Original Article

Validated High Performance Liquid Chromatography Method for the Quantification of Loteprednol Etabonate in Self Micro Emulsifying Drug Delivery Systems

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ABSTRACT

Background: The objective of this work is to develop and validate a reverse-phase HPLC method for determining Loteprednol Etabonate in self micro emulsifying drug delivery systems at nanogram level. **Methods:** A systematic approach has been implemented for the optimization of chromatographic conditions. The developed reverse-phase HPLC method was validated for linearity, accuracy, precision, limit of detection and quantification, extraction recovery, specificity (matrix effect and forced degradation studies), and robustness. The method was also investigated for the estimation of Loteprednol under forced degradation for acid, base, thermal and oxidative conditions. **Results:** The developed method showed linearity between 200 and 12000 ng/mL with R^2 value of 0.998. The detection and quantification limits were determined to be 60 and 180 ng/mL, respectively. The % recovery and % RSD of the developed method were 99.00 % to 101.79 % and 0.0459 % - 0.6470 %, respectively. The validated method was able to identify the degradants and formulation excipients interactions at the drug peak retention time (4.7 min). The assay determination in lipid emulsion formulations was successfully carried out using the established method. The % recovery of loteprednol etabonate from formulations has shown good agreement without any interference. **Conclusion:** The validated stability-indicating analytical method was useful for the quantification of loteprednol etabonate in self micro emulsifying drug delivery systems.

Key words: Loteprednol etabonate, HPLC, stability indicating, analytical method, Self micro emulsifying systems

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INTRODUCTION

Topical corticosteroids are an important therapeutic option for the management of ocular inflammation of the anterior segment. However, their use is associated with adverse events, including escalated intraocular pressure, cataract development, and increased susceptibility to viral infections.[1] To control these adverse effects, Bodor and colleagues employed retrometabolic drug design to develop loteprednol etabonate (Figure 1).

Loteprednol etabonate (LE) has enhanced hydrophobicity, which allows it to permeate the cells efficiently. LE has demonstrated 10 times greater hydrophobicity compared to that of dexamethasone. Moreover, it binds with the glucocorticoid receptor with 4.3 folds greater affinity than dexamethasone.[2] LE is sparingly soluble in aqueous buffers. It has a maximum solubility of 0.2 mg/mL in a 1:1 solution of PBS: ethanol at pH 7.2. LE is soluble in organic solvents such as dimethyl sulfoxide, ethanol, and dimethylformamide, with a 30 mg/mL solubility.[3] The mechanism of action of LE is similar to that of other topical

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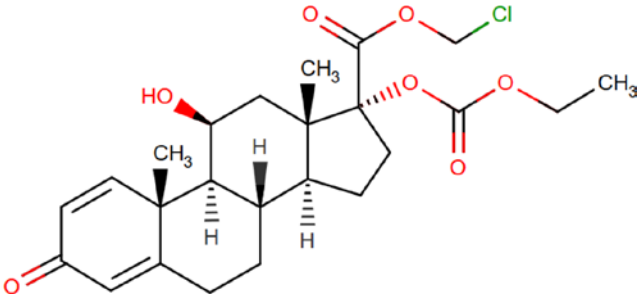

Figure 1: Structure of loteprednol etabonate.

corticosteroids. LE is used as a first-line corticosteroid to treat ocular inflammation of the anterior segment because of its outstanding safety profile. It is usually administered as eye drops with LE in the concentrations of 0.2 % or 0.5 %.[1] It is used in the treatment of acute uveitis routinely. It is also used in the preventive management of chronic recurrent uveitis.[4,5]

Self-Micro emulsification drug delivery system (SMEDDS) is one of the good approaches for enhancement of the bioavailability of LE.[6] SMEDDS must undergo characterisation for drug content, as well as *in vitro* and *ex vivo* drug release investigations. A few reverse-phased high-performance liquid chromatographic (RP-HPLC) methods have been reported in the literature for the estimation of LE. Han *et al.* have reported an RP-HPLC method for the quantification of LE in eye drops. The mobile phase consisted of 34.5 % water, 65 % acetonitrile, and 0.5 % acetic acid, run at 1 mL/min. The retention time was obtained at 6.71 mins. Upon validation of the method, it was found that the calibration curve was linear in the range of 30-70 µg/mL with regression value 0.999. The accuracy data showed satisfactory recovery 100.78 %.[7] Samir *et al.* developed an HPLC method for the simultaneous quantification of LE and its metabolites. The HPLC method was based on acetic acid gradient.[8] Yang and his team developed an analytical method for LE and its related substances determination in ophthalmic suspension using the gradient elution method. The mobile phase consisted of 0. 25 % acetic acid solution-acetonitrile (80/20) and acetonitrile, with a linearity range of 0. 001-1. 02 mg/mL and a flow rate of 2 mL/min.[9] Divya *et al.* developed HPLC to estimate LE using 0.05 M buffer (pH 5) and acetonitrile in the ratio of 40:60 at a flow rate of 1.0 mL/min. The concentration range 10-90 µg/mL were determined at a retention time of 5.60 min. The method development was sensitive with LOD and LOQ of 0.82 µg/mL and 2.49 µg/mL.[10] Patel *et al.*, developed a rapid reverse phase HPLC using methanol: phosphate buffer (pH, 6.0): Acetonitrile (65:25:10 v/v/v) with linearity range of 2 - 35 µg/mL.[11] The reported methods were applied for conventional formulations and applied to limited samples only. Additionally, some methods were not sensitive enough to determine the LE in nanogram concentration. There is no HPLC method for estimating LE in self micro-emulsifying drug delivery systems (SMEDDS) based on the literature. The present study has developed an HPLC method for estimating LE in SMEDDS that is rapid, simple, accurate, precise, and sensitive. The method is further validated as per the guidelines suggested by the International Conference on Harmonization (ICH Q2(R1)).[12-14]

MATERIALS AND METHODS

Chemicals

Loteprednol etabonate (LE) was received as a gift sample from Slay back pharma (Hyderabad, Telangana). HPLC grade methanol and acetonitrile were procured from Merck Pvt. Ltd. Potassium dihydrogen phosphate was procured from Hi media. Sodium hydroxide and hydrochloric acid were procured from Merck Pvt. Ltd, Mumbai. Hydrogen peroxide was from Sigma Aldrich. Milli Q water was used throughout the analysis (Millipore, MA, and USA). 0.22 µm Membrane Filter was from Merck Limited (Mumbai, India).

Chromatographic Conditions

The HPLC system comprised of Shimadzu LC-2010CHT (Kyoto, Japan) equipped with LC-2010CHT sample cooler, dual-wavelength UV-visible detector, and high-efficiency 5-line degasser. LC solutions software was used to integrate the obtained peak areas. Prior to use, the aqueous phase was filtered through 0.22 µ membrane filter and degassed with ultrasonic bath for 15 min. 20 µL injection volume was given at a temperature of 30°C. The detector was functioned at dual wavelengths (set at 243 nm and 246 nm) for the data acquisition of loteprednol etabonate. A systematic approach i.e., various buffers with different columns and organic phases was tried to get desired peak properties.

Stock, Standard, and Quality Control Solutions

The stock solution with concentration of 1 mg/mL of LE was prepared in acetonitrile. Further, serial dilutions were made with acetonitrile to prepare the concentrations of LE in the range of 200–12000 ng/mL from stock solution. The quality control (QC) samples were prepared in three different concentration level, i.e. the lowest limit of quantification (LLOQ, 200 ng/mL),

low (600 ng/mL, LQC), medium (5000 ng/mL, MQC) and high quality control (10000 ng/mL, HQC).

Validation

Following the International Conference on Harmonization (ICH Q2(R1)), the established RP-PLC method was validated.[12]

System suitability

System suitability test was carried out to ensure that the system performance is preferable for the intended application, i.e., estimation of LE in SMEDDS in this case. Various parameters *viz.* retention time, column efficiency, tailing factor, peak area, resolution, and column efficiency were determined for six replicates of the same concentrations. These parameters helped in the verification of the complete recital of the HPLC system. The acceptance criteria to establish system suitability of LE resolution > 2, tailing factor < 2, RSD ≤ 2% for the retention time and peak area, and theoretical plate > 2000.

Linearity and range

The method is considered linear if the obtained test findings are directly proportional to the analyte concentration. Six standard LE calibration solutions in the concentration range of 200 ng/mL to 12000 ng/mL were produced. LE's peak area. The data obtained for LE was used to plot the linearity curve i.e peak area of LE on y-axis and the LE standard solutions concentration on the x-axis.

Limits of detection and quantification

The lowest concentration of analyte that can be identified and separated from the noise is known as the limit of detection (LOD). The lowest level of analyte concentration that can be detected with an acceptable level of precision and accuracy is known as the limit of quantification (LOQ). The signal-to-noise ratio (S/N) of 3:1 and 10:1 were used to determine LOD, and LOQ. The lower limit of quantification (LLOQ) is represented by the first point on the calibration curve. It was analyzed by using 6 replicates.

$$\text{LOD and LOQ} = \frac{\text{Concentration of standard}}{\frac{S}{N}\text{ value of standard}} \times \text{Desired}\frac{S}{N}\text{value}$$

Precision and accuracy

The method was said to be precise when multiple replicates can be analyzed repetitively at three different points of time. The method was accurate if the observed value is in close agreement with the true value. Six replicates of the three QC and LLOQ samples were analysed on the same day to determine intraday precision and accuracy. Six replicates of the QC samples were analysed for three days along with LLOQ to find out interday precision and accuracy. The percent RSD (relative percentage deviation) was determined. Their acceptance criterion was limited to RSD ≤2 %. The % bias (≤2 %) and % recovery (≤2 %) was calculated for accuracy determination.

Specificity and forced degradation studies

The specificity of LE was determined by spiking a 100 µg/mL standard solution of LE into the self-micro emulsifying excipients (oleic acid, tween 80, and propylene glycol). The obtained chromatogram was assessed for the existence of interfering peaks related to LE. Moreover, the specificity of LE was also confirmed by conducting forced degradation studies. Briefly, forced degradation conditions include acidic or basic pH, thermal, and oxidation. Initially, 5 mg of LE was dissolved in 5 mL of acetonitrile and kept as a standard stock solution. For acid and base degradation, 1 mL of LE stock solution was spiked individually into 0.1 N hydrochloric acid solution, 0.5 M sodium hydroxide, 3% hydrogen peroxide solution, and Milli-Q water and refluxed for 12 hr.[15,16]

Carryover effect

The carryover effect takes place when an analyte from a previous HPLC run reappears in a subsequent run due to sample overloading. It was determined by injecting three concentrations of higher concentrations continuous injections followed by a blank sample. A carryover effect of less than 20% of LLOQ was acceptable.

Robustness

The robustness of the developed analytical method was evaluated by variation in chromatographic conditions, including analytical instruments from Shimadzu (model number LC 2010CHT), Kyoto, Japan to Shimadzu (model number LC-10AT), change in column oven temperature, and change in the organic: Aqueous phase ratio in the mobile phase. The chromatographic parameters *viz.* retention time, efficiency, and tailing factor were evaluated.

Applicability of Method

Preparation of self-micro emulsifying drug delivery systems of loteprednol etabonate

Formulation I: Accurately weighed LE (500 mg) was placed in the glass vial into which Oleic acid (10 %), Polysorbate 80 (60 %), and Propylene glycol (30 %) were added. Then the mixture was magnetically stirred for

3 min at a speed of 200 rpm. The obtained LE loaded SMEDD was stored at room temperature for further characterization.[6,17]

Formulation II: Accurately weighed LE (500 mg) was placed in the glass vial into which LabrafilM2125 (10 %), Polysorbate 80 (62.1 %), and Transcutol P (27.9 %) were added. Then, for the mixture was magnetically stirred for 3 min at a speed of 200 rpm. The obtained LE loaded SMEDD was stored at room temperature for further characterization.[6,17]

Determination of amount of LE in Self micro emulsifying drug delivery systems

The developed method was employed for the quantification of LE content in different SMEDDs formulation. To estimate the amount of drug present in the LE loaded SMEDDs formulation, an aliquot of 1 g of the formulation was added to 5 mL of methanol. The drug dissolved by vortexing and further subjected to centrifugation at 15000 rpm for 15 min at 25°C. The supernatant of 200 µL was diluted by the diluents and filtered through a 0.22 µ membrane filter before HPLC analysis.

RESULTS AND DISCUSSION

To achieve adequate chromatographic conditions for LE, a systematic approach was employed for analytical technique development. The experimental conditions were carried out using Thermo scientific BDS Hypersil C_{18} column (250 × 4.6 mm, 5 µm) with mobile phase composition of acetonitrile – Milli Q water (pH: 5.5 ± 0.1) (50:50, v/v) at a flow rate of 1.0 mL/min. A good chromatogram peak was obtained at 15.79 min with a tailing factor of 1.161. The ability to speed up an HPLC run has evident benefits by reducing analysis time and, cost. Thus to reduce the run time, Supelco, Ascentis C_{18} column (5 cm × 4.6 mm, 5 µm particle size) has been used with different elution systems. Table 1 presents different elution systems with their responses. Table 2 represents the optimized chromatographic conditions.

After six replicate injections of the 5 µg/mL concentrations, the system suitability data revealed that there was no significant change in peak area, retention time, or peak tailing of LE. The tailing factor value was found to be < 2, and RSD ≤ 2 %, thus confirmed the HPLC system's high degree of accuracy. The linearity between the LE and peak area concentration was obeyed in the range of 200 ng/mL to 12 µg/mL with a good R^2 of 0.998. The obtained linear regression coefficient was y = 35.93x + 4903.5. In this 'x' represents the LE concentration, and 'y' represents the peak area at 243 nm in the regression equation. The linearity plot of loteprednol etabonate is shown in Figure 2. The analytical method's LOD and LOQ were estimated to be 60 ng/mL and 180 ng/mL, respectively. The LLOQ

Table 1: Different elution systems with their responses.

Organic phase	Aqueous phase	Ratio of organic to aqueous phase	Tailing factor	Retention time	Observation
Methanol	MilliQ water	50:50 v/v	-	-	No chromatogram was observed with 30 min run time
Acetonitrile	Acetate buffer (pH 5.5)	50:50 v/v	0.847	4.8	Peak fronting
Acetonitrile	Acetate buffer (pH 5.5)	45: 50 v/v	0.923	7.4	-
Acetonitrile	Phosphate buffer (pH 5.5)	50:50 v/v	0.876	4.7	Peak fronting
Acetonitrile	MilliQ water (pH 5.5)	50:50 v/v	0.981	4.7	Good peak chromatogram

Table 2: Chromatographic condition.

Chromatographic Parameter	The optimised condition for loteprednol etabonate
HPLC system	Shimadzu LC-2010HT (Kyoto, Japan)
Column	Supelco, Ascentis C_{18} column (5 cm × 4.6 mm, 5 µm particle size)
Wavelength	243 nm
Mobile Phase	Acetonitrile: milliQ water (pH:5.0±0.2); 50:50 (Isocratic mode)
Flow rate	1 mL/min
Injection volume	20 µL
Run Time	7 min

(*n*=6) was estimated to be 200 ng/mL. The findings showed that the suggested approach was capable of detecting and quantifying LE at nanogram levels. As a result, this approach would be useful for routine LE analysis in SMEDDs containing a low concentration of a drug. The calibration curve data of LE are mentioned in Table 3. The statistical data of the regression equations and validation parameters are presented in Table 4.

Calculating the percent recovery of the Quality control samples of LE was used to determine the correctness of the developed procedures (LLOQ, LQC, MQC, and HQC). Table 5 summarises the findings of the recovery investigations. The percent recovery ranged from 99.00 % to 101.79 %. LE's intraday and interday precision values are presented in Table 5. The RSD of the developed approaches was in the range of 0.0459 % to 0.6470 %. The % RSD obtained in both diluents was 2, and the percent bias was within the acceptable range. This confirmed the consistency of the developed analytical method. Intra and inter-day Precision and Accuracy results are shown in Table 5. Chromatogram representing overlay of LQC, MQC, and HQC (Figure 3). The chromatogram of LE using ACN: Milli-Q water (pH 5.5) in 50:50 ratio and 1 mL/min flow rate demonstrated the absence of interference of any other peak equivalent to formulation excipients at 243 nm compared to standard LE solution. Also, no interference

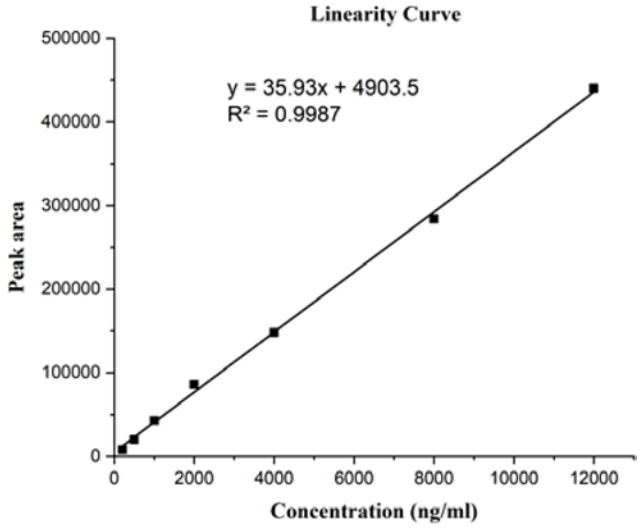

Figure 2: Linearity plot of loteprednol etabonate.

Table 3: Data of Calibration curve peak area.

Concentration (ng/ml)	Peak area ± SD
200	8077 ± 540.23
500	20190 ± 1353.40
1000	42821 ± 1563.41
2000	86098 ± 2486.18
4000	148185 ± 625.08
8000	283945 ± 6085.36
12000	440257 ± 5592.51

SD: Standard deviation

Table 4: Statistical data of the regression equations and validation parameters (*n* = 3).

Regression analysis	
Equation	y = a + b*x
Intercept (a)	4903.55885 ± 3330.7935
Slope (b)	35.92953 ± 0.58197
Pearson's r	0.99934
R-Square	0.99869
Adj. R-Square	0.99843
Validation analysis	
LOD (ng/mL)	60 ng/ml
LOQ (ng/mL)	180 ng/ml
Linearity	0.2 – 12 µg/ml

LOD: Limit of detection; LOQ: Limit of quantification

Table 5: Intra and inter day Precision and Accuracy results (*n*=6).

Sample	Intraday Precision			Accuracy
QC	Amount found (ng/ml) ± SD	% Recovery ± SD	% RSD	% bias
LLOQ	209.46 ± 8.61	104.73 ± 1.4	1.732 ± 0.0084	- 4.5 ± 0.057
LQC	597.6 ± 0.6	99.52 ± 0.075	0.0837 ± 0.0023	0.4778 ± 0.138
MQC	4981.37 ± 33.11	99.62 ± 0.705	0.6470± 0.0184	0.373 ±0.352
HQC	10179.9 ± 27.71	101.79 ± 0.226	0.2686± 0.0075	-1.799 ± 0.357
	Interday Precision			
LLOQ	209.61 ± 4.54	104.8 ± 1.2	1.722 ±0.0054	-4.5 ± 0.047
LQC	594.06 ± 25.72	99 ± 0.14	0.0988± 0.064	0.990 ± 0.033
MQC	4989.5 ± 117.22	99.77 ± 0.510	0.6365± 0.0045	0.230 ± 0.075
HQC	10168.5 ± 169.95	101.69 ± 0.05	0.0459± 0.0014	-1.684 ± 0.024

LLOQ: Lowest limit of quantification (200 ng/ml); LQC: Low quality control (600 ng/mL); MQC: Middle quality control (5000 ng/mL); HQC: High quality control (10000 ng/mL)

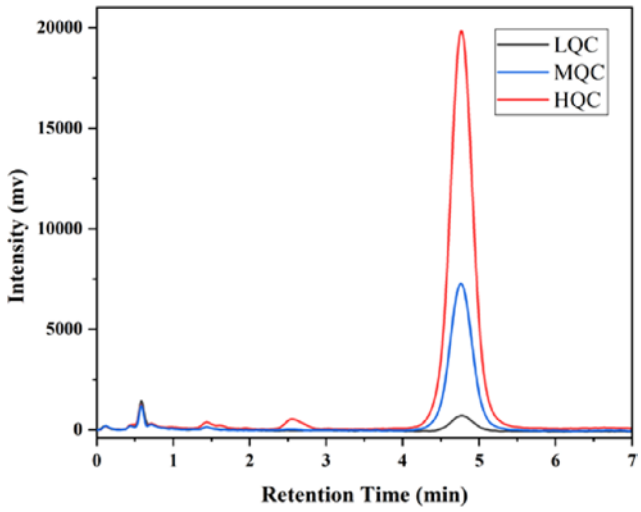

Figure 3: Chromatogram representing overlay of LQC, MQC and HQC.

Table 6: Data of stress testing (*n*=3).

Type of reaction	% Degradation	Other peaks Retention times
Acid degradation	54.99 ± 0.16	0.8, 1.25, 1.75
Base degradation	51.68 ± 0.50	1.2, 1.8, 2.5, 3.2
Oxidation	45.00 ± 0.45	0.8, 1.5
Thermal degradation	22.62 ± 0.63	1.0, 1.2, 1.8, 2.5

of degradants was observed during forced degradation studies at a retention time of 4.7 min. It was observed that when LE was subjected to acid, basic, thermal, and oxidation conditions led to elution of LE degradants. The % recovery and retention time of LE during forced degradation study are presented in Table 6. HPLC chromatograms for stress testing are shown in Figure 4. Figure 5 presents the specificity of LE chromatogram in the existence of lipid excipients.

The robustness of the developed analytical method was evaluated by variation in chromatographic conditions, including analytical instruments from Shimadzu (model number LC 2010CHT), Kyoto, Japan to Shimadzu (model number LC-10AT), column oven temperature to ±5°C and mobile phase ratio to 52: 48. The above said conditions were maintained for the analysis of chromatograms of LQC, MQC, and HQC. There is an insignificant effect observed compared to the standard chromatogram conditions. This obscure that the developed method was stable when inherent parameters changed slightly. To verify the carryover effect of the chromatographic system for the proposed analysis, the carryover effect was performed by three continuous injections of high concentrations of linearity range of LE. Then followed by a blank sample was performed. At the 4.7-min retention time, there was no LE peak in the blank. This demonstrates that this method may be used in a continuous run with a many number of samples.

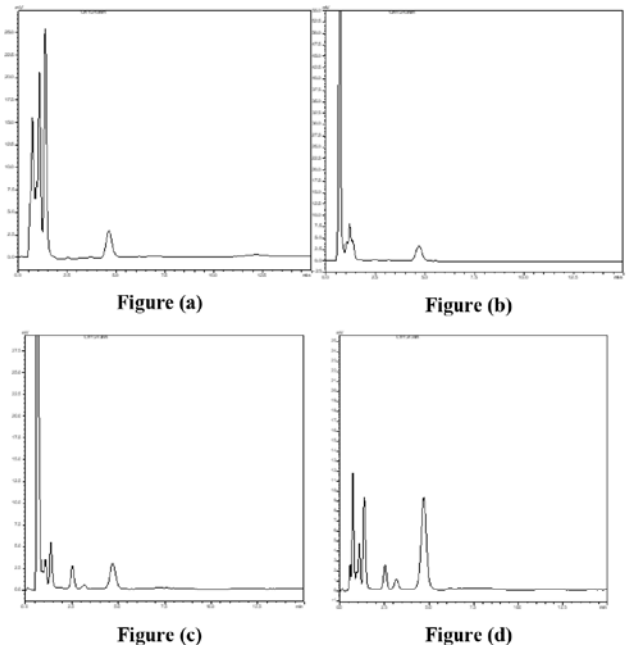

Figure 4: HPLC chromatograms for stress testing (a) acid hydrolysis (b) base hydrolysis (c) oxidation (d) thermal conditions.

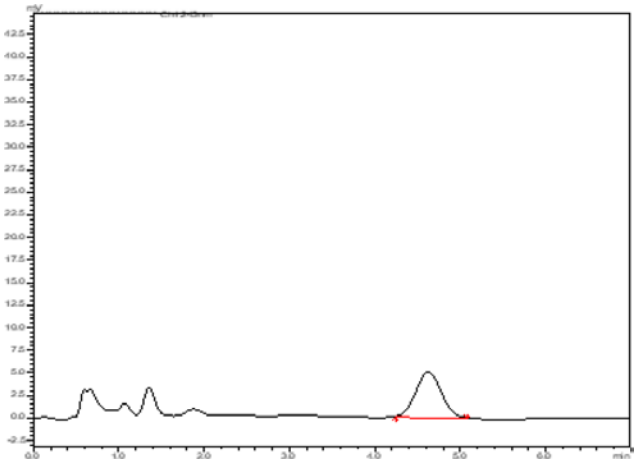

Figure 5: Chromatogram of LE in the presence of lipid excipients.

Table 7: Assay of LE in SMEDDs.

Formulation	% assay ± SD
Formulation I	100.02 ± 0.078
Formulation II	100.5± 0.062

SD: Standard deviation

LE loaded different SMEDDs based formulations were evaluated for assay (*n*=3). The assay results attained from LE loaded SMEDDs are presented in Table 7. Results

demonstrated high reproducibility (% RSD<0.01) and good % recovery (100 ± 0.065 %). The results showed that the validated HPLC method could determine the LE content accurately and precisely without the interference of excipients or the SMEDDs formulation matrix. Furthermore, it also reflected that the method can determine the drug content in complex lipid-based formulation also.

CONCLUSION

An RP-HPLC method was developed and validated as per ICHQ2 (R1) guidelines for LE's quantification. The validated analytical method was simple, robust, and sensitive enough to identify the degradants. Also, the method was successfully applied for the assay study in lipid emulsion formulations. The recovery from formulations has shown good agreement without any interference. Upon application of this validated analytical method for the estimation of LE in self micro emulsifying drug delivery systems demonstrated that the method can determine the degradants and also formulation interferences. This stipulates that the validated analytical method could be useful as stability-indicating method for quantifying LE in pharmaceutical dosage forms in the academic and industrial sectors.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

LE: Loteprednol etabonate; **RP-HPLC:** Reverse phase high performance liquid chromatography; **LOD:** Limit of detection; **LOQ:** Limit of quantification; **ICH:** International Conference on Harmonization; **LLOQ:** Lower limit of quantification; **LQC:** Low quality control; **MQC:** Middle quality control; **HQC:** High quality control; **S/N:** Signal to noise; **RSD:** Relative standard deviation; **SMEDDS:** Self micro-emulsifying drug delivery systems.

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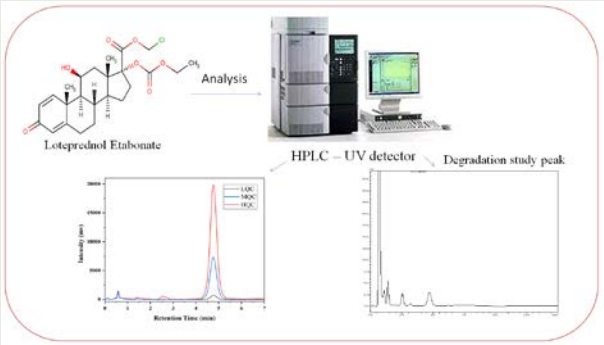
PICTORIAL ABSTRACT



SUMMARY

- Loteprednol etabonate is used as a first-line corticosteroid to treat ocular inflammation of the anterior segment
- This research work optimized the stability-indicating method using RP-HPLC as per ICH guideline and evaluated the stability of Loteprednol etabonate under various stress conditions such as acidic, alkaline, oxidative, and thermal conditions
- The developed method was applied for the determination of Loteprednol etabonate in self-micro emulsifying drug delivery systems
- The developed method is able to detect the degradant peaks and excipient peaks thus this can be implemented for routine analysis of Loteprednol etabonate in the bulk and pharmaceutical dosage forms.

About Authors

Prof. Ranendra Narayan Saha, is a Senior Professor in the Department of Pharmacy, BITS. He has more than 35 years of teaching, research and administrative experience. He is expert in pharmacokinetic and dosage form design. He has successfully completed several industrial and government-funded projects. He has transferred five product technologies to the pharma industry.

Dr. Gautam Singhvi, is working as an Assistant Professor in the Department of Pharmacy, BITS, Pilani. He has more than 14 years of teaching and research experience. Currently, he is involved in industrially feasible nanocarriers-based formulation development and optimization for various therapeutic agents. He has many publications in reputed international and national peer-reviewed journals and filed 6 patents.

Ms. Gorantla Srividya, is currently pursuing Ph.D. under the supervision of Dr. Gautam Singhvi and Prof. R. N. Saha. She has qualified AP-PGECET 2016, achieved rank 97. She has been selected for the DST-INSPIRE fellowship award (INSPIRE Fellowship Registration No. IF190259). She has research experience in the development and characterization of lipid-based nanoformulations for topical delivery, ophthalmic delivery and Targeted drug delivery systems for the treatment of rheumatoid arthritis.

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