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Stability indicating HPLC method for the simultaneous analysis of Gatifloxacin and Loteprednol in eyedrop formulation using design of experiment approach

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Design of experiment (DOE) assisted simple, rapid, precise and accurate stability indicating HPLC method has been developed for simultaneous estimation of Gatifloxacin (GTF) and Loteprednol (LOT) along with their forced degradation products. The developed method has been optimized and developed by using central composite design (CCD) in response surface methodology (RSM). Trails have been undertaken and ratio of phosphate buffer in mobile phase, *p*H of buffer and flow rate are selected as factors. Resolution, tailing factor (GTF) and tailing factor (LOTE) are selected for determining the system response in the process of method optimization. The responses have been optimized using the Derringer's desirability function. The effective separation is achieved on Phenomenex EVO-C18 column (250 mm x 4.6 mm i.d, 5 µm particle size) with mobile composed of 10 mM phosphate buffer, *p*H 3.5 and organic phase composed of mixture of acetonitrile and methanol 60:40 % v/v, the flow rate was 1.0 mL/min, the signals were detected at 267 nm. The developed method was validated for linearity, accuracy, precision, and robustness. The method was applied successfully for stability samples.

Keywords: Central composite design, Gatifloxacin, HPLC, Loteprednol

A combination of Gatifloxacin (GATI) and Loteprednol (LOTE) have been introduced in an eyedrop formulation for treating eye infections with inflammatory conditions. The combination is regarded ideal for treating infected ocular conditions associated with inflammation, GATI is a fourth-generation fluroquinolone antibiotic and LOTE is a steroid useful in reducing inflammation. Gatifloxacin chemically is 1-cyclopropyl-6-fluoro-8-methoxy-7-(3-methylpiperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid[1] (Fig. 1). The antibacterial action of GATI is attributed to its topoisomerase II inhibitory action, the inhibition results in halting replication, transcription, recombination and repair of DNA[2]. Loteprednol (LOTE) chemically is chloromethyl 17-ethoxycarbonyloxy- 11-hydroxy- 10,13- dimethyl-3-oxo- 7,8,9,11,12,14,15, 16-octahydro- 6H-cyclopenta[a] phenanthrene-17-carboxylate[3] (Fig. 1). LOTE is novel glucocorticoid molecule intended for topical application. It is structurally similar compound to prednisolone having low side effects. The molecule consists of a carboxylic ester at 17β-position in its structure with the possibility that upon hydrolysis by enzyme esterases would convert it into acarboxylic acid metabolite of steroid[4]. LOTE is a choice of drug for countering topical inflammatory and allergic conditions of ocular cavity in an eye drop formulation with 0.2 or 0.5% strength[5].

Literature reports number of analytical methods for GATI and LOT in pharmaceuticals. For GATI analytical methods such as UV-visible spectroscopy[6,7], capillary electrophoresis[8], HPLC-fluorometry[9], HPLC stability indicating method for GATI and its impurities[10] and titrimetry[11]. However, the analytical profile of LOTE has not been extensively explored, a UV-Spectrophotometry[12], Stability indicating HPLC[13] and HPTLC[14] method have been reported for this steroidal compound. The combination of GATI and

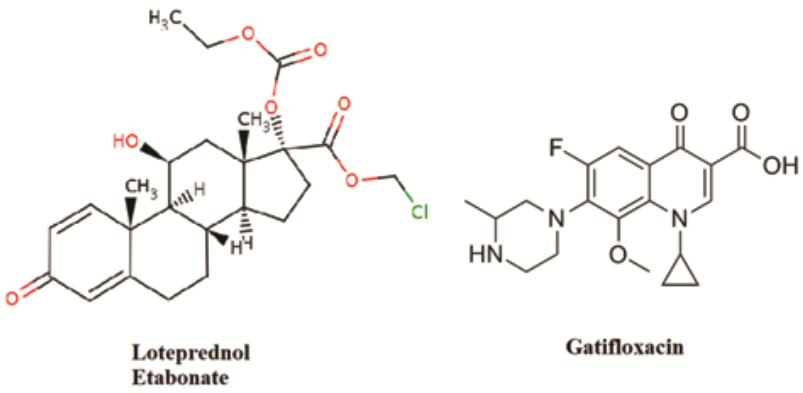

Fig. 1 — Chemical structure of Gatifloxacin and Loteprednol.

LOTE is not official in pharmacopeia, in addition to this there are number of HPLC methods reported for simultaneous estimation of GATI and LOTE.

However, there are reports presenting HPLC analysis of both GATI and LOTE in combined dosage form. A HPLC method involving phosphate buffer 0.02 M: acetonitrile (75:25) using a C18 column, the method was applied for analysis of a laboratory mixture with GATI eluting at 3 and LOTE at 7 min[15]. In another method acetonitrile: phosphate buffer in the ratio of 40: 60 with GATI showing retention time 3.177 and LOTE at 6.977 min was used for analysing both in a laboratory mixture[16]. A HPLC method reported for estimation of GATI and LOTE in an ophthalmic formulation utilized mobile comprising methanol: phosphate buffer: acetonitrile (65:25:10) indicating high amount of organic phase, GATI was eluted at 2.31 and LOTE at 5.36 min, the method analysed both in an eye drop formulation[17].

All the reported HPLC methods were targeted for analysing laboratory mixtures or eye drop formulationswithout demonstrating the ability to separate the degradation products of GATI and LOTE when present in combined dosage form. Also, the methods suffered disadvantage with elution times of target analyte GATI being too early i.e around the void volume peak. In the present study we report a stability indicating HPLC method for analysis of GATI and LOTE in an eye drop formulation employing a systematic central composite design approach.

Experimental Section

Reagents and Chemicals

The working standards Gatifloxacin (99.68%) and Loteprednol Etabonate (99.31%) were supplied by Arrow chem, Mumbai India and were utilized without any further purification steps. HPLC grade solvents Methanol, Acetonitrile and Water were provided by Sigma Aldrich USA. Ortho-phosphoric acid, potassium dihydrogen orthophosphate was bought from Sigma Aldrich USA. The solvents and working standards were filtered using 0.2 µm syringe nylon filter (Sigma Aldrich USA). The commercial formulation containing 3 mg of GATI and 5 mg LOTE was obtained from local market.

Buffer and diluent for GATI and LOTE

Phosphate buffer 10 mM was prepared by dissolving 3.2 g of Potassium Dihydrogen Ortho phosphate in 1000 mL HPLC grade water and the *p*H was adjusted to 3.5 using ortho phosphoric acid. The final mobile phase was submitted to ultra-sonicator for 3 min and filtered through 0.45 µ nylon membrane filter prior to use. The mixture of methanol and water 60:40 % v/v was used as diluent.

Chromatographic conditions and instrumentation

The separation for GATI and LOTE was achieved on a Water's 600 quaternary gradient HPLC system (Waters Milford MA) equipped with 515 autosampler and 996 diode array detectors. Empower Pro-2 was used for system controls, processing and collection of chromatographic data. The effective separation of GATI and LOTE along with their degradation products was achieved on Phenomenex Kinetex EVO C18 column (5 µm particle size, 4.6 mm internal diameter). The effective chromatographic separation of GATI and LOTE was attained by a gradient elution program. The gradient program comprised of 10 mM phosphate buffer *p*H

Table 1 — Variables and levels for HPLC method optimization.

Variables	Levels				
	-1.68179	-1	0	+1	+168179
Ratio of phosphate buffer in gradient program (%)	70	75	80	85	90
Flow rate of mobile phase (mL/min)	0.8	0.9	1.0	1.1	1.2
*p*H of mobile phase	2.5	3.0	3.5	4.0	4.5

3.5 modified with ortho-phosphoric acid (A) and the organic phase composed of mixture of acetonitrile and methanol in 60:40 % v/v (B). The five-step gradient program started with step-1 initially with 75 % of phosphate buffer (*p*H 3.5) and organic phase 25 % for first 8 min. In step2 the volume of buffer changed linearly to 25 % for next 2 minutes, in step3 buffer volume was maintained for 25 % for next 5 min, in step-4 the buffer achieved initial volume of 75 % in next 2 min and was maintained in step-5 for next 3 minutes thus concluding the gradient run. The column oven temperature was set at 30°C whereas the sample cooler temperature was 25°C, the volume of injection was 20µL whereas the signals were monitored 267 nm.

Standard and sample solution

An Accurate quantity 30.0 mg of GATI and 50.0 mg of LOTE was added to 100 mL volumetric flask followed by addition of diluent and was sonicated to solubilise both GATI and LOTE, the diluent was added to make the final volume. The prepared solution was filtered using 0.2 µm filter, some of the first volumes of filtrate was discarded, 5 mL of resultant was transferred to 50 mL volumetric flask, the volume was made up to the mark. The final solution contained 30 µg/mL of GATI and 50 µg/mL of LOTE.

For preparing sample, 1 g of test sample (containing 6 mg of GATI and 10 mg of LOTE) was transferred to 50 mL volumetric flask followed by addition of diluent to make the final volume 50 mL. The final solution was filtered through 0.2 µm filter, first few mL of resultant was discarded and 5 mL from remaining was transferred to 20 mL volumetric flask, the volume was made up to the mark with diluent. The resulting sample solution contained 30 µg/mL of GATI and 50 µg/mL of LOTE.

Design of experimental approach and optimization of HPLC conditions

Design of experiment approach involving central composite design with response surface methodology was opted for systematic optimization of various chromatographic parameters involved in separation of GATI and LOTE along with their degradation products in combination. All the statistics for optimization of chromatographic parameters were performed using Design-Expert software version 13 (stat-Ease USA). For optimization of method parameters, factors selected were volume of phosphate buffer in gradient run (65, 70, 75, 80 and 85 %), second factor was pH of buffer (2.5, 3, 3.5, 4 and 4.5), the third factor was flow rate (0.8, 0.9, 1.0, 1.1 and 1.2 mL/min) (Table 1). The response factors were tailing factor and resolution. For performing and generating the experimental design we used 3 variables at 5 points in 19 experiments (Table 2).

Validation of HPLC method

The developed HPLC method was validated for various parameters in accordance to ICH guidelines.

System suitability

System suitability studies were carried to assess the system performance of the developed HPLC method. The system suitability of the method was evaluated by injecting 6 replicates injections of GATI and LOTE standard solution followed by recording mean peak area, retention time, USP plate count, purity angle, purity threshold and USP tailing

Specificity and selectivity studies

To assure that there were no interferences involved from solution matrix and they were well resolved from target analytes specificity studies were done. Specificity and selectivitywere carried to evaluate the analytes in presence of various components which might be present in the sample preparation matrix, whereas selectivity was done to assure that the peaks of analytes do not have any other components from sample matrix hiding with them. The specificity studies were done by injecting blank as well as placebo under developed HPLC parameters whereas for selectivity peak purity was assessed for recording purity angle and purity threshold values.

Table 2 — Central composite design with three independent variables studied along with the responses observed for separation of GATI and LOTE.

Run	%Phosphate buffer	Flow rate (mL/min)	*p*H	Response factors		
				Resolution	Tailing (GATI)	Tailing (LOTE)
1	1.68179	0	0	3.2	2.6	3.1
2	-1	1	1	3	2.2	2.1
3	0	0	-1.68179	4.1	2.6	2.7
4	0	0	0	4.95	1.2	1.1
5	0	0	0	4.75	1.1	1.2
6	0	1.68179	0	2.95	1.4	1.7
7	1	-1	-1	3.66	3	3.2
8	0	-1.68179	0	3	2.5	2.7
9	-1	-1	-1	2.85	2.6	2.6
10	0	0	1.68179	2.8	2.4	2.5
11	0	0	0	5.1	1.1	1.3
12	-1	1	-1	2.65	2.4	2.3
13	0	0	0	4.95	1.1	1.2
14	1	1	1	3.75	1.9	2.2
15	1	-1	1	3.6	2.1	2.6
16	-1	-1	1	2.95	2.3	2.7
17	-1.68179	0	0	2.25	2	2.5
18	1	1	-1	3.6	2.2	2.1
19	0	0	0	5.1	1.1	1.3

Linearity and range studies

Linearity studies were performed in the range from 25 to 175% of concentration of analytes in target formulation. The dilutions were prepared making serial dilutions from stock solution for obtaining aliquots with concentration of GATI ranging from 7.5 to 52.5 % µg/mL and that of LOTE 12.5 to 87.5 µg/mL. Each of the dilution was injected in triplicate in developed chromatographic parameters, calibration curve was constructed by plotting mean peak area against drug concentration.

Precision studies

The precision power of developed HPLC method was studied by performing system precision and method precision. The system precision was assessed by injecting mix standard solution of GATI and LOTE under optimized chromatographic parameters. After injecting the system precision dilutions mean retention time and peak area were recorded followed by RSD of ten runs. For method precision, dilution of sample (marketed formulation) was carried under optimized chromatographic parameters. About six samples were taken into account and injected followed by recording of mean retention time and peak area.

Accuracy studies

Accuracy of the method was assessed by standard addition method at 3 different levels for GATI and LOTE. The placebo was spiked with standard of known concentration GATI and LOTE at 50 %, 100 % and 150 % levels. About 6 samples for each spike level were analysed. The injections were evaluated for mean % recovery and % RSD.

Limit of detection (LOD) and limit of quantitation (LOQ)

The LOD and LOQ of both the selected analytes GATI and LOTE was done as per the ICH guidelines opting the signal-to-noise ratio method[18]. The study was performed by injecting the dilutions of mix standard solutions of GATI and LOTE into developed chromatographic parameters followed by evaluation of signal-to-noise (S/N) ratio at each concentration.

Storage stability

The stability of analytical dilutions was assessed for both standard as well as sample by storing them at ambient temperature for 24 h. Both, standard as well as samples were analysed at various time points such as 0, 5, 10, 15 and 24 h. The mean peak areas were recorded followed by recording the difference (%) between the areas of fresh runs.

Forced degradation studies

The stability indicating capability of developed HPLC method was assessed by performing forced degradation studies as ICH guidelines Q1AR[18]. The drug substance was submitted to forced degradation by acid hydrolysis, alkaline hydrolysis, thermal degradation and hydrogen peroxide oxidation

studies[19]. The standard drug substances, sample and placebo were submitted to stress conditions such as acid degradation (2N HCl, at 80° C for 2 h), alkaline degradation (1N NaOH, at room temperature for 30 min), Hydrogen peroxide (6% H_2O_2 at 80° C for 3 h) and for thermal degradation heating at 80° C for 3 h. All the forced degradation samples were analysed by the proposed method.

Results and Discussion

Preliminary optimization

In the initial chromatographic development stage inclusion of any single organic phase i.e methanol or acetonitrile was unable to provide efficient resolution with good peak symmetries, hence a mixture of acetonitrile and methanolin the ratio 60:40 % v/v produced peaks with good symmetry and was hence selected for our study ahead. Buffers (Phosphate and acetate) of varied strengths and *p*H were screened, it was observed that phosphate buffers provided reproducible results compared to acetate buffers. Hence, we selected phosphate buffer for our studies ahead. Optimum *p*H is required for achieving effective separation of analytes, literatures suggested acidic range *p*H ranging between 2.5 to 4.5 for effective separation.

Experimental design for optimization of chromatographic conditions

Optimization of final chromatographic conditions needed for separation of GATI and LOTE were achieved by experimental design utilizing central composite design (CCD) approach. The CCD composed of three variables (*p*H of mobile phase, concentration of phosphate buffer and flow rate) at five levels and nineteen experiments (Table 1). The efficiency of developed CCD model and effect of various variables were verified by analysis of variance (ANOVA), f-value and p-value (Table 3). Effect of selected variables were studied on response factors such as purity angle, purity threshold, mean peak area and tailing factor. The three variables and response factors against them are detailed in Table 2. The quadratic modeldeveloped using CCD is presented in Table 3.

The quadratic model developed, looking into the results of ANOVA the statistical description of the model was as follows, the p-value and f-value recorded for the variable resolution were 0.0007 and 80.55, tailing factor (GATI) were 0.0001 and 72.86, for tailing factor (LOTE) the values were 0.0002 and 86.84. It was observed that in all the variables the p-values were less than 0.001 suggesting the correctness of developed quadratic model (Table 3).

The 3D surface plots were generated (Fig. 2), the results (Table 3) suggested that among the selected variablespH and % phosphate buffer in mobile phase were effective in causing alterations in tailing factor and resolution between the peaks of GATI and LOTE. The phosphate buffer concentration and *p*H of buffer in mobile phase were crucial for as alteration in both resulted in improved resolution with peaks having low tailing factors for both GATI and LOTE. The CCD quadratic model fit statistics showed closeness of predicted R^2 values with adjusted R^2 indicating a perfect fit quadratic model acceptable to the present study.

From the regression model using the dependent variables (Tailing factor (GATI), Tailing factor (LOTE) and Resolution) against the two independent variables (keeping the third variable constant) the 3D response surface plots were generated (Fig. 2). A 3D surface plot and contour plot (Fig. 3) shows a relation between each of the dependent variables and the two independent variables. The 3D surface plots clearly indicated that the optimum resolution between the peaks having low tailing values for GATI and LOTE could be achieved at 75 %phosphate buffer having *p*H 3.5, the 3D plots also concluded that flow rate was not a crucial factor in the selected ranges of 0.8 to 1.2 mL/min, hence we selected 1 mL/min as flow rate in present chromatographic conditions.

Table 3 — Effect of variables and the efficiency of central composite design model.

Variables	ANOVA for quadratic model		Fit statistics of quadratic model	
	P-value	*F*-value	Predicted R^2	Adjusted R^2
Resolution	0.0007	80.55	0.7682	0.8511
Tailing factor (GATI)	0.0001	72.86	0.8111	0.8977
Tailing factor (LOTE)	0.0002	86.84	0.8458	0.9339

Note: 1. The *F*-value implies the model is significant, there is only *P*-value/100 % chance that *F*-value this large occur due to noise.
2. The predicted R^2value difference with adjusted R^2 value is less than 0.2 suggesting agreement between predicted and adjusted R^2 values.

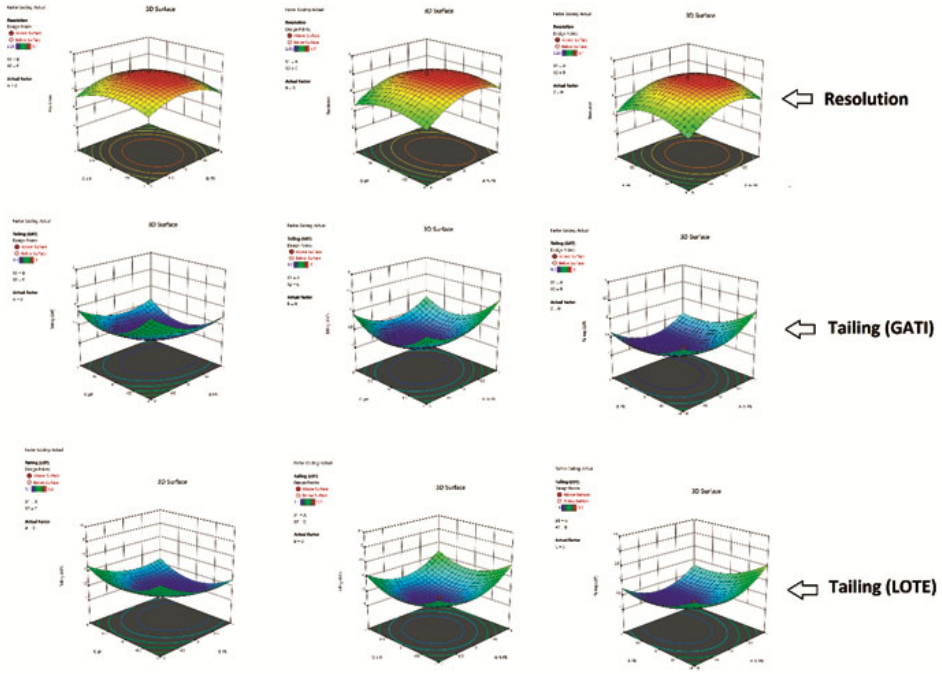

Fig. 2 — Central composite design response surface plots A-C: Effect of % phosphate buffer, flow rate and *p*H of mobile phase on resolution of Gatifloxacin and Loteprednol. D-F: Effect of % phosphate buffer, flow rate and *p*H of mobile phase on tailing factor of Gatifloxacin. G-I: Effect of % phosphate buffer, flow rate and *p*H of mobile phase on tailing factor of Loteprednol.

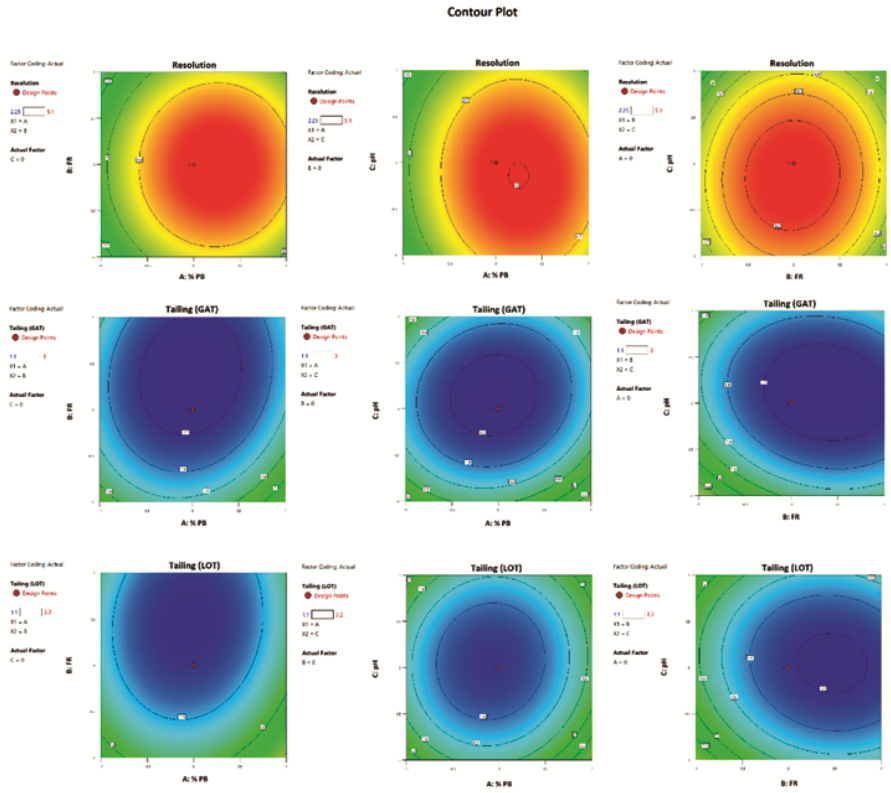

Fig. 3 — Central composite design showing contour plots. The plot shows relation between flow rate, *p*H and % phosphate buffer in separation of Gatifloxacin and Loteprednol.

Table 4 — Final optimized gradient program

Time	Mobile phase A	Mobile phase B	Flow rate (mL/min)
0	75	25	1
8	75	25	1
10	25	75	1
15	25	75	1
17	75	25	1
20	75	25	1

Mobile phase A: Phosphate buffer 10 mM *p*H 3.5, Mobile phase B: Mixed Acetonitrile and Methanol in 60:50 v/v ratio and sonicated to degas.

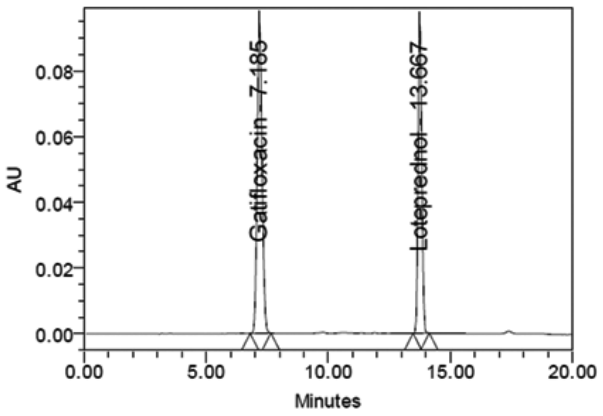

Fig. 4 — Optimized HPLC chromatogram showing separation of Gatifloxacin and Loteprednol under developed HPLC parameters.

The developed quadratic model for separation of GATI and LOTE suggested the optimum chromatographic conditions required for separation of GATI and LOTE with highest resolution having lower tailing factor values. The optimum chromatographic conditions comprised of 75 % phosphate buffer in the step 1 of gradient program having *p*H of 3.5 with flow rate of 1.0 mL/min (Table 4). An, chromatogram showing optimum separation of GATI and LOTE is presented in Fig. 4.

Method validation

The developed HPLC method was validated as per the ICH recommended guidelines [19]. The validation was performed to test whether the method was appropriate for analysing the target molecules GATI and LOTE in combination and in presence of their degradation products.

System suitability test

System suitability was carried to assess the suitability of developed chromatographic parameters in analysing the target molecules. About six replicate injections were made under optimized chromatographic parameters and %RSD of mean peak area was calculated. The outcomes suggested that the %RSD values were less than 2 % (Table 5) suggesting precision and suitability of the method.

Table 5 — Method validation data of developed HPLC method.

Validation parameters	GATI	LOTE
Specificity[#]	No interferences	No interferences
Linearity (correlation coefficient) ^	0.9998	0.9991
Linearity equation^	Y=21993X-1411.8	Y=18528X-4217.8
Repeatability (% Assay) *	100.5	100.02
Repeatability (% RSD) *	0.13	0.80
System precision (% RSD) *	0.143	0.087
Method precision (% RSD) *	1.6	1.1
Accuracy (% RSD) ǂ	0.4	1.7
Accuracy (% Recovery) ǂ	99.8	99.9
LOD (µg/mL)	0.04	0.04
LOQ (µg/mL)	0.132	0.132
Stability-24 h (%) ¤	100.2	99.90

Note:
*Determined by six replicate injections.
#Determined by running placebo and peak purity data.
^ Determined by running seven levels of dilution ranging from 25-175%.
ǂ Determined by injecting 3 levels (50, 100 and 150%)
¤ Compared to fresh solution

Specificity of method

The specificity of method was studied for assuring that the eluted peaks of target molecules do not encounter any interferences. The method specificity was assessed by 2 approaches, in the first approach placebo and the blank were injected under the optimized chromatographic conditions. It was found that no interferences were seen at the retention times of target analytes GATI and LOTE (Data not presented). In the other method for determining the specificity, peak purity study was done with the photo diode array detector, the purity angle and purity threshold of both GATI and LOTE were studied and found that the purity angle of GATI and LOTE were less than the purity threshold (Table 5).

Linearity and range studies

The developed HPLC method was tested for linearity and range studies from 25 to 175 % of the target labelled concentration of GATI and LOTE in formulation. The results indicated correlation coefficient values greater than 0.99 suggesting linearity of method. The equation of regression and

Table 6 — Results of stress degradation of Gatifloxacin and Loteprednol

Gatifloxacin Stress condition	Peak area ± %RSD	Purity angle ± % RSD	Purity threshold ± RSD	% Degradation
Unstressed	1428022 ± 2.2	0.039 ± 1.2	0.220 ± 1.20	NA
Acid Degradation (5N HCl, 80°C, 3h heating)	1419544 ± 2.1	0.030 ± 2.2	0.221 ± 1.30	0.3
Alkali Degradation (5N NaOH, 80°C, 3h heating)	1419589 ± 1.6	0.031 ±1.85	0.221 ± 1.40	0.3
Peroxide Degradation (6 % H_2O_2, 80°C, 3h heating)	1329499 ± 2.1	0.027 ± 1.45	0.218 ± 1.45	6.8
Loteprednol				
Unstressed	1952064 ± 2.4	0.141 ± 1.5	0.214 ± 1.35	NA
Acid Degradation (5N HCl, 80°C, 3h heating)	1408430 ± 1.8	0.271 ± 2.1	0.210 ± 1.80	27.9
Alkali Degradation (5N NaOH, 80°C, 3h heating)	1740676 ± 1.9	0.175 ±1.35	0.213 ± 1.50	10.8
Peroxide Degradation (6 % H_2O_2, 80°C, 3h heating)	1790234 ± 2.4	0.173 ± 1.85	0.210 ± 1.65	8.3

the values of correlation coefficient are depicted in Table 5.

Precision studies

The precision power of developed chromatographic method was assessed, the results of system and method precision are depicted in Table 5. The % RSD values of peak areas of GATI and LOTE were near to 1 (less than 2.5) indicating high degree of method and system precision for developed HPLC method.

Accuracy studies

Method accuracy was assessed by standard addition method. The sample was spiked with standard at 3 different concentrations ranging from 50, 100 and 150% of the labelled claim of GATI and LOTE in target formulation. The mean percent recoveries were 99.8 % for GATI and 99.9 % for LOTE. The outcomes clearly suggested accuracy of method also confirming no interferences from the sample matrix of formulation (Table 5).

LOD and LOQ

Limit of detection (LOD) and limit of quantification (LOQ) was carried out by signal/noise (S/N) ratio method. The LOD the sound/noise ratio of at least 3 and for LOQ 10 was confirmed, the values were as per requirements of ICH guidelines. The results are depicted in Table 5.

Stability studies

For establishing stability studies solution stability of sample was carried out. The results sample solution stability showed no notable variations in % recoveries upon storing the sample solutions at room temperature conditions for 24 h, the results are depicted in Table 6.

Forced degradation studies

The stability indicating power of developed HPLC method was assessed by submitting the samples to various stress conditions such as acidic and alkali hydrolysis and peroxide stress. The degraded samples were analysed by developed HPLC method, the outcomes suggested major degradation of LOTE under all the three stress conditions. It was noted that GATI was relatively stable under all the three stresses (Fig. 5). The chromatograms (Fig. 5) show GATI and LOTE under various stress conditions, the chromatograms show well separated peaks of degradation products. The peak purity plots suggest no interferences or hidden peaks thus confirming peaks to be pure. The developed HPLC method was specific and was suitable for separating the degradation products of GATI and LOTE in combination, the results are presented in Table 6.

Literature reports number of reports presenting HPLC analysis of GATI and LOTE in combined dosage forms[15-17]. However, no reports presenting HPLC method for GATI and LOTE in combined dosage form demonstrated the ability to separate their degradation products, present study reports a stability indicating LC method. The basis of selecting initial chromatographic conditions was based upon reported HPLC methods for GATI and LOTE individually or in combinations.A stability indicating HPLC method for GATI, the mobile phase composed of acetonitrile-methanol and ammonium acetate using a C18 column[21]. Razzaq *et al*.[22] reported an HPLC method for GATI along with Dexamethasone using an C8 column opting Phosphate buffer pH 3 and methanol as composition of mobile phase. A HPLC stability indicating method analysing GATI and flurbiprofen involved phosphate buffer *p*H 3 and methanol as

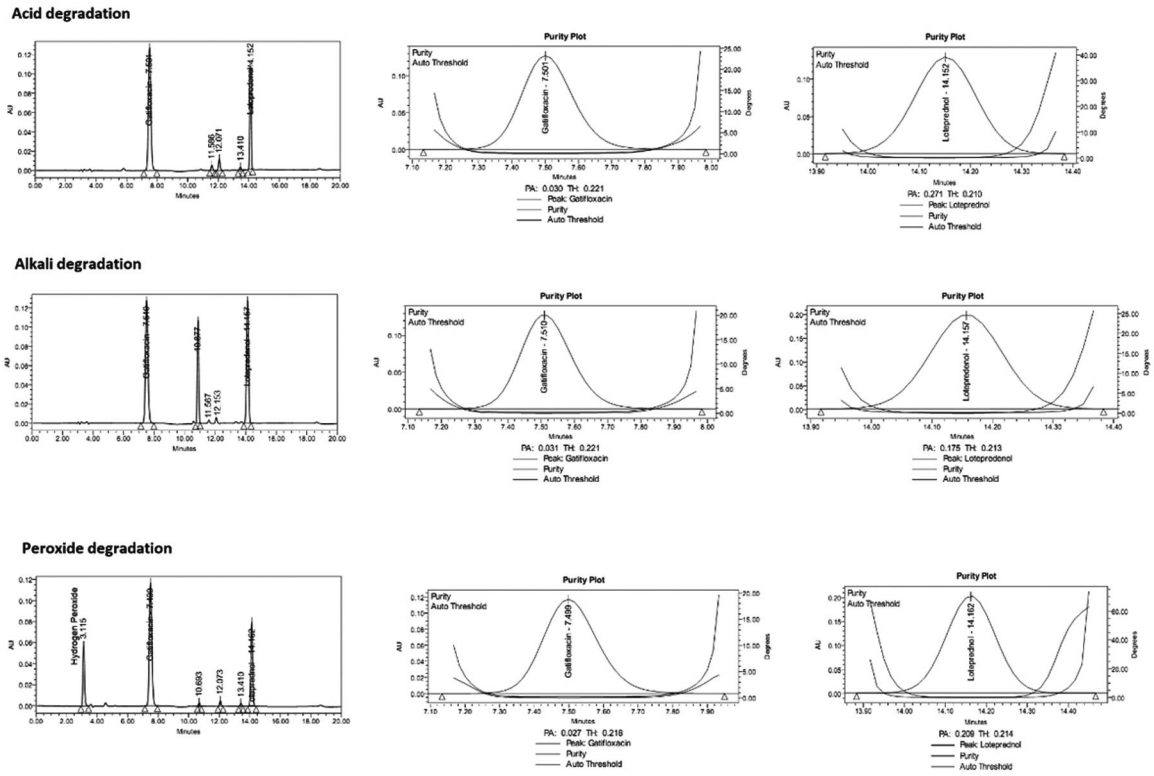

Fig. 5 — Forced degradation studies showing degradation of Gatifloxacin and Loteprednol under various stress conditions such as acid, alkaline and peroxide.

composition of mobile phase using a C8 column[23]. Major of the methods utilized C8 column and phosphate buffer of *p*H 3 as part of chromatographic conditions for analysing GATI. In addition to all of these studies our earlier experiments reporting analysis of Ciprofloxacin along with Loteprednol in presence of their degradation products[24] were also helpful in selecting the initial chromatographic conditions. We selected C18 as stationary phase looking into our applicability for separating both GATI and LOTE along with their degradation products. We started our study utilizing phosphate buffer in acidic range along on a C18 column. During initial study it was experienced that mixture of acetonitrile with methanol resulted in peaks of proper symmetry hence mixture of methanol and acetonitrile in the ratio 60:40 was selected as mobile phase component for study ahead. Also, the earlier studies suggested gradient approach instead of isocratic mode as choice because the target was to elute all the degradation products of GATI and LOTE when present in a combined dosage form.

Experimental design is a systematic approach in defining and optimizing various chromatographic parameters in analytical method development. The approach reduces the number of trials and thus preventing un-necessary chromatographic runs thus saving resources and valuable time. Number of reports have emerged recently which confirm the usefulness of design of experiment approach in developing complex analytical methods specially which involve critical variables such as gradient approach for achieving separation of target analytes. In the present study we applied the design of experiment approach using response surface methodology. Looking into output of initial runs ratio of phosphate buffer in mobile phase in the initial step of gradient run, its *p*H and flow rate of mobile phase were identified as controlling variables, whereas the response factors were the tailing factor for both GATI and LOTE and their resolution. The central composite design was prepared at five levels which as -1.68179, -1, 0, +1, +1 and 1.68179, a total of 19 experiments were selected in the model. The first variable i.e the ratio of phosphate buffer in the initial step of gradient program was defined at 5 levels i.e 65, 70, 75, 80 and 85 %, the second factor *p*H of buffer the was 2.5, 3, 3.5, 4 and 4.5, the third factor was flow rate i.e 0.8,

0.9, 1.0, 1.1 and 1.2 mL/min. A study reported earlier involved experimental design approach for optimization of chromatographic variables in the process of analysing pharmaceuticals, the authors achieved successful separation of analytes by varying the three variables at 5 levels in a gradient program[25]. In the present study we assessed the effect of selected variables in a gradient program and the efficiency of experimental design model. The values of ANOVA in the quadratic model showed p-value and f-value recorded for the variable resolution were 0.0007 and 80.55, tailing factor (GATI) were 0.0001 and 72.86, for tailing factor (LOTE) the values were 0.0002 and 86.84. The fit statistics of the developed quadratic model are in good agreement between the predicted and adjusted R^2 values. The parameter resolution demonstrated predicted and adjusted R^2 values as0.7682 and 0.8511, for tailing factor (GATI) the values were 0.8111 and 0.8977, for tailing factor LOTE the values were 0.8458 and 0.9339. The values clearly indicated good agreement between the predicted and adjusted R^2 values.

The effective separation of both GATI and LOTE along with their degradation products was achieved on Phenomenex C18 EVO column opting a five-step gradient program. The phosphate buffer (*p*H 3.5) concentration after optimization was as follows, step-1 75% for first 8 minutes, step-2 25% for next 2 min and maintained for next 5 min (step-3), in step-4 the buffer achieved concentration of 75 % in 2 minutes and was maintained in step-5 for 3 min and thus ending with total run time of 20 min. The organic phase composed of mixture of acetonitrile and methanol in the ratio 60:40 %v/v. The flow rate was 1 mL/min, column temperature was maintained at 30°C and that of sample cooler was 25° C, the volume of injection was 20µl and the signals were monitored 267 nm. The method successfully separated GATI and LOTE in presence of their degradation products which was evidenced by peak purity data suggesting no hidden peaks coinciding with the peaks of GATI and LOTE. The method suggested major degradation of LOTE under all the three stressors selected for the study (alkali, acid and peroxide). The outcomes also suggested that GATI was relatively stable under all the three stressors.

The developed stability indicating HPLC method for GATI and LOTE was validated as per ICH guidelines. The selectivity studies were carried out by performing peak purity data by observing the purity angle and purity threshold values which suggested the peaks to be pure and hence the method to be selective[18,26]. Also, method linearity, specificity, precision, repeatability, accuracy, limit of detection and limit of quantification was carried. The method was found to be linear as the values of R^2 were near to 1 i.e0.9998 for GATI and 0.9991 for LOTE. The method showed good repeatability as seen %RSD values of 0.13 and 0.80 for GATI and LOTE respectively. The method was accurate as it showed excellent recovery mean values of 99.8 % and 99.9 % for GATI and LOTE respectively. The method was precise with both system and method precision experiments, the %RSD values for method precision were 1.6 % and 1.1 %, for system precision were 0.143 % and 0.087 % for GATI and LOTE respectively. The method was sensitive which was observed from the LOD and LOQ values which were 0.04 and 0.04, 0.132 and 0.132 for GATI and LOTE respectively

Conclusion

Present study reports a simple, specific and accurate high performance liquid chromatographic method involved with diode array detection. The method which was optimized by a systematic experimental design approach was successfully applied for analysing the stability samples and was able to separate the degradation products of GATI and LOTE in an eye drop formulation. The experimental design approach offered advantage over the trial-and-error methodology by minimizing the unwanted trials and thus reducing the overall cost of the method. The method was validated as per ICH guidelines and was successfully applied for stability samples.

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Conflict of interest

Authors declare no conflict of interest.

References

1 Aljuffali I A, Kalam M A, Sultana Y, Imran A & Alshamsan A, *Saudi Pharm J*, 23 (2015) 85.
2 Diekema D J, Jones R N & Rolston K V, *Diagn Microbiol Infect Dis*, 34 (1999) 37.
3 Han Y K & Segall A I, *J Chromatogr Sci*, 53 (2015) 761.

4 Williams J R, Moore R H, Li R & Weeks C M, *J Org Chemistry*, 45 (1980) 2324.
5 Martindale, *The Complete Drug reference*, 32th Edn. Pharmaceutical Press, London, (1999) 1045.
6 Amin A S, El-Fetouh G A A, El-Sheikh R & Zahran F, *Spectrochim Acta Part A*, 67 (2007) 1306.
7 Fetouh G A A, El-Sheikh R & Amin A S, *Chem Pharm Bull*, 56 (2008) 34.
8 Flurer C L, *Electrophoresis*, 18 (1997) 2427.
9 Tasso L& Dalla C T, *J Pharm Biomed Anal*, 44 (2007) 205.
10 Motwani S K, Khar R K, Ahmad F J, Chopra S, Kohli K, Talegaonkar S& Iqbal Z, *Anal Chim Acta*, 576 (2006) 253.
11 Belal F, Al-Majed A A & Al-Obaid A M, *Talanta*, 50 (1999) 765.
12 Solanki R, Patel N, Patel M & Kothari C, *NIRMA Univ J Pharm Sci*, 5 (2018) 27.
13 Han Y K & Segall A I, *J Chromatogr Sci*, 53 (2015) 761.
14 Premakumari K B & Murugan V, *Am J Pharm Tech Res*, 5 (2015) 565.
15 Saxena V & Singh A, *Int J Sci Res*, 5 (2013) 252.
16 Joshi K A, Pradhan P K, Dey S D & Upadhyay U M, *Asian J Pharma Res*, 3 (2013) 9.
17 Patel Ankit B & Patel Dipti B, *Asian J Res Chem*, 6 (2013) 393.
18 *International Conference on Harmonization* (ICH); *Validation of analytical procedures: Text and methodology*. Q2 (R1), Geneva, Switzerland, (2005).
19 Bakshi M & Singh S, *J Pharm Biomed Anal*, 28 (2002) 1011.
20 *International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use. Validation of Analytical Procedures: Text and Methodology*, Q2 (R1), Geneva, Switzerland, (2005).
21 Grace A C, Prabha T, Jagadeeswaran M, Srinivasan K & Sivakumar T, *Int J Pharma Pharm Sci*, 11 (2019) 1814.
22 Razzaq S N, Ashfaq M, Khan I U, Mariam I, Razzaq S S, Mustafa G, Zubair M, Braz *J Pharm Sci*, 53 (2017) 15177.
23 Khan I U, Razzaq S N, Mariam I, Ashfaq M & Razzaq S S, *Quím Nova*, 37 (2014) 349.
24 Dewani A P &Vekariya H J, *J Chromatogr Sci*, 16 (2022) 1.
25 Kashyap R & Srinivasa U, *Int J Drug Develop Res*, 6 (2014) 99.
26 Negi V, Chander V, Singh R, Sharma B, Singh P & Upadhaya K, *Indian J Chem Technol*, 24 (2017) 441.