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Article

A Validated Specific Stability-Indicating RP-HPLC Assay Method for the Determination of Loteprednol Etabonate in Eye Drops

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A new stability-indicating RP-HPLC assay method was developed and validated for quantitative determination of loteprednol etabonate in bulk drugs and in ophthalmic suspensions in the presence of degradation products generated from forced degradation studies. The system consisted of Agilent Technologies Zorbax Eclipse XDB-Phenyl 5 μm 4.6 × 250 mm, and detection was performed at 244 nm. The mobile phase consisted of water–acetonitrile–acetic acid (34.5:65.0:0.5, v/v/v) run at a flow rate of 1 mL/min and maintained at room temperature. The calibration curve was linear from 30 to 70 μg/mL with $r > 0.999$. Accuracy (mean recovery 100.78%) and precision were found to be satisfactory. Stress conditions including acid and alkali hydrolysis, water stress, oxidation, photolysis and heat were applied. The degradation products did not interfere with the detection of loteprednol etabonate, thus the method can be considered as a stability-indicating method. The proposed method can be used for quality control assay of loteprednol etabonate in bulk drug and in ophthalmic suspensions and for stability studies as a result of the ability of the method to separate loteprednol etabonate from its degradation products and excipients.

Introduction

Loteprednol etabonate, chloromethyl-17α-[(ethoxycarbonyl) oxy]-11β-hydroxy-3-oxandrosta-1,4-diene-17β-carboxylate (Figure 1), is a new glucorticoid drug that was developed for topical use. This compound was designed based on prednisolone and to reduce side effects, a carboxylic ester functionality was introduced at 17β-position, in the expectation that hydrolysis by non-specific esterases would transform it into the indicative steroid carboxylic acid metabolite (1). Loteprednol etabonate is used in the topical management of inflammatory and allergic disorders of the eye. It is usually employed as eye drops containing 0.2 or 0.5% (2). Coffey and coworkers describe a novel ophthalmic gel formulation of loteprednol etabonate in the treatment of ocular inflammatory conditions. The gel formulation is non-settling, eliminating the need to shake the product to re-suspend the drug, has a pH close to the tears and has a low preservative concentration (3). Prolonged application to the eye of preparations containing corticosteroids has caused raised intra-ocular pressure and reduced visual function (2).

A literature survey revealed two high-performance liquid chromatographic methods for loteprednol etabonate in bulk material, using a phenyl column, mobile phase consisting of different proportions of acetonitrile–water–acetic acid, in the presence of its impurities or degradation products. Shirasaki and coworkers described the photodegradation products of loteprednol etabonate after an exposition to white fluorescent light at 3000 lx for 18 days and near UV fluorescent light at 0.5 mW/cm^2 for 40 h at 25 ± 2°C (4). Yasueda and coworkers described a method for the quantitation of loteprednol etabonate in the presence of its four related substances and four degradation products (5). They also found more degradation products with fluorescent light and UV exposition. Despite these findings, we found that loteprednol etabonate was extremely sensible to alkaline exposition.

This paper describes a simple, rapid, precise and accurate isocratic reversed-phase HPLC method for the determination of loteprednol etabonate in eye drops in the presence of its degradation products and excipients. The proposed HPLC method utilizes economical solvent system, better retention time, very sharp and symmetrical peak shapes.

The method was validated by following the analytical performance parameters suggested by the International Conference on Harmonization (ICH) (6).

Experimental

Chemical and reagents

The loteprednol etabonate working standard (99.50%) was provided by Sun Pharmaceutical Industries Ltd (Bharuch, India), calculated with reference to the dried substance. An eyed-drop formulation was studied. Its composition was loteprednol etabonate 500 mg, in a matrix of disodium EDTA, benzalkonium chloride, glycerin, polyvinylpyrrolidone and tyloxapol.

Acetonitrile used was of HPLC grade (Sintorgan, Buenos Aires, Argentina), and Acetic acid was of AR grade (Sintorgan). Distilled water was passed through a 0.45-μm membrane filter.

Equipment

The HPLC system that consisted of a dual-piston reciprocating Thermo Finnigan pump, a Rheodyne injector, and a DAD Dionex Ultimate 3000 with operating software Chromeleon 6.8 was used during the study.

Chromatographic conditions

The LC method was carried out on an Agilent Technologies Zorbax Eclipse XDB-Phenyl 5 μm 4.6 × 250 mm column (USA), maintained at room temperature. The mobile phase consisted of water–acetonitrile–acetic acid (34.5:65.0:0.5, v/v/v) run at a flow rate of 1 mL/min and using ultraviolet detection at 244 nm.

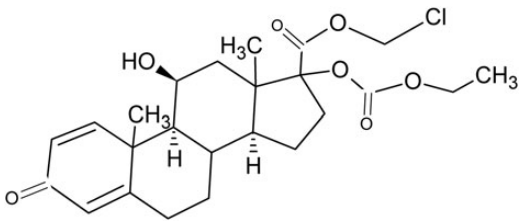

Figure 1. Loteprednol etabonate.

The chromatographic separation was obtained with a retention time of 6.71 min. The injection volume was 20 μL.

Preparation of standard solution

An accurately weighed quantity of 100 mg of loteprednol etabonate was dissolved in 100 mL of mobile phase. Then, 5 mL was withdrawn in a 100 mL volumetric flask. The volume was made with mobile phase. The solutions were passed through a 0.45-μm nylon membrane filter before injection (25 mm disposable filter; Cat. No. R04SP02500, Osmonics Inc., Minnesota, USA).

Sample preparation

Approximately 5 mL of eye drops loteprednol etabonate suspension 0.5% (5 mg/mL) were exactly weighed, placed into a 100-mL volumetric flask, taken to volume with mobile phase. Then, 5 mL was withdrawn in a 25-mL volumetric flask. The volume was made with mobile phase.

The solutions were passed through a 0.22-μm nylon membrane filter before injection (25 mm disposable filter; Cat. No. R04SP02500, Osmonics Inc.).

Method validation

System suitability

The relative standard deviation (RSD) values of the peak area, tailing factor, theoretical plates and retention time were the chromatographic parameters selected for the system suitability test (7). The described reversed-phase liquid chromatography method was developed to provide a rapid quality control determination of loteprednol etabonate in eye drops. The analytical column was equilibrated with the eluting solvent system used. After an acceptable stable baseline was achieved, the standards and then the samples were analyzed.

Specificity

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities. All stress degradation studies were performed at an initial drug concentration of 1 mg/mL, with acid (0.5 N HCl), base (0.1 N NaOH), water and 100% hydrogen peroxide. Acid degradation was maintained at 60°C for 15 min and water and oxidative degradation for 30 min. Alkaline degradation was maintained only with stirring during 15 min. The drug was subjected to thermal degradation (either in the solid state or in solution in an open container in an oven at 100°C for 24 h) and photochemical degradation (a solution was transferred to a container and exposed to daylight for 24 h). After the degradation procedure, samples were allowed to cool at room temperature and diluted, if necessary, to the same concentration as that of the standard solution, after being neutralized. Further, samples were analyzed using the methodology and the chromatographic conditions described.

Linearity

Aliquots of the standard solution were suitably diluted in such a way that the final concentration of the drug was in the range of 30–70 μg/mL. Triplicate injections of 20 μL were made, and a chromatograph was obtained under the above-mentioned conditions. The drug was evaluated and peak areas were recorded. A calibration curve was plotted by taking the peak area on the *y*-axis and the respective concentration of the drug on the *x*-axis. The calibration curve was constructed and evaluated by its coefficient of determination (r^2) and by least-squares linear regression analysis.

Precision

Six replicates of standard solution were analyzed to assess system precision. The assay method precision was evaluated by carrying out six independent assays of a commercial formulation of loteprednol etabonate against qualified working standard and the mean and the RSD were calculated. Intermediate precision was assessed by comparing the results obtained from six samples prepared by two different analysts on two different days.

Accuracy

The accuracy of the assay method was evaluated in triplicate at three concentration levels, i.e., 30, 50 and 70 μg/mL of a commercial formulation of loteprednol etabonate. The percentage recoveries were calculated from the slope and Y-intercept of the calibration curve.

Robustness

The robustness of the assay method was carried out by using the variation in the flow rate and mobile phase ratio. The retention time, tailing factor and efficiency were evaluated.

Results

A number of HPLC chromatographic systems were investigated to optimize the separation of loteprednol etabonate and its degradation products. The retention time for loteprednol etabonate function of stationary phase Zorbax Eclipse XDB-Phenyl 5 μm 4.6 × 250 mm (Agilent Technologies) and the mobile phase consisted of water–acetonitrile–acetic acid (34.5:65.0:0.5, v/v/v) run at a flow rate of 1 mL/min and using ultraviolet detection at 244 nm.

System suitability

System suitability is the checking of a system to ensure system performance before or during the analysis of unknowns. The system suitability test is an integral part of method development and is performed to evaluate the behavior of the chromatographic system. Plate number (*N*), tailing factor (*T*) and RDS were evaluated for six replicate injections of the drug at a concentration of 50 μg/mL.

The system suitability results were calculated according to the USP 35 <621> (7) from typical chromatograms. Instrument precision as determined by six successive injections of the standard preparation provided a RSD below 1.0%. Peak asymmetry or tailing factor, T, was calculated using the formula: $T = W_{0.05}/2f$, where $W_{0.05}$ is the distance from the leading edge to the tailing edge of the peak, measured at 5% of the peak height from the baseline, and f is the distance from the peak maximum to the leading edge of the peak. The tailing factor did not exceed 1.5. The column efficiency should be more than 11,000 theoretical plates.

The stability of the standard solution was studied by injecting the prepared solution at periodic intervals into the chromatographic system up to about 7 days, which is stored in a refrigerator. The solution was maintained at least 99.92% of their initial concentration under the test conditions.

Method validation

No peak was observed in the chromatogram of placebo solution at the retention time of loteprednol etabonate. It was stable under thermal conditions (solid). Significant degradation of the drug substance was observed under acid, alkaline, oxidative, hydrolysis, photolytic and thermal conditions (solution) (Figure 2). Table I shows the degradation conditions and the relative retention time of the degradation products with respect to the principal peak of loteprednol etabonate.

Degradation was indicated in the stressed sample by a decrease in the expected concentration of the drug and increased levels of degradation products.

The DAD data show that the purity angle is less than the purity threshold and spectra at peak start, at end, at apex exactly matching in the range 200–400 nm for all the stressed samples. Hence, the peak purity test results confirm that the loteprednol etabonate peak is homogeneous and pure in all the stress samples analyzed. The peak purity from the sample solution was >0.990 that indicates the method specificity. Degradation products did not interfere with the detection of the drug. The mass balance is a process of adding together the assay value and the levels of degradation products to see how closely these add up 100% of the initial value with due consideration of the margin of analytical error. The mass balance of stressed samples was 100.0%, except

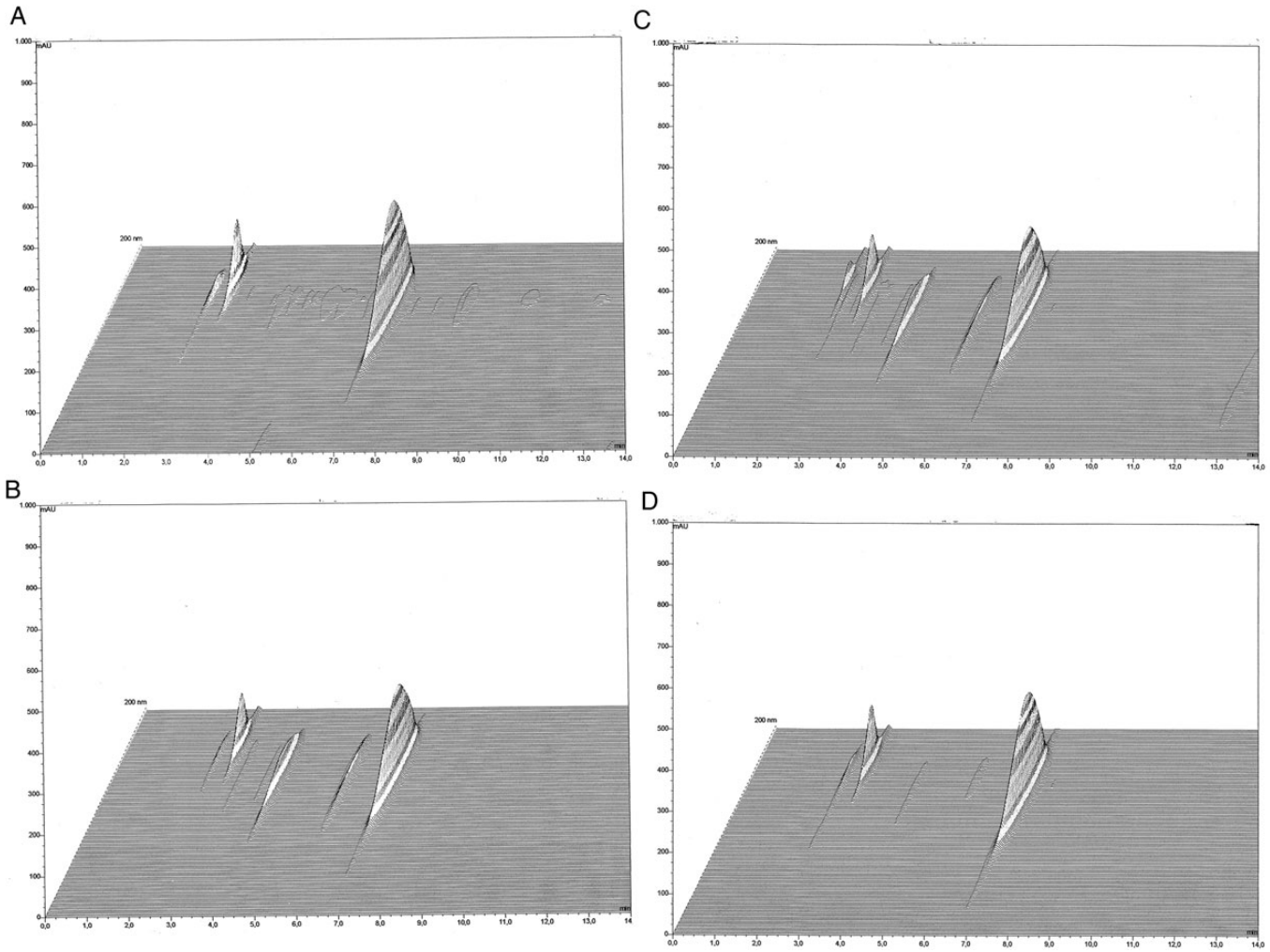

Figure 2. Chromatograms of loteprednol etabonate. (A) Standard; (B) hydrolysis (water, 60°C, 0.5 h); (C) oxidation (hydrogen peroxide 100%, 60°C, 0.5 h); (D) daylight exposure (24 h); (E) heat dry, 100°C (solution, 24 h); (F) heat dry, 110°C (solid, 24 h); (G) acid hydrolysis (0.5 N HCl, 60°C, 0.25 h) and (H) alkaline hydrolysis (0.1 N NaOH, cold, 0.25 h).

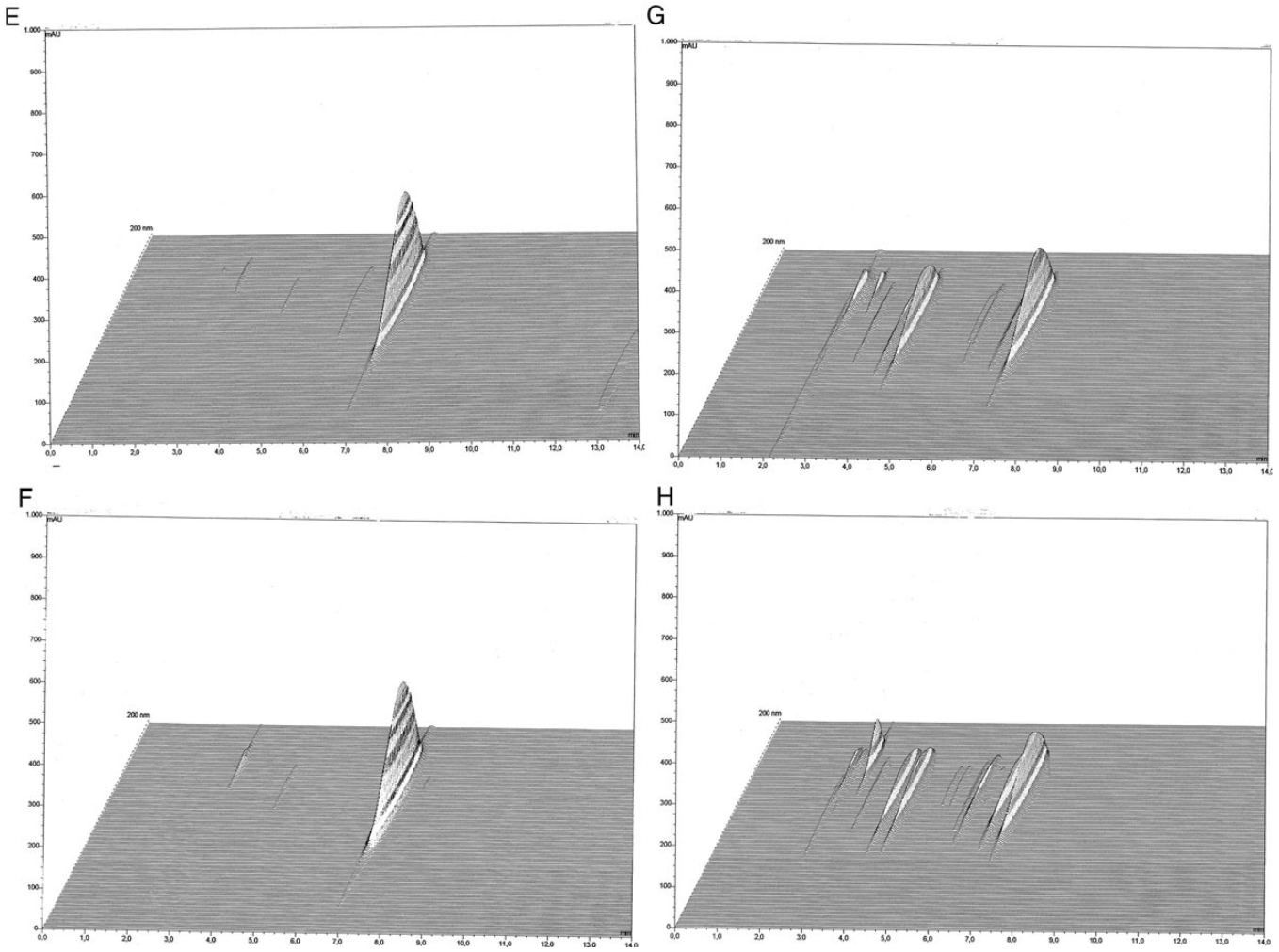

Figure 2. *Continued*

Table I
Selectivity: Degradation Conditions of Loteprednol Etabonate

Condition	Time (h)	Percentage of loteprednol etabonate	RRT of degradation products
Acid (0.5 N HCl, 60°C)	0.25	58.3	0.54, 0.59, 0.84, 0.94
Base (0.1 N NaOH, cold)	0.25	46.1	0.45, 0.54, 0.59, 0.84, 0.94
Hydrogen peroxide 100**%** (60°C)	0.5	78.7	0.59, 0.84
Water (60°C)	0.5	78.3	0.59, 0.84
Heat dry, 100°C (solution)	24	97.7	0.84
Heat dry, 100°C (solid)	24	99.1	Non detectable
Daylight exposure	24	93.4	Non detectable

RRT, relative retention time.

the photolytic degradation. Shirasaki and coworkers found that the principal photolytic degradation product shows a little adsorption at 244 nm.

The selectivity was demonstrated by showing that loteprednol etabonate was free of interference from degradation products, and that no interference from the sample excipients was observed at the detection wavelength; thus, the proposed method can be used in a stability assay (Figure 3).

The linearity of measurement was evaluated by analyzing different concentrations of the standard solutions of loteprednol etabonate. The calibration curve was constructed by plotting the average peak area against concentration, and then the regression equation was computed.

A linear calibration plot for the assay method was obtained over the calibration ranges tested, i.e., 60–150% of the assay analyte concentration and the correlation coefficient obtained was >0.9993. Linearity was checked for the assay method over the same concentration range for two consecutive days. The results show that an excellent correlation existed between the peak area and the concentration of the analyte.

The regression line was calculated by using the formula $y = 0.7618x - 0.2829$ with a determination coefficient (r^2) of 0.9986. The linearity of the calibration graphs was validated by the high value of the correlation coefficient and the intercept value that was not statistically ($P = 0.05$) different from zero (Table II and Figure 4).

The precision was demonstrated at two levels: repeatability and intermediate precision. The precision is usually expressed as the RSD of a series of measurements. The RSD of peak area response and retention time showed the satisfactory repeatability

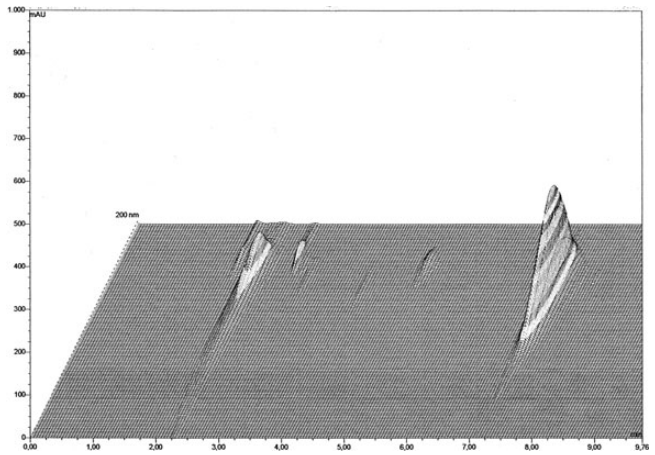

Figure 3. Typical chromatogram of loteprednol etabonate eye drop suspension.

Table II
Linearity Data of Loteprednol Etabonate

Percentage of nominal value	Concentration (μg/mL)	Average peak area response	RSD
60	30.48	22.9245	0.43
80	40.64	30.6646	0.20
100	50.80	37.9485	0.31
120	60.96	47.0000	0.03
150	76.20	57.4139	0.04
Slope[a]	0.7618 ± 2.0309		
Intercept[b]	−0.2829 ± 110.0602		

[a]Confidence limits of the slope ($P = 0.05$).
[b]Confidence limits of the intercept ($P = 0.05$).

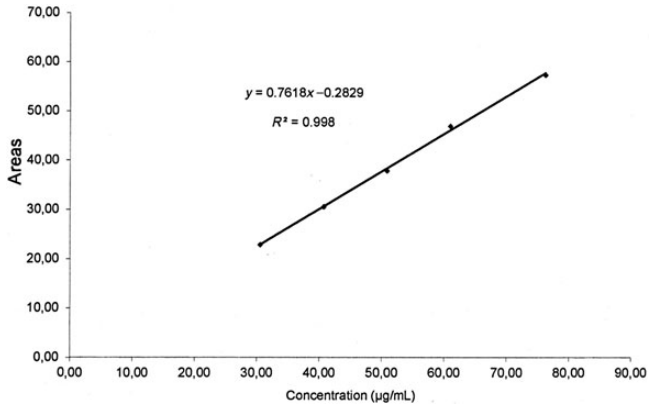

Figure 4. Linearity of the assay method.

of the system (<1%). The intermediate precision was performed by assaying the samples on two different days by two different analysts. The results were given both individually and as the average. For each precision assay, the results were as follows: the mean values 0.5281 and 0.5313%, and RSD 0.70 and 0.27%. Test "*t*" comparing two samples with 95% confidence interval for 10 degrees of freedom disclosed that both results were not significantly different *inter se* ($t_{n-2,\ \alpha:0.05}$) = 2.23 (Table III)

To study the reliability, suitability and accuracy of the method, recovery experiments were carried out. The accuracy of a

Table III
Precision of the Assay Method for Loteprednol Etabonate

Analyst 1 Sample no.	%	RSD (%)	Analyst 2 Sample no.	%	RSD (%)
1	0.5314	0.37	1	0.5317	0.75
2	0.5240	0.37	2	0.5313	0.75
3	0.5320	0.37	3	0.5305	0.75
4	0.5292	0.37	4	0.5297	0.75
5	0.5232	0.37	5	0.5338	0.75
6	0.5288	0.37	6	0.5308	0.75
Mean	0.5281	0.70	Mean	0.5313	0.27

Table IV
Recovery Analysis of Loteprednol Etabonate

Percentage of nominal value	Added amount (g)	Found amount (g)	Recovery (%)	Average recovery ($n = 3$) (%)	RSD (%)
	4.2433	4.224	99.55		
80	4.2175	4.1985	99.55	99.74	0.40
	4.2288	4.2341	100.12		
	5.3042	5.2542	99.06		
100	5.2719	5.2396	99.39	99.28	0.32
	5.2860	5.2541	99.40		
	6.3414	6.5178	102.78		
120	6.3557	6.5703	103.38	103.32	0.52
	6.3757	6.6188	103.81		
Mean ($n = 9$)				100.78	1.89

Table V
Robustness

Mobile phase	RT, loteprednol etabonate (min)	Tailing	*N*
Water–acetonitrile–acetic acid (34.5:65.0:0.5 v/v) Flow: 1 mL/min	6.71	1.22	11,543
Water–acetonitrile–acetic acid (34.5:65.0:0.5 v/v) Flow: 0.8 mL/min	8.89	1.28	12,737
Water–acetonitrile–acetic acid (34.5:65.0:0.5 v/v) Flow: 1.2 mL/min	5.94	1.21	10,699
Water–acetonitrile–acetic acid (32.5:67.0:0.5 v/v) Flow: 1 mL/min	6.42	1.24	11,497
Water–acetonitrile–acetic acid (36.5:63.0:0.5 v/v) Flow: 1 mL/min	7.84	1.20	12,220

RT, retention time; N, efficiency.

method is expressed as the closeness of agreement between the value found and the value that is accepted as a reference value. It is determined by calculating the percent difference between the measured mean concentrations and the corresponding nominal concentrations. The accuracy of the proposed method was tested by recovery experiments of one commercial formulation studied ($n = 3$ for 80, 100 and 120%). The mean recovery was 100.78% and the RSD was 1.89%. The experimental *t* of the recovery percentage value was 1.239, being it far below the 2.306 established in the tabulated *t* (95% level of probability, 8 df). (Table IV)

The robustness of the analytical procedure, which is the measure of the method's capacity to remain unaffected by small but deliberate variations in method parameters, is shown in this study. The robustness of the proposed method was found after altering the parameters deliberately: the mobile phase ratio variants ± 1% of water and acetonitrile and flow ± 20%. The

retention time, tailing factor and efficiency of the compound were evaluated. The results are provided in Table V. When the change in the mobile phase proportion of water–acetonitrile was of 1%, the retention time changes similarly. It also affects the efficiency. The change in the flow rate also affects the retention time, tailing factor and efficiency.

Discussion

During method development, different options were evaluated to optimize sample extraction, detection parameters and chromatography. A mobile phase containing water, acetonitrile and acetic acid in varying combinations was tried during the initial development stages. The sensitivity and peak shape were also checked. The best signal and peak shape for loteprednol etabonate were achieved using a stationary phase Zorbax Eclipse XDB-Phenyl 5 μm 4.6 × 250 mm and the mobile phase consisted of water–acetonitrile–acetic acid (34.5:65.0:0.5, v/v/v) and ran at a flow rate of 1 mL/min.

The proposed method was validated as per the ICH guidelines for its selectivity, linearity, precision, accuracy, and robustness. No peak was observed in the chromatogram of placebo solution at the retention time of loteprednol etabonate.

A significant degradation of the drug substance was observed under acid, alkaline, oxidative, hydrolysis, photolytic and thermal conditions (solution). The degradation products of stress tests did not interfere with the main peak. In contrast to the studies reported in the literature, we found that loteprednol etabonate is more sensitive to alkaline degradation to photolysis.

The method is very simple and specific, as the peak is well separated from its impurities and excipient peaks with a total runtime of 10 min, which makes it especially suitable for routine quality control analysis.

Conclusion

The present method is sensitive, rapid, robust, precise and accurate. The mobile phase was easy to prepare. The recovery from formulations was in good agreement and suggested no interference in the estimation.

Application of this method for the analysis of eye drops reveals that neither the degradation products nor the excipients interfere with the analytical determination. This indicates that the proposed method could be used as a stability-indicating method for the determination of loteprednol etabonate either in bulk or in pharmaceutical formulations (ophthalmic suspensions).

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