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VALIDATED SPECTROPHOTOMETRIC AND STABILITY INDICATING RP-HPLC METHODS FOR THE SIMULTANEOUS ESTIMATION OF GATIFLOXACIN AND DEXAMETHASONE IN OPHTHALMIC DOSAGE FORM

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ABSTRACT

The main objective of the present work is to develop simple, precise, accurate and reproducible UV-spectrophotometric and stability indicating RP-HPLC methods for simultaneous estimation of gatifloxacin (GAT) and dexamethasone (DEX) in ophthalmic dosage form. Dual wavelength spectrophotometric method, which involves solving of simultaneous equations based on the measurement of absorbances at 279 nm and 239 nm, which are the absorption maxima (λmax) of GAT and DEX respectively. The RP-HPLC analysis is carried out on Shiseido C18 column (250 mm × 4.6 mm I. D.), using 0.1% orthophosphoric acid in water and acetonitrile in the ratio of (50:50 v/v) as the mobile phase with a flow rate of 1.0 mL/min. The detection was carried out at a wavelength of 241 nm. The retention times were found to be 2.124 ± 0.5 min and 4.578 ± 0.5 min for GAT and DEX respectively. The linearity range was found to be 6-18 µg/mL and 2-6 µg/mL for GAT and DEX, respectively by UV method and 15-75 µg/mL and 5-25 µg/mL for GAT and DEX respectively by HPLC method. The percentage recoveries of both the drugs GAT and DEX from the ophthalmic form were 99.46% and 98.71%, respectively by UV method and 99.65% and 99.16%, respectively by HPLC method. The correlation coefficients of both the drugs were found to be more than 0.999 by two methods. Other parameters like ruggedness, robustness were well within the acceptance criteria. Both UV-spectrophotometric and stability indicating RP-HPLC methods were found to be accurate, rapid, precise and simple. These methods can be used for the simultaneous estimation of GAT and DEX in bulk and in ophthalmic dosage form.

Key words: Gatifloxacin, Dexamethasone, Simultaneous equation, Validation, RP-HPLC.

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INTRODUCTION

Gatifloxacin (GAT) (Fig. 1) is chemically 1-cyclopropyl-6-fluoro-8-methoxy-7-(3-methylpiperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid. It is an antibiotic of the fourth-generation fluoroquinolone family, which inhibits the bacterial enzymes DNA gyrase and topoisomerase IV. It is mainly used to treat respiratory tract infections[1].

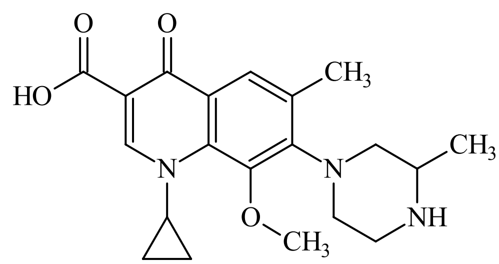


Fig. 1: Chemical structure of Gatifloxacin

Dexamethasone (DEX) (Fig. 2) is chemically 9-fluoro-11β, 17, 21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione. Anti-inflammatory actions of DEX are thought to involve phospholipase A2 inhibitory proteins, lipocortins, which control the biosynthesis of potent mediators of inflammation such as prostaglandins and leukotrienes. It is mainly used to treat many different inflammatory conditions such as allergic disorders, skin conditions, ulcerative colitis, arthritis, lupus, psoriasis, or breathing disorders[2,3].

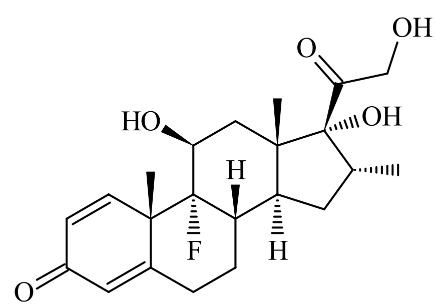


Fig. 2: Chemical structure of Dexamethasone

Detailed literature survey revealed analytical methods like spectrophotometric[4-9], HPTLC[10,11] and HPLC[12-20] are available for the estimation of these drugs individually or in combination with other drugs. But very few methods[21,22] are available for the simultaneous estimation of these drugs. Hence, we tried to develop new and simple spectrophotometric and RP-HPLC methods for the simultaneous estimation of these drugs. The developed methods were validated as per the guidelines of ICH[23]. To establish stability indicating[24] natures of the RP-HPLC method, forced degradation of drug substances was performed under various stress conditions.

EXPERIMENTAL

Instrumentation and analytical conditions

The UV method was performed on a double-beam LABINDIA UV-visible spectrophotometer 3092, with spectral bandwidth of 2 nm. Wavelength accuracy 0.5 nm and a pair of 1 cm matched quartz cells were used to measure absorbance of solution. RP-HPLC method was performed on the Shimadzu HPLC system consisting of binary gradient pump and UV detector (LC-20AD) was employed for analysis and rheodyne injector with 20 µL fixed loop was used for the present study.

Chemicals and reagents

GAT and DEX, working standards were procured from Yarrow Chemicals Ltd., Mumbai. Commercially available ENGATT DX Eye drops were purchased from the local pharmacy. HPLC grade acetonitrile and methanol were purchased from Merck Specialties Pvt. Ltd., Mumbai. HPLC grade water was purchased from Thermo Fisher Scientifics Ltd., Mumbai. Orthophosphoric acid, hydrochloric acid, sodium hydroxide pellets purified and hydrogen peroxide 30% of AR grade were procured from Merck Specialties Pvt. Ltd., Mumbai.

Preparation of solutions

Preparation of standard solutions

UV method

Standard stock solution of GAT and DEX were prepared by transferring accurately weighed GAT (10 mg) and DEX (10 mg) to a 100 mL volumetric flask separately, dissolved and diluted to a mark with the solvent consisting of acetonitrile:water in the ratio of 50:50 v/v, to obtain a standard solution of GAT (100 µg/mL) and DEX (100 µg/mL). From these solutions, standard stock solutions were prepared in 10 mL volumetric flasks and made up the volume with the same solvent, to get the concentration of 12 µg/mL of GAT and 4 µg/mL of DEX.

HPLC method

Standard stock solution of GAT and DEX were prepared by transferring accurately weighed GAT (10 mg) and DEX (10 mg) to a 100 mL volumetric flask separately, dissolved and diluted to a mark with the solvent consisting of acetonitrile:water in the ratio of 50:50 v/v, to obtain a standard solution of GAT (100 µg/mL) and DEX (100 µg/mL). From these solutions, standard stock solutions were prepared in 10 mL volumetric flasks and made up the volume of the mobile phase to get the concentration of 45 µg/mL of GAT and 15 µg/mL of DEX.

Preparation of the sample solutions

UV method

1 mL of the test sample (ENGATT DX Eye drops) contains 0.3 w/v of GAT and 0.1% w/v of DEX as per the labeled claim. 1 mL of the formulation was taken into 100 mL volumetric flask and diluted up to the mark with the solvent consisting of acetonitrile:water in the ratio of 50:50 v/v, to obtain a concentration of 30 µg/mL of GAT and 10 µg/mL of DEX. 4 mL of the above solution was taken in 10 mL volumetric flasks and diluted to 10 mL with the same solvent to obtain a final concentration of 12 µg/mL of GAT and 4 µg/mL of DEX.

HPLC method

1 mL of the test sample (ENGATT DX Eye drops) contains 0.3 w/v of GAT and 0.1% w/v of DEX as per the labeled claim. 1 mL of the formulation was taken into 10 mL volumetric flask and diluted up to the mark with the solvent consisting of acetinitrile:water in the ratio of 50:50 v/v, to obtain a concentration of 300 µg/mL of GAT and 100 µg/mL of DEX. 1.5 mL of the above solution were taken in 10 mL volumetric flasks and diluted to 10 mL with the same solvent to obtain a final concentration of 45 µg/mL of GAT and 15 µg/mL of DEX.

Optimized analytical methods

UV method

The method is based upon determination of GAT at 279 nm and DEX at 239 nm. The method employed was simultaneous equation method for the determination of concentration of drugs. Acetonitrile : water in the ratio of 50:50 v/v was used as solvent. The absorption spectrum of GAT and DEX in acetonitrile:water (50:50 v/v) is shown in Fig. 3.

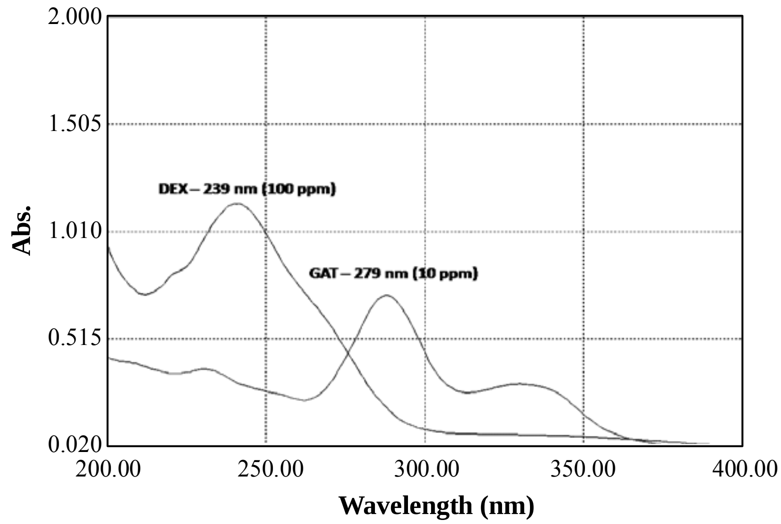


Fig. 3: Overlain spectrum of GAT and DEX

HPLC method

Shiseido C18 column (250 mm × 4.6 mm I.D.) was used as stationary phase. GAT and DEX were eluted isocratically with a flow rate of 1.0 mL/min using a mobile phase consisting of 0.1% orthophosphoric acid in water and acetonitrile in a proportion of 50:50 v/v, respectively. The wavelength of the UV detector was set at 241 nm. The prepared mobile phase was filtered through 0.45 µm membrane filter (Millipore) and sonicated before use. A representative chromatogram was presented in Fig. 4.

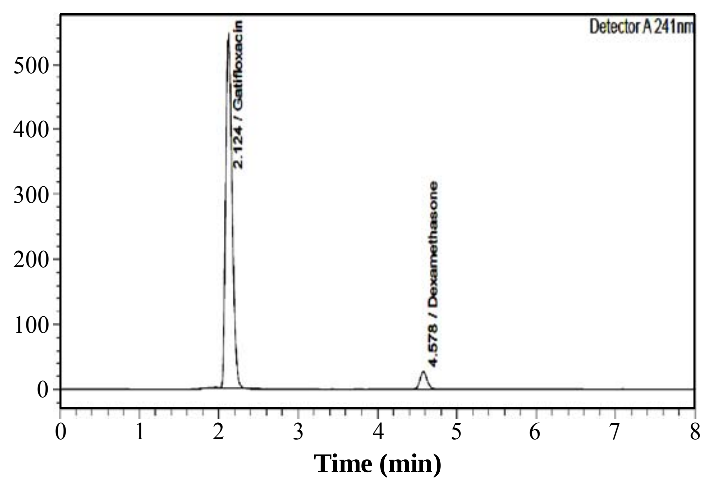


Fig. 4: Chromatogram showing well resolved peaks of GAT and DEX

Method validation

The developed methods were validated according to International Conference on Harmonization guidelines for validation of analytical procedures.

Specificity

It is the ability to assess unequivocally the analyte in the presence of impurities, degradants and matrix. To determine this, 20 µL of blank, standard and sample solutions were injected separately in triplicate and respective chromatograms were recorded under the optimized conditions.

Linearity

The calibration curves for UV method were obtained with concentrations of the standard solutions of 6-18 µg/mL and 2-6 µg/mL for GAT and DEX respectively and for RP-HPLC method 15-75 µg/mL and 5-25 µg/mL for GAT and DEX, respectively. The solutions were prepared in triplicate. Linearity was evaluated by regression analysis, which was calculated by the least square regression method.

Accuracy

To check the degree of accuracy of UV and RP-HPLC method, recovery studies were performed in triplicate by the standard addition method at 80%, 100% and 120% levels.

Precision

Precision of UV and RP-HPLC methods were checked by analyzing the samples at different time intervals of the same day (intra-day precision) as well as on different days (inter-day precision).

Limit of detection and limit of quantitation

Limit of detection (LOD) and limit of quantitation (LOQ) of UV and RP-HPLC methods were calculated by using the values of slopes and intercepts of the calibration curves for both the drugs.

Robustness

Robustness for RP-HPLC method was determined by analysis of samples under deliberately changed chromatographic conditions. The flow rate of the mobile phase was changed from 0.9 mL/min to 1.1 mL/min. The ratio of the organic phase was changed by 5%, i.e., 45%, 50%, 55% of acetonitrile. The effect of retention time and peak parameter was studied.

Assay of ophthalmic formulation

20 µL of each standard and sample solution were injected and from the peak areas of GAT and DEX amount of each drug in samples was computed.

Stability studies

Degradation studies were performed in sample solutions containing 45 µg/mL of GAT and 15 µg/mL of DEX.

Acidic degradation

1 mL of 0.1M, 0.5M, 1M and 2M HCl were added individually to the final drug solution in different volumetric flasks and they were refluxed for 1 hr. at 60°C. After 1 hr. these solutions were injected under optimized chromatographic conditions.

Alkaline degradation

1 mL of 0.1M, 0.5M, 1M and 2M NaOH were added individually to the final drug solution in different volumetric flasks and they were refluxed for 1 hr. at 60°C. After 1 hr. these solutions were injected under optimized chromatographic conditions.

Oxidative degradation

1 mL of 1%, 3%, 5% and 10% H_2O_2 were added individually to the final drug solution in different volumetric flasks and they were refluxed for 1 hr. at 60°C. After 1 hr. these solutions were injected under optimized chromatographic conditions.

Photolytic degradation

The final drug solution was kept at room temperature and exposed to sunlight for 8 hrs. After 8 hrs, this solution was injected under optimized chromatographic conditions.

Thermal degradation

The final drug solution was kept at a temperature of 60°C for 6 hrs. After 6 hrs. This solution was injected under optimized chromatographic conditions.

Neutral degradation

The final drug concentration is refluxed for 1 hr. at 60°C. After 1 hr. this solution was injected under optimized chromatographic conditions.

The stress degradation study was conducted on the 1st day, 3rd day and the 5th day for the above mentioned solutions and the degradation was studied.

RESULTS AND DISCUSSION

System suitability

Twenty micro liters of working standard solution was prepared and injected into the system under optimized chromatographic conditions. Chromatograms were recorded and studied for different system suitability parameters like tailing factor, theoretical plates and resolution were studied. Six different working standard solutions were injected to study this parameter and all the suitability parameters were found to be within the limits. The system suitability parameters were shown in Table 1.

Table 1: RP-HPLC System suitability parameters

Parameter	Observation*	
	GAT	DEX
Retention time (min.)	2.124	4.578
No. of theoretical plates	3080	10780
Tailing factor	1.192	1.090

*Average of six readings

Specificity

The UV spectra and HPLC chromatograms were recorded for blank and sample under optimized analytical conditions, compared them with that of standard solution and found no additional peaks. The two peaks were completely separated in HPLC chromatogram and the resolution was found to be more than 2. Even in presence of excipients of the sample no interfering peaks were found in UV spectra or HPLC chromatogram.

Linearity

For UV method, calibration curves were constructed in the concentration range of 6-18 µg/mL and 2-6 µg/mL for GAT and DEX, respectively. Beer's law obeyed over this concentration range, for HPLC method, the calibration curves for GAT and DEX were constructed in the concentration range of 15-75 µg/mL and 5-25 µg/mL of GAT and DEX, respectively and the correlation coefficient for both the drugs was found to be nearer to 1 (Table 2).

Table 2: Linearity values of GAT and DEX

Method	Parameter	GAT	DEX
UV	Regression equation	y = 0.042x - 0.001	y = 0.016x - 0.001
	Linearity (µg/mL)	6-18	2-6
	Correlation coefficient	0.997	0.998
HPLC	Regression equation	y = 69694x + 20915	11452x - 182.2
	Linearity (µg/mL)	15-75	5-25
	Correlation coefficient	0.999	0.999

Accuracy

The accuracy for proposed methods were determined for both the methods, recovery studies were performed in mentioned levels and recorded (Table 3), obtained results were found to be within the limits of 98-102%, indicating an agreement between the true value and found value.

Table 3: Recovery values of GAT and DEX

UV method						
Drug	**Recovery**			**% RSD**		
	80%	**100%**	**120%**	**80%**	**100%**	**120%**
GAT	100.025	99.18	98.95	0.05	0.10	0.29
DEX	98.35	99.13	98.66	0.35	0.29	0.28
HPLC method						
Drug	**Recovery**			**% RSD**		
	80%	**100%**	**120%**	**80%**	**100%**	**120%**
GAT	99.26	100.48	99.21	0.28	0.72	0.51
DEX	98.78	100.14	98.57	0.19	0.73	0.81

Precision

Precision was calculated as intra-day and inter-day variations for the drugs for both analytical methods. Percent relative standard deviations for estimation of GAT and DEX under intra-day and inter-day variations were found to be less than 2 (Table 4).

Table 4: Precision values of GAT and DEX

Method	Drug	Conc. (μg/mL)	Intra-day (% RSD)	Inter-day (% RSD)	System precision (% RSD)
UV	GAT	6	0.14	0	0.04
		12	0.14	0	
		18	0.22	0.20	
	DEX	2	0.25	0.26	0.09
		4	0.26	0.15	
		6	0.33	0.26	
HPLC	GAT	22.5	1.45	0.23	1.19
		45	0.60	0.13	
		67.5	1.51	0.23	
	DEX	7.5	1.34	0.07	1.05
		15	0.62	0.08	
		22.5	1.18	0.17	

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

For both methods, the LOD and LOQ were calculated using the values of slopes and intercepts of the calibration curves for both the drugs (Table 5).

Robustness

For robustness studies, conditions like flow rate and concentration of organic phase were changed and method was performed. In all deliberately varied conditions, percent relative standard deviations for peak areas, retention times, theoretical plates and tailing factor were found to be less than 2% (Table 6).

Table 5: LOD and LOQ of GAT and DEX

Method	Drug	LOD (µg/mL)	LOQ (µg/mL)
UV	GAT	0.314	0.952
	DEX	0.169	0.512
HPLC	GAT	2.69	8.16
	DEX	0.79	2.40

Table 6: Robustness parameters of GAT and DEX

Parameter	GAT	DEX
	R_t (min)	R_t (min)
Initial flow	2.105	4.516
Flow 0.9 mL/min	2.353	5.047
Flow 1.1 mL/min	1.916	4.116
Organic phase, 10 % more (55 %)	1.991	4.313
Organic phase, 10 % less (45 %)	1.961	4.839

Assay

The percent of assay was calculated using absorbances of standard and sample for UV method and by using peak areas of standard and sample for HPLC method. The experimental values obtained for the determination of GAT and DEX in ophthalmic formulation were within the claimed limits for both the methods (Table 7).

Table 7: Assay data of marketed formulation

Method	Drug	Amount labeled	Amount found	% Label claim	% RSD
UV	GAT	3 mg	2.980 mg	99.33	0.13
	DEX	1 mg	0.991 mg	99.10	0.21
HPLC	GAT	3 mg	2.990 mg	99.66	0.23
	DEX	1 mg	0.985 mg	98.50	0.62

Stability studies

Degradation studies were performed for GAT and DEX in various conditions like acidic degradation, alkaline degradation, oxidative degradation, photolytic degradation, thermal degradation and neutral degradation.

Acidic degradation

GAT showed good stability in acidic conditions compared to DEX. GAT was stable in 0.1M HCl and 0.5M HCl. But, showed degradation in 1M HCl and 2M HCl. The same pattern was observed in 3rd & 5th day also. DEX show stability in 0.1M HCl, but degraded in other acidic conditions.

Alkaline degradation

GAT showed in stability in 0.1M NaOH and 0.5M NaOH. But, % degradation increased in 1M NaOH and 2M NaOH. Whereas, DEX showed stability in 0.1M NaOH to degradation in other alkaline conditions.

Oxidative degradation

Both the drugs underwent degradation in appreciable amounts in all peroxide conditions except in 1% H_2O_2.

Photolytic degradation

Both the drugs showed good stability under photolytic conditions with very less degradation. DEX showed more degradation in 3rd & 5th day compared to GAT.

Thermal degradation

Both the drugs showed good stability under thermal conditions with very less degradation.

Neutral degradation

Both the drugs showed good stability under neutral conditions with very less degradation. The percent amount of drug degraded after degradation studies are given in (Table 8a & 8b).

Table 8a: Degradation data* of stress studies

Day	GAT				DEX			
	Acid hydrolysis with HCl (% DEG ± SD)							
	0.1 M	**0.5 M**	**1 M**	**2 M**	**0.1 M**	**0.5 M**	**1 M**	**2 M**
Day-1	1.03 ± 0.51	2.38 ± 0.96	9.90 ± 1.37	14.16 ± 0.98	2.47 ± 1.77	20.58 ± 0.79	35.40 ± 1.10	55.02 ± 1.33
Day-3	1.66 ± 0.65	3.98 ± 0.34	17.65 ± 0.98	19.80 ± 1.20	12.97 ± 0.89	33.66 ± 0.24	42.87 ± 1.15	61.12 ± 0.19
Day-5	4.41 ± 0.95	9.78 ± 1.71	23.99 ± 0.64	36.84 ± 0.65	14.00 ± 1.78	48.25 ± 0.21	52.80 ± 0.50	62.69 ± 1.16
	Base hydrolysis with NaOH (% DEG ± SD)							
	0.1M	**0.5M**	**1M**	**2M**	**0.1 M**	**0.5 M**	**1 M**	**2 M**
Day-1	0.50 ± 0.12	3.59 ± 0.88	17.63 ± 1.04	28.58 ± 1.41	3.48 ± 0.51	15.04 ± 0.55	59.05 ± 1.01	97.31 ± 0.87
Day-3	4.87 ± 0.71	6.89 ± 0.84	28.58 ± 1.64	40.24 ± 1.37	4.12 ± 0.18	17.06 ± 0.35	82.63 ± 0.44	99.21 ± 0.34
Day-5	10.08 ± 0.53	13.28 ± 0.92	30.95 ± 0.77	55.14 ± 0.75	6.93 ± 1.08	21.55 ± 0.85	86.75 ± 1.00	99.41 ± 0.85
	Oxidative hydrolysis with H_2O_2 (% DEG ± SD)							
	1%	**3%**	**5%**	**10%**	**1%**	**3%**	**5%**	**10%**
Day-1	3.23 ± 1.52	9.14 ± 1.08	13.76 ± 1.67	25.59 ± 0.37	5.00 ± 0.38	49.46 ± 0.23	58.31 ± 0.33	75.89 ± 0.98
Day-3	15.40 ± 1.32	23.75 ± 1.74	54.44 ± 0.38	71.85 ± 0.61	28.35 ± 0.96	51.21 ± 1.05	62.09 ± 0.64	76.88 ± 0.71
Day-5	17.52 ± 0.85	42.28 ± 1.00	55.34 ± 0.13	68.97 ± 0.10	29.05 ± 0.38	52.38 ± 0.59	62.57 ± 0.70	76.93 ± 1.03

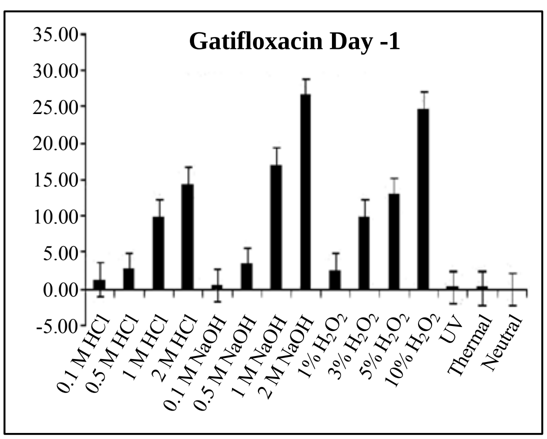
Table 8b: Degradation data* of stress studies

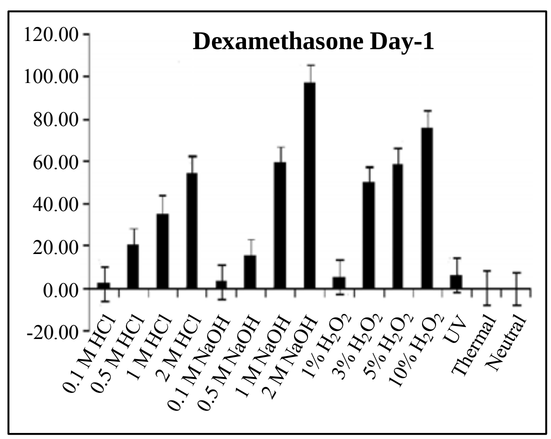
	Other hydrolysis methods (% DEG±SD)					
	GAT			**DEX**		
	UV	**Thermal**	**Neutral**	**UV**	**Thermal**	**Neutral**
Day-1	0.70 ± 0.02	0.64 ± 0.01	0.50 ± 0.03	6.97 ±1.08	0.62 ± 0.51	0.98 ± 0.00
Day-3	1.23 ± 0.27	2.32 ± 1.33	2.10 ± 0.75	9.82 ± 0.31	0.88 ± 0.43	0.68 ± 0.18
Day-5	3.22 ± 0.28	2.73 ± 0.20	6.40 ± 0.43	15.20 ± 1.66	1.99 ± 1.70	1.50 ± 0.46

*Average of three determinations (each condition), DEG: Degradation, SD: Standard deviation

The pattern of degradation of the drugs individually in all the conditions and in different days along with the error bars (taking standard errors into consideration) was well portrayed in the Fig. 5.

The proposed UV method, allows a rapid and accurate quantification of GAT and DEX in ophthalmic preparation without any time consuming sample preparation. Moreover, the spectrophotometric method involves simple instrumentation compared with other instrumental techniques. Wavelengths selected for analysis are 279 nm (λ_{max} of GAT) and 239 nm (λ_{max} of DEX). For HPLC method, different proportions of acetonitrile and orhophosphoric acid (OPA) were tried for selection of the mobile phase. Ultimately, 0.1% OPA in water and acetonitrile in a proportion of 50:50 v/v respectively was finalized as the mobile phase. The elution order was GAT (R_t = 2.124 min) and DEX (R_t = 4.578 min), at a flow rate of 1.0 mL/min.





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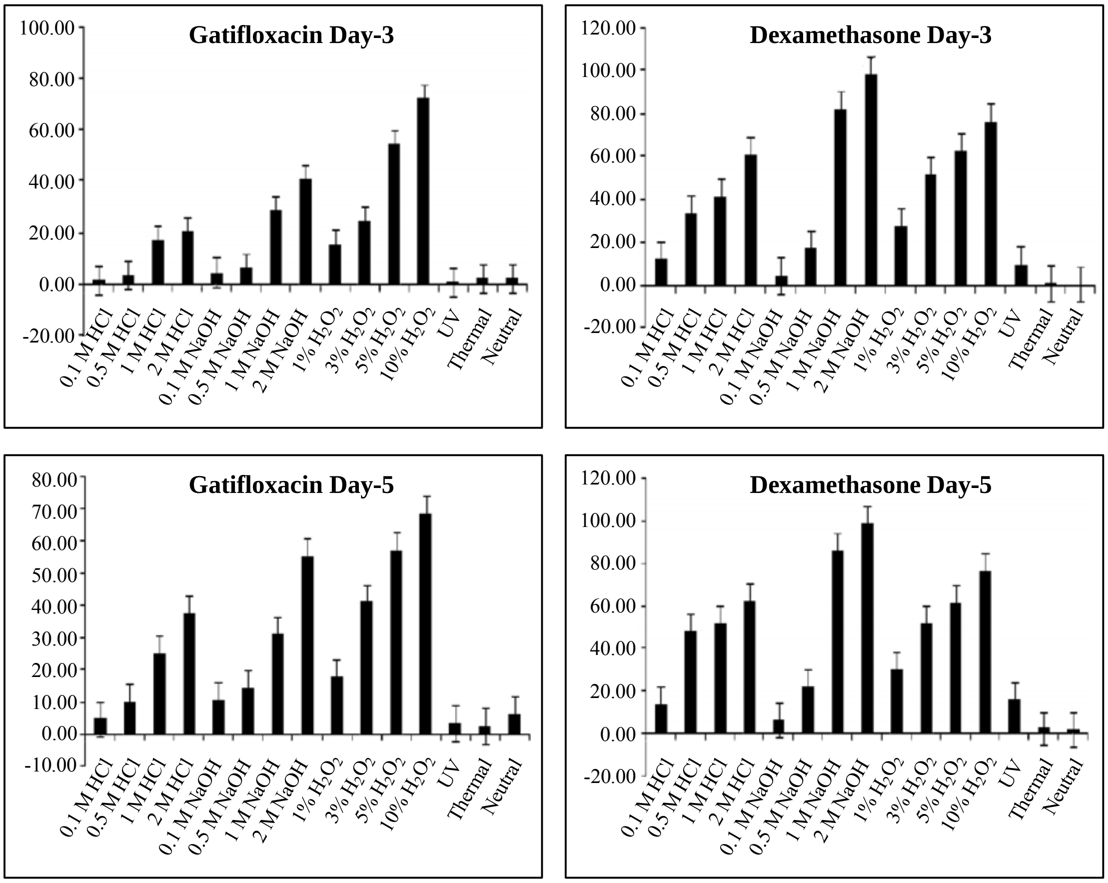

Fig. 5: Graphs showing degradation pattern of GAT and DEX in different conditions

The chromatogram was recorded at 241 nm. The developed method was validated as per ICH guidelines. Parameters like precision, accuracy, specificity, ruggedness, robustness were done and found to be within the acceptance criteria. LOD and LOQ were determined and the developed method was applied for determination of assay of ENGATT DX eye drops. The stability of the drugs was examined under different stress conditions such as acidic, alkaline, peroxide, thermal, photolytic and under neutral conditions. Moreover, we have even used different concentrations of the above mentioned conditions so that we could find out the extent of degradation in different conditions.

CONCLUSION

The two proposed methods based on the spectrophotometry and RP-HPLC were developed and validated as per ICH guidelines. The standard deviation and % RSD calculated for the proposed methods are low, indicating a high degree of precision of the methods. The results of the recovery studies performed show the high degree of accuracy of the proposed methods. The RP-HPLC method could selectively quantifies GAT and DEX in presence of its degradation products hence; it can be employed as a stability indicating method. From the found experimental data, it can be concluded that the developed spectrophotometric and stability indicating high performance liquid chromatographic methods are accurate, precise and selective and can be employed successfully for the estimation of GAT and DEX in ophthalmic dosage form.

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