleods Pvt. Ltd. India. HPLC grade methanol was purchased from Merck (India). Deionised and ultrapure water was used in all experiments was obtained from Milli-Q system (Millipore Inc., USA).Potassium dihydrogen orthophosphate used as buffer AR grade (Qualigens). Acetic acid used for adjusting the PH of buffer solution AR grade (S. D. Fine chemicals).

2.2. Equipment

PH of the mobile phase was checked on a pH/ion analyser (Lab India, PHAN, India). Refluxing of drug in hydrolysis condition was carried out in round bottom flask-condenser

assembly. The HPLC system employed in method development, forced degradation studies, and assay method validation was Jasco PU-2080 Plus Intelligent HPLC pump (Japan) and Rheodyne 7725i manual injector. Jasco MD-2010 Plus Multiwavelength Detector (Japan) and Chrompass software as data integrator.

2.3. Preparation of mobile phase

600mL of 50m potassium dihydrogen orthophosphate buffer solution was prepared and pH was adjusted to 4.5 with acetic acid. The final volume was adjusted by adding this 600mL of buffer to 400mL methanol, which resulted in pH4.5 for final mobile phase. The mobile phase was sonicated for 15min.

2.4. Preparation standard solution

Stock solution of Pazufloxacin mesylate (1mg mL⁻¹) was prepared in methanol. Standard solutions were prepared by dilution of stock solution with mobile phase to give solution in concentration of 5.000 to 15.000 μ g mL⁻¹. The stock solution used for the degradation studies was 15 μ g mL⁻¹.

2.5. Optimized chromatographic condition

The chromatographic separation was achieved on HIQ SIL RP-C₁₈ column (250×4.6 mm, 5 μ m particle size) using a mobile phase consisting of mixture of methanol: 50m potassium dihydrogen orthophosphate (40:60, v/v), adjusted pH4.5 with acetic acid. All reagents were filtered through the 0.45 μ m filter paper and sonicated before use. The injection volume was 20 μ L. The PDA detector was set at the wavelength 249nm. The assay was performed at 25^oC and the flow rate was fixed at 1mg mL⁻¹.

2.6. Validation of method

The stock solution of the drug was prepared at strength of 1mg mL⁻¹. It was diluted to prepare solutions containing 5.000 to 15.000 μ g mL⁻¹ of the drug Pazufloxacin mesylate. The solution was injected in triplicates into a HPLC column keeping the injection volume constant (20 μ L).

Accuracy is usually demonstrated by spiking an accepted reference standard into the product matrix. Percent recovery (observed/expected x 100%) should ideally be demonstrated over the $\pm 30\%$ Assay at selected analysed concentration.

Interday precision indicates how the test results are under ideal condition (same sample, operator, Instrument, and Day). Intraday precision indicates how precise test results are on any given day.

Twelve injections of three different concentration LQC ($8.000\mu g mL^{-1}$), MQC ($10.000\mu g mL^{-1}$), HQC ($12.000\mu g mL^{-1}$)], were given at the same day and the values of relative standard deviation were calculated to determine intra-day precision. These studies were also repeated on different days to determine inter-day precision.

Accuracy was calculated for the known concentration of the drug. The recovery of the added drug was determined. The method is specific as it is well resolved and distinguished from the degradation products.

The LOD and LOQ were determined at a signal to noise ratio of 3:1 and 10:1 respectively by

injecting the dilute solution with known concentrations.

Robustness of method was investigated by varying the chromatographic condition such as change of flow rate ($\pm 10\%$), organic content in mobile in phase ($\pm 2\%$), wavelength of detection ($\pm 5\%$), and the pH of buffer in the mobile phase ($\pm 0.2\%$). Robustness of the developed method was indicated by the overall %RSD between the data at each variable condition.

The solution stability was carried out by leaving both test solution of sample and reference standard in tightly capped volumetric flask at -20° C for 7 days. The sample solution was assayed after 7 days with fresh sample.

2.7. Stress degradation studies

Acid induced stress degradation was performed by adding 10mL of stock solution (5mg mL⁻¹) of Pazufloxacin mesylate to 5mL of 0.1 M HCl and refluxing the mixture at 80° C for approximately for 8 Hrs. The solution was left to reach ambient temperature, neutralised to pH7 by addition of 0.1 M NaoH.

Base induced stress degradation was performed by adding 10mL of stock solution (5mg mL⁻¹) of Pazufloxacin mesylate to 5mL of 0.1M NaoH and refluxing the mixture at 80° C for approximately 8 Hrs. The solution was then left to reach the ambient temperature. Neutralised to pH 7 by addition 0.1 M HCl.

2.8. Oxidative degradation

To study the effect of oxidizing conditions, 10mL of stock solution (5mg mL⁻¹) of Pazufloxacin mesylate was added to 5mL of 3% H_2O_2 solution and the mixture was refluxed at 60^oC for 60min. The solution was then left to reach the ambient temperature.

2.9. Thermal degradation

To study the effect of temperature, approximately 10mL of stock solution (5mg mL⁻¹) of Pazufloxacin mesylate was stored at $80^{\circ}C$ for 48 hour then it was adjusted up to 100mL with mobile phase.

2.10. Photolysis

To study the effect of UV light, approximately 10mL of stock solution (5mg mL⁻¹) of Pazufloxacin mesylate was exposed to UV fluorescent lamp having spectral distribution from 320 to nm to 400 nm with a maximum energy emission between 350nm to 370nm for 7 days then volume was adjusted up to 100mL with mobile phase. 20μ L of resulting solution was injected into HPLC and chromatograms were recorded. The stability samples were recorded using PDA detector, as the method was found to be rugged in nature.

3. Result and discussion

3.1. Degradation behavior

HPLC studies on Pazufloxacin mesylate under different stress condition suggested the following degradation behaviour (Table 1).

| No. | condition | Retention time Of drug, min | Peak area μv.s | Mass conc. μg/mL | Degradation of drug % |
|-----|--|--------------------------------|--|---------------------|--------------------------|
| 1 | Untreated Stock solution (500µg/mL) | 9.27 | 58737423.8 | 500 | |
| 2 | Acid hydrolysis | 3.08 5.57 9.71 | 1258159.9 860832.9 54531233.1 | 464.19 | 7.16 % |
| 3 | Base hydrolysis | 9.71 | 46736810.0 | 397.84 | 20.43 % |
| 4 | Oxidation | 2.44 2.74 4.56 9.47 | 122991.0 10376953.8 527692.7 54041807.0 | 460.02 | 7.99 % |
| 5 | Thermal | 9.33 | 53403106.5 | 454.59 | 9.08 % |
| 6 | Photolytic | 9.66 | 55819450.3 | 475.16 | 4.96 % |

Table 1. Percent degradation of pazufloxacin mesylate

The % degradation was calculated by following formula:

Actual initial area of - reduced area of

% degradation = $\frac{\text{Untreated stock solution treated stock solution x 100}}{\frac{100}{100}}$

Actual initial area of untreated stock solution

The chromatogram of untreated stock solution shown in given figure (Figure 2 (a)).

The rate of hydrolysis in acid was fast and significant reduction in peak area, with degradation product was observed in sample.

It was observed that on heating at 80° C in 0.1 M HCl area of drug decreased with respect to time in solution, with an additional peak at 3.08min, 5.57min, this indicate drug is hydrolysed under strong acidic condition. Pazufloxacin mesylate found to be degraded 7.16% in acidic conditions. (Figure 2 (b)).

The drug was found to be susceptible to alkaline hydrolysis. The reaction in 0.1 N NaOH at 80° C was so rapid that 20.43% of the drug was degraded. (Figure 2 (c)).

The drug was found to be highly susceptible to oxidation with 3% hydrogen peroxide at 60° C temperature. It was decomposed to 7.99%. Major degradation products were observed at Rt 2.44min, 2.74min, 4.56min, which indicated that the drug is degraded in oxidative conditions (Figure 2 (d)).

In comparison to acid and alkali hydrolysis the drug was reasonably stable to thermal and photolysis where no degradation was found. (Figure 2 (e) and (f)).

3.2. Establishment of stability-indicating method

Pazufloxacin mesylate is weak acidic in nature so reversed phase chromatography was considered as the best choice. Separation of Pazufloxacin mesylate from its degradation product has been performed on C18 column. The mobile phase was optimised with different ratio of

potassium dihydrogen orthophosphate buffer and methanol. The proportion of methanol in the mobile phase was alter to get good resolution and desired retention time. Increasing the methanol ratio was accompanied by decreased in retention time of different component; however the separation was still achieved. Since pKa of Pazufloxacin mesylate is 6.0 so in the acidic pH probability of drug being in ionised form is more, which in turn has an effect on peak shape and retention time. This statement supported when improved peak shape, tremendous decrease in tailing and reproducible response observed between pH ranges of 4.5 to 5.5. In order to ensure complete separation and high resolution, the chosen ratio was methanol: 50m potassium dihydrogen orthophosphate (pH 4.5) (40:60%, v/v) as a mobile phase. Final pH of the mobile phase was 4.5.

Pazufloxacin mesylate showed maximum absorbance at 249 nm and the average retention time of Pazufloxacin mesylate for 10 replicates was 9.5 ± 0.05 minutes. Construction of calibration curve was performed by transferring the aliquots of Pazufloxacin mesylate stock and working standard solution into a series of 10mL volumetric flask and diluting to volume with mobile phase to obtain solution in concentration range of $5.0\mu g$ ml⁻¹ to $15.0\mu g$ ml⁻¹. $20\mu L$ volumes from each solution were injected in multiple of 5 injections. Chromatographic separation was run under previously mentioned conditions. All determination was performed at ambient temperature. The average peak area obtained for each concentration was plotted versus concentration.



(a)



(b)



Figure 2. (a) Chromatogram of untreated stock solution; (b) acid degradation; (c) base degradation; (d) oxidation degradation; (e) thermal degradation; (f) photolytic degradation

3.3. Validation of method

The method was validated with respect to linearity, precision, accuracy, specificity and robustness. The response for the drug was linear in the studied concentration range ($r^2=0.9998$). The mean ($\pm R.S.D.$) value of slope and correlation coefficient were 111130 and 0.9998, respectively. (Table 2)(Figure 3).

| Linearity and range | Pazufloxacin mesylate |
|---------------------|-----------------------|
| Range (µg/ml) | 5.00 to 15.00 |
| r2 | 0.9998 |
| Slope | 111130 |
| Intercept | 36833 |

Table 2. Linearity and range



Figure 3. Calibration curve of standard solution of pazufloxacin mesylate

The R. S. D. values for intra and inter-day precision were < 3%, thereby indicating that the method was sufficiently precise .The method was found to be specific to drug. The drug peak was free from any co eluting peak. The result indicated that the method was highly precise (Table 3). Good separations were always achieved which suggested that the method was selective for all components under the test. The LOD and LOQ concentrations were found to be $0.05\mu g ml^{-1}$ and $0.2\mu g ml^{-1}$ (Table 4).

| | Precision | | | |
|-------------------------|--|---------------------|------------|-----------|
| Actual Concentration | Actual Concentration (µg/mL)Measured Concentration (µg/mL) ± S.D.; % R.S.D. | | % Recovery | |
| (µg/mL) | | | | |
| | Intra-day | Inter-day | Intra-day | Inter-day |
| 8.00 | 8.029±0.023; 0.290 | 8.022±0.017; 0.232 | 100.37 | 100.28 |
| 10.00 | 10.00±0.0416; 0.416 | 10.016±0.041; 0.418 | 100.00 | 100.16 |
| 12.00 | 12.006±0.114; 0.954 | 12.014±0.065; 0.543 | 100.05 | 100.12 |

Table 3. Precision and recovery data

| SN | LOD | LOQ |
|------|-----------|----------|
| 1 | 6231.8 | 34699.6 |
| 2 | 6156.3 | 32967.8 |
| 3 | 6277.3 | 33696.1 |
| 4 | 6211.6 | 34179.2 |
| 5 | 6218.1 | 34107.9 |
| MEAN | 6219.02 | 33930.12 |
| SD | 43.444183 | 645.4787 |
| RSD | 0.6985696 | 1.902377 |

Table 4. Calibration data for LOD and LOQ

Influence of all small changes in chromatographic conditions such as change in flow rate $(\pm 10\%)$, Organic content in mobile phase $(\pm 2\%)$, wavelength of detection $(\pm 5\%)$, and pH of buffer in mobile phase $(\pm 0.2\%)$, studied to determine the robustness of the method are also in favour (%R. S. D. < 2%) of the developed HPLC method for the analysis of Pazufloxacin Mesylate.

The % R. S. D. of the assay of Pazufloxacin Mesylate during solution stability experiments were within 2%. No significant changes were observed during solution stability. The solution stability data confirms that the sample solutions were stable at least for 7 days.

4. Conclusions

The study shows that the developed HPLC Method is fast, precise, specific, accurate and stability indicating. The stability-indicating method resolved the drug peak and also the peaks of degradation products formed under variety of conditions. After exposure of Pazufloxacin Mesylate to stress conditions, it was concluded that the drug is susceptible to acid; and oxidation, with maximum degradation observed. Therefore this method can be employed for monitoring the stability of Pazufloxacin Mesylate drug substance commercially.

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