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Research Article

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A New Validated *UV* Spectrophotometric Method for Quantitative Determination of Pefloxacin Mesylate in Bulk Form and Developed *In-Situ* Gelling Ocular Formulations

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Abstract The present study was conducted to develop a new simple, rapid, precise, sensitive, eco-friendly *UV*-spectroscopic method for the quantitative determination of Pefloxacin Mesylate in bulk form and prepared *in-situ* gelling ocular formulations. Method was successfully developed in simulated tear fluid pH 7.4 and further validated in accordance with International Conference on Harmonization (ICH) Q2B guidelines. In line to this, it was tested for linearity, accuracy, precision, detection limit, quantification limit, stability testing, Sandell's sensitivity and molar absorptivity. Finally developed method was applied to conduct assay of Pefloxacin Mesylate and recovery study in developed *in-situ* gelling ocular formulations. The absorption maximum of the drug was found to be 272 nm and linearity was observed from 0.5-20 µg/ml with a regression coefficient of 0.999. Validation and statistical results strongly suggested that the developed *UV* spectroscopic method was accurate, precise, sensitive, versatile and stable. It could be a feasible eco-friendly alternate for the rapid analysis of Pefloxacin Mesylate in bulk as well as *in-situ* gelling ocular formulations.

Keywords Pefloxacin Mesylate, *In-Situ* Gelling, UV Spectrophotometric Method, Ocular.

INTRODUCTION

Pefloxacin is a synthetic broad-spectrum third generation fluoroquinolone antibiotic. Chemically pefloxacin is 1-ethyl-6-fluoro-7- (4-methyl- 1-piperazinyl)-4-oxo- 1, 4- dihydro-3-quinolone carboxylic acid and structurally it is analog of norfloxacin [1]. Dihydrate mesylate salt of pefloxacin has been frequently used worldwide in the form of oral tablet, parentral infusion and topical eye drops [2]. It is effective against most gram-negative (*Enterobectar, E. coli, klebsiella* [3], *Pseudomonas aeruginosa* [4], *Acinetobacter spp., Alcaligenes* [1], Pseudomonas spp. including *Xanthomonas maltophilia* [1], *H. influenza* [1], *N. gonorrhoeae* [5] and *G. vaginalis* [6]) and gram-positive bacteria (*S. aureus* [7], *S. epidermidis* [7], *S. pneumonia* [6] and many other mycobecteria [8]).

It inhibits the bacterial enzymes DNA gyrase and topoisomerase IV, which are responsible for transcription and replication of bacterial DNA. DNA gyrase is considered as primary quinolone target for gram-negative bacteria while topoisomerase IV for gram-positive organisms. Systemically PM is prescribed in uncomplicated cystitis in women, uncomplicated gonococcal urethritis in males and for gram-negative bacterial infections in gastrointestinal

system and genitourinary tract [8]. Furthermore it is also used to treat external infections of the eye, such as acute and subacute conjunctivitis [9, 10], bacterial keratitis and kerato-conjunctivitis [11].

Although some methods have been reported for determination of Pefloxacin in bulk, pharmaceutical formulation and biological fluids such as vibrational spectroscopy (VIB) [12], atomic absorption spectroscopy (AAS) [13-14], spectrofluorimetry (SF) [13], capillary electrophoresis (CE) [15], NIR-spectroscopy [16], *UV*-spectroscopy (UV) [17], visible spectroscopy (VS) [18-25], voltammetric study (VL) [24, 26-27], microbiological assay (MA) [28-30], potentiometric titration [31-32], enzyme-linked immunosorbent assay (ELISA) [33] and high performance liquid chromatography (HPLC) [33-39]. All the above methods have their own utility, applications and limitations. Some of analytical methods (AAS, CE, MA, ELISA and HPLC) even have good sensitivity and selectivity but are complex, costly and time consuming and can't be suitable for assay of Pefloxacin in non biological samples. Besides this, in visible spectroscopic and colorimetric methods various dyes and organic solvents frequently used which is not eco-friendly, time consuming and sometimes carcinogenic too.

Hence in this present study we developed a simple, rapid, precise, sensitive, robust *UV*-spectroscopic method for the quantitative determination of Pefloxacin Mesylate (PM) in bulk form and prepared *in-situ* gelling ocular formulations.

EXPERIMENTAL WORK

Instrumentation

UV-visible double beam spectrophotometer, Labindia 3000 model connected to UV Win software with spectral bandwidth of 0.1 nm, and a pair of 1 cm matched quartz cells was used.

Materials

Pefloxacin Mesylate reference standard was purchased from Sigma Aldrich. Simulated tear fluid (STF) pH 7.4 was used for the preparation of stock and further dilutions. Sodium Chloride (Merck), Sodium bicarbonate (Merck), Calcium Chloride (Merck) and distilled water were used for the preparation of STF. All chemical used for analysis were of analytical grade.

In addition to this, Poloxamer 407 (Sigma Aldrich), low viscosity grade Chitosan (Sigma Aldrich), Methocel E4M premium (Colorcon, India ltd), Pluronic F-68 (Sigma Aldrich), Sodium Chloride, Calcium Chloride, Benzalkonium Chloride (Merck) and Sterile Water for Injection were used for the preparation of *in-situ* gelling ocular formulations.

Methods

Preparation of simulated tear fluid of pH 7.4

For the preparation of STF we used previously reported formula [40]. In our study it was prepared by simply dissolving sodium chloride (0.670 g), sodium bicarbonate (0.200 g) and di-hydrated calcium chloride (0.008 g) in to small proportion of distilled water. Resulting solution was sonicated for 10 minutes. Finally weight was adjusted to 100g by using distilled water and pH was confirmed to 7.4.

Preparation of the stock solution

A standard stock solution containing 1mg/ml was prepared by dissolving 50 mg of PM in 50 ml of STF pH 7.4. Resulting solution was sonicated for 10 minutes. From this solution 5 ml was taken and diluted to 50 ml with STF pH 7.4 to get a stock solution containing 100 µg/ml of drug. This stock solution used for further preparation of working standards.

Identification of absorption maxima

The stock solution was diluted to get 18 µg/ml using STF 7.4. Further scanning was made between 200- 400 nm by using *UV* spectrophotometer. While determination of absorption maxima STF was used as a blank and all the reading were taken in triplicate.

Preparation of calibration curve

Twelve working standard of concentration 0.5, 1, 2, 4, 6, 8, 10, to 20 µg/ml were prepared from 100 µg/ml stock solution by proper dilution with STF pH 7.4. Further respective absorbance of above solutions was measured at 272 λ_{max} and a calibration curve was plotted for the determination of linearity and R^2. Triplicate measurements were made to meet minimum statistical requirements.

Preparation of *in-situ* gelling ocular formulations

Total Four *in-situ* forming eye drops containing 0.3 % w/w PM (equivalent in dose to earlier marketed formulation Proflox eye drops of cipla) were prepared by using different proportions of Poloxamer 407, low viscosity grade Chitosan, Methocel E4M (as viscosity imparting agent), calcium chloride (as cross linking agent) and Benzalkonium chloride (0.01 %) in sterile water for injection. All the formulations were sterilized by autoclave at 121°C and 15 lb pressure for 15 minutes.

Assay of Pefloxacin Mesylate in prepared ocular formulations

Weighed amount (5 g) of prepared formulation was diluted to approximately 10 ml using STF in 50 ml volumetric flask. It was further sonicated for 10 minutes and finally volume was made up to 50 ml. Resulting mixture was filtered through Whatman filter paper No.41 and the filtrate was suitably diluted to produce the desired concentration using STF. Appropriate aliquots of PM within the Beer's range were taken and samples were tested in triplicate manner for percentage purity. Further results were statistically analyzed for significant difference among all formulations by applying one way ANOVA post hoc Tukey's multiple comparison test at $P<0.05$ as significance level.

Validation

The above developed method was validated in accordance with International Conference on Harmonization (ICH) Q2B guidelines [41]. It was further tested for linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ) and robustness or stability testing. Sandell's sensitivity and molar absorptivity were also calculated for added information regarding sensitivity of developed method.

Linearity and range: linear relationship was evaluated across the range for developed method. For mathematical expression of linearity correlation coefficient, y-intercept, slope of the regression line was reported [41].

Accuracy: To determine the accuracy of the proposed method, recovery study was carried out by standard addition method. In prepared formulation solution known concentration of PM was added at three different levels (80%, 100% and 120%). All three levels were measured in triplicate and results were reported as percentage recovery and relative standard deviation [41].

Precision: For evaluating precision repeatability and intermediate precision was accessed. Repeatability was tested by 15 (5 different concentrations and each triplicate) determinations covering the linearity range for the developed method. While intermediate precision was evaluated as inter day, intraday and analyst variations and results were reported in terms of standard deviation, relative standard deviation (RSD) [41].

Limit of detection and Limit of quantification: The limits of quantification (LOQ) and limit of detection (LOD) were evaluated based on the standard deviation of the response and the Slope. They were determined using calibration standards. LOD and LOQ were calculated as $3.3\sigma/S$ and $10\sigma/S$ respectively, where S is the slope of the calibration curve and σ is the standard deviation of intercept of calibration curve at detectable limit [41].

Stability testing: Stability study for PM sample was subjected under room temperature. Change in absorbance was observed at all the concentration points of the calibration curve at 1, 3 and 5th day which was prepared using the stock solution. All the measurements were triplicate to meet minimum statistical requirements.

Statistical analysis

All the data for accuracy, precision and stability testing were analyzed statistically by two-way ANOVA followed by Tukey's post hoc multiple comparison tests using statistical software graph pad prism 6. The calculated value $P<0.05$ was considered as level of significance.

RESULTS

The *UV* spectroscopic method for the determination of PM was successfully developed using STF at pH 7.4. The absorption maxima of PM was found to be 272 nm in simulated tear fluid at pH 7.4 as represented in figure 1.

Developed method for PM showed linearity for the concentration range 0.5-20 µg/ml with R^2 value of 0.999 which suggested versatility of the method which is essential for *in vitro* drug release study of formulations. The intercept

on y axis and slope for the regression line was found to be 0.000, 0.083 respectively for the developed method. Goodness to fit was found 0.0137 at 95 % confidence interval which confirmed good linearity.

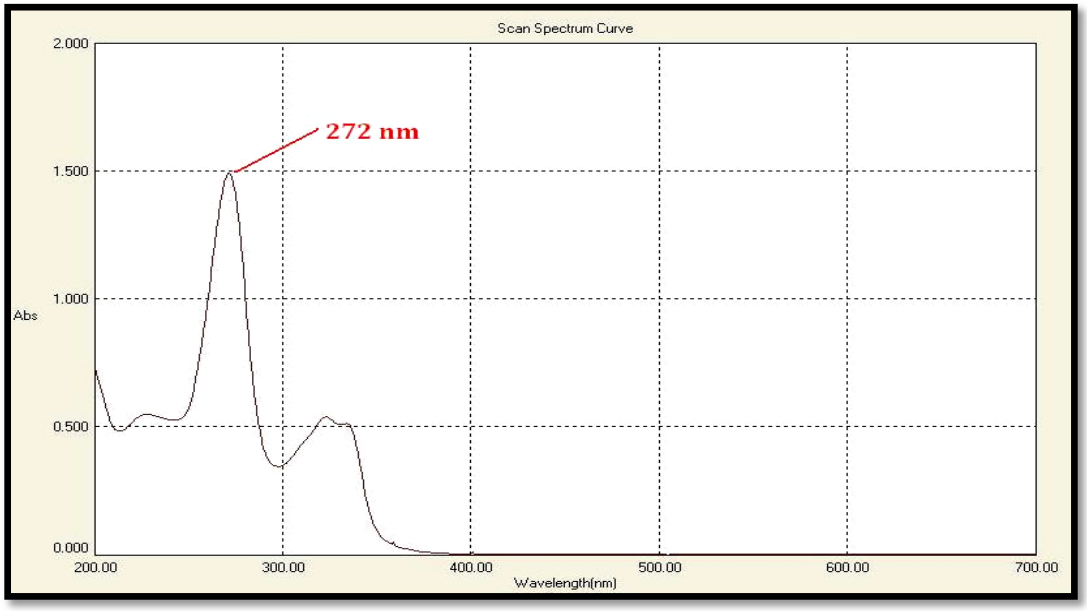

Figure 1: *UV spectra of pefloxacin mesylate (18µg/ml) in simulated tear fluid at 272 nm*

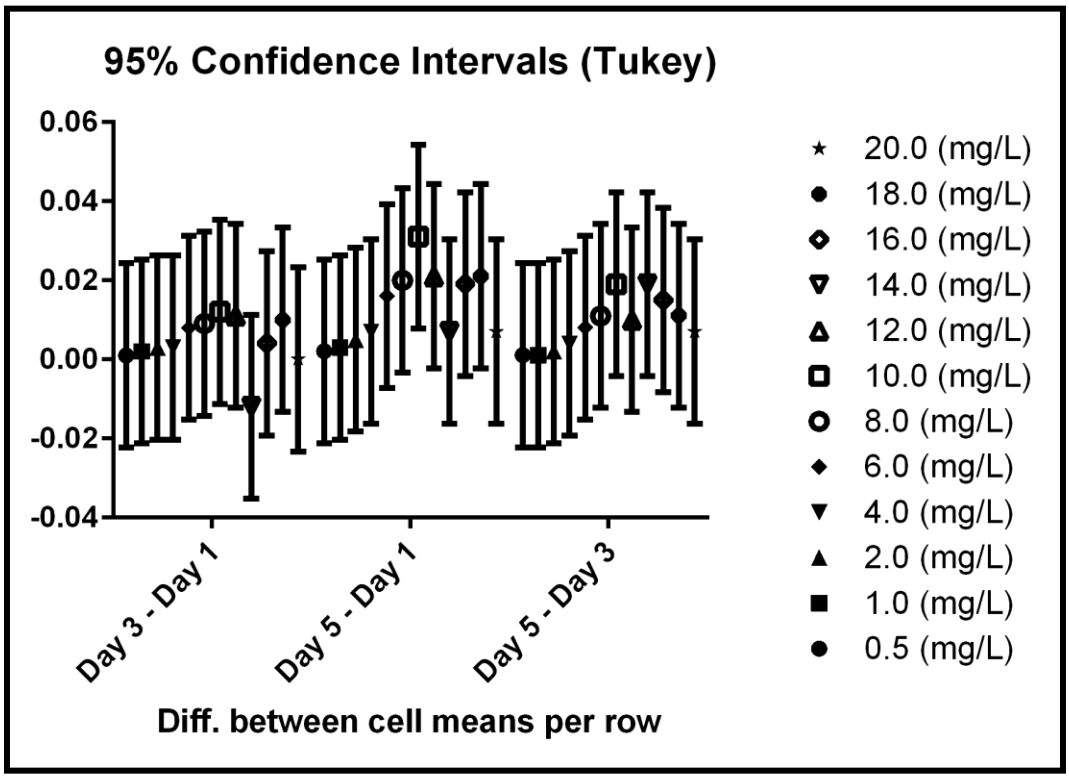

Figure 2: *Stability study comparison by Tukeys confidence interval graph (95 %)*

Assay results for developed formulation in the form of percentage (%) purity were found to be in the range of 98.44-99.88 %. The results of one way ANOVA (with P = 0.370, F= 1.199) suggested that there was no statistically significant differences among means of all the formulations. Furthermore, Tukey's multiple comparison tests also

strongly recommended that no significant difference exist between means of the percentage purity for all prepared formulation with P values 0.599-0.998 at 8 degree of freedom.

Table 1: Results of Accuracy test of the developed method

Formulation Code	PM in solution of formulation (µg)	Standard addition of PM (µg)	Recovery of PM (%)*	RSD (%)
A	9.97	8.00	98.68±0.85	0.85
	9.97	10.00	99.14±0.50	0.50
	9.97	12.00	99.07±0.52	0.53
B	9.98	8.00	98.99±0.55	0.56
	9.98	10.00	99.43±0.28	0.29
	9.98	12.00	98.78±0.89	0.91
C	9.95	8.00	98.79±0.85	0.86
	9.95	10.00	99.08±0.28	0.29
	9.95	12.00	98.86±0.45	0.46
D	9.96	8.00	98.55±0.55	0.56
	9.96	10.00	98.68±0.50	0.51
	9.96	12.00	99.54±0.65	0.65

*Values represented as Mean±SD (n=3)

Table 2: Results of repeatability and intermediate precision test of PM in STF at pH 7.4

Concentration of PM (µg/ml)	Repeatability		Intraday precision		Inter day precision		Analyst variation	
	SD	**RSD (%)**	**SD**	**RSD (%)**	**SD**	**RSD (%)**	**SD**	**RSD (%)**
0.50	0.01	1.14	0.01	1.15	0.01	1.96	0.01	1.17
2.00	0.02	1.07	0.01	0.54	0.03	1.34	0.01	1.09
10.00	0.04	0.36	0.02	0.18	0.19	1.86	0.04	0.40
16.00	0.04	0.23	0.03	0.19	0.12	0.75	0.05	0.31
20.00	0.02	0.09	0.28	1.39	0.05	0.24	0.03	0.15

Accuracy was evaluated by performing recovery study with known standard addition in all four prepared *in-situ* gelling formulations at three different levels. The results (table 1) suggested that percentage (%) recovery was found in between 98.55-99.54 for, all three different levels of standard addition among the all prepared formulations. Further, the result of the two way ANOVA, failed to reject the null hypothesis hence, no significant difference was found in percentage (%) recovery of different formulations at different levels (80, 100, 120 %) of standard addition with P value 0.3477.

The change in the formulations did not affect the accuracy of the method with P value 0.942, and even interaction among levels were found to be non significant hence, change in the level of addition did not affect the accuracy of the method, with P value 0.485.

In addition to this, Tukey's multiple comparison tests (α=0.05) also strongly recommended, no significant difference between means of the percentage recovery from different formulations at, all three levels with adjusted P 0.692 to >0.999. In nutshell, the results suggested that developed method was found to be significant accurate.

Precision was evaluated by performing repeatability and intermediate precision test. Repeatability was tested in five different concentrations among the working range and each concentration point was analyzed in three replicates. Further, intermediate repeatability also tested using intraday (thrice in a day); inter day (I, III and V day) and using analyst (three different) variation. The results (table 2) stated that the relative standard deviation (% RSD) for repeatability test was found to be less than 2 %, which confirmed good precision for the method. The RSD (%) for intra-day and inter day precision was found to be within the official limit (2 %) which suggested the good intra and inter day precision of the method.

For the confirmation of sensitivity of the developed method, we determined LOD, LOQ, Sandell's Sensitivity and Molar Absorptivity (ε). The results suggested that method was sensitive enough as the value of LOD and LOQ were 0.041, 0.125 µg/ml respectively. Sandell's sensitivity was 0.011 µg/cm^2/0.001 absorbance unit for the developed method. In addition to this, molar absorptivity (ε) was found 3.841 x 10^4 L.mol^{-1}.cm^{-1}, which suggested moderate sensitivity of the developed method which favors its suitability towards analysis of PM.

Finally, stability study was performed using PM solution in STF at pH 7.4 at room temperature and was found significant stable (P=0.912, F=0.60) up to 5 days. The results of Tukeys's multiple comparison tests (Figure 2) with confidence interval at 95% was also suggested that, all the mean values of concentration at different days was within the limit.

CONCLUSION

The results revealed that the developed *UV* spectroscopic method for the quantitative determination of PM was simple, rapid, accurate, precise, sensitive and eco-friendly. It can be a viable alternate for the quantitative determination of pefloxacin mesylate in bulk form, *in-situ* gelling ocular formulations and even others formulations in future.

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