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Original Article

SIMULTANEOUS ESTIMATION OF BEPOTASTINE BESILATE AND BENZALKONIUM CHLORIDE IN OPHTHALMIC FORMULATION BY RP-HPLC METHOD

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ABSTRACT

Objective: Development and validation of stability indicating RP-HPLC method for the simultaneous determination of bepotastine besilate (Bepo B) and benzalkonium chloride (BKC) in an ophthalmic dosage form.

Methods: A chromatographic separation of the drug, as well as a preservative, was achieved using Shimadzu HPLC 1100 series consisted of binary pump LC-10 ADvp, Rheodyne universal injector 7725i and Shimadzu SPD-10 UV–Visible detector. The chromatographic separations were performed using Analytical® Hyperchrome ODS C18, 5 µm, 250 mm X 4.6 mm i.d. column with isocratic mobile phase Acetonitrile: phosphate buffer (60:40) pH 5.5. The drug and a preservative were monitored at an ambient temperature and detection wavelength of 210 nm with a flow rate of 1 ml/min and an injection volume of 20 µl.

Results: The mean % recovery at the 80, 100 and 120% level for Bepotastine and benzalkonium chloride was found to be 100.09 and 100.81% respectively and % RSD was found to be 0.21 and 0.85% respectively, which meets the established acceptance criteria. Forced degradation of bepotastine besilate was carried under alkaline, acidic, neutral, oxidative, humidity, thermal and photodegradation conditions and it was analyzed by proposed method. The drug degrades to some extent in all forced degradation condition.

Conclusion: The developed method was validated as per ICH guidelines using validation parameters such as accuracy, precision, linearity and range, robustness, ruggedness, LOD, LOQ, specificity, and system suitability testing. The proposed method can be used for routine analysis stability testing and assay of bepotastine besilate ophthalmic solution in quality control laboratories.

Keywords: Bepotastine besilate (Bepo B), Benzalkonium chloride (BKC)

INTRODUCTION

Bepotastine besilate (Bepo B) is a selective histamine H1-receptor antagonist. Bepreve™ (bepotastine besilate ophthalmic solution), 1.5% is a sterile ophthalmic solution of bepotastine besilate proposed for the treatment of itching associated with signs and symptoms of allergic conjunctivitis in patients aged 3 y or older. The proposed dosage and route of administration for Bepreve™ (bepotastine besilate ophthalmic solution), 1.5% is as follows: instill one drop into the affected eye(s) twice a day (BID). Bepotastine besilate (also known as TAU-284 and SNJ1773) was originally developed in Japan by Ube Industries, Ltd. and Tanabe Seiyaku Co., Ltd. as a treatment for allergic rhinitis. An oral preparation of bepotastine besilate (Talion® tablets, Mitsubishi Tanabe Pharma Corporation [formerly Tanabe Seiyaku Company, Ltd.]) was approved in Japan in July 2000 and launched in October 2000. In January 2002, the additional indication of pruritus/itching accompanying urticaria and other skin diseases was approved in Japan [1-2].

A clinical trial study showed that bepotastine, cetirizine, fexofenadine, and olopatadine inhibit the histamine-induced wheal-and-flare response of humans *in vivo* and induce a variable systemic sedative effect and impaired psychomotor activity [3]. Bepotastine was generally well tolerated in adult and paediatric patients with Allergic conditions. It was also noticed that bepotastine (20 mg/day) was significantly more effective than terfenadine (120 mg/day) in patients with perennial allergic rhinitis. Although a number of studies have been made to evaluate clinical efficacy and safety of bepotastine, scarcely any literature is available for its estimation [4].

Bepotastine besilate (Bepo B) chemically known as ({d-(S)-4-[4-[(4-chlorophenyl) (2-pyridyl) methoxy] piperidino} butyric acid monobenzene sulphonate), is a new second-generation antihistamine developed in Japan. It reduces the natural chemical histamine in the body which can produce allergic symptoms of itching or watery eyes. The chemical structure of bepotastine besilate is depicted in fig. 1.

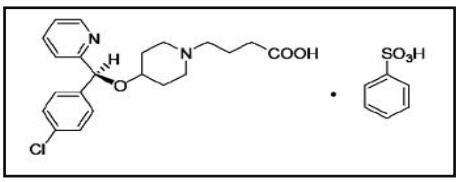

Fig. 1: Structure of bepotastine besilate

Benzalkonium chloride (BKC), a typical quaternary ammonium salt, is often used as an antiseptic. Its structure is shown in fig. 2, and the C12 homolog is the major species in a benzalkonium chloride preparation [5]. The mode of antiseptic action of quaternary ammonium compounds appears to be associated with their effect on the cytoplasmic membrane that controls cell permeability, and the C12 homolog is most effective against yeast and fungi [6].

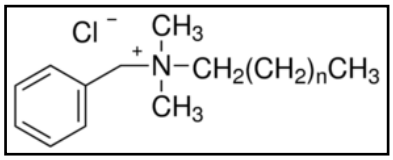

Fig. 2: Structure of benzalkonium chloride

A literature survey reveals that Mamta D *et al.*,[7] developed LC-MS/MS method for estimation of bepotastine besilate. Sharath P *et al.*,[8] developed RP-HPLC method for estimation of Bepotastine Besilate only. Narasimha K *et al.*,[9] has given stability indicating HPLC method for the quantification of bepotastine besilate and its related substances. It is necessary to estimate % purity of drug as

well as a preservative in an ophthalmic formulation, hence it was thought worthwhile to develop and validate the stability indicating HPLC method for simultaneous estimation of bepotastine besilate and benzalkonium chloride.

Literature survey revealed that separate chromatographic conditions have been used for determination of drug and preservative in ophthalmic dosage forms. So, it was thought worthwhile to develop a method in which both drug and preservative content can be determined in the same chromatographic condition.

MATERIALS AND METHODS

Chemicals

API (bepotastine besilate-100.4% purity) was supplied by Bal Pharma Ltd. Bommasandra Industrial area, Bangalore, India. Bepotastine besilate ophthalmic solution 1.5% is available under the brand name Bepreve™ by ISTA Pharmaceuticals, Inc. Irvine, CA. Methanol; Acetonitrile (HPLC grade) was obtained from Finar Limited, Ahmadabad, India. Furthermore, Triethylamine (HPLC grade) procured from Himedia Laboratories, Mumbai, India. Potassium dihydrogen phosphate, sodium hydroxide, hydrochloric acid and hydrogen peroxide (AR grade) obtained from Qualigens Fine Chemicals, Mumbai, India.

Chromatographic condition and equipment

The HPLC system consisted of Shimadzu HPLC 1100 series consisted of binary pump

LC-10 ADvp, Rheodyne universal injector 7725i and Shimadzu SPD-10 UV–Visible detector. The chromatographic separations were performed using Analytical® Hyperchrome ODS C18, 5 µm, 250 mm X 4.6 mm i.d. column, at ambient temperature, eluted with mobile phase at the flow rate of 1.0 ml/min. The mobile phase consisted of acetonitrile and potassium dihydrogen phosphate buffer (60:40, v/v), apparent pH adjusted to 5.5±0.1 with phosphoric acid solution, filtered through 0.45 µm nylon filter and degassed in ultrasonic bath prior to use. Wavelength was selected by scanning standard solutions of both drugs over 200 to 400 nm wavelengths using Shimadzu model 1601 double beam UV–visible spectrophotometer with a pair of 10 mm matched quartz cells. Measurements were made with injection volume 20 µL and ultraviolet (UV) detection at 210 nm, as both components show a reasonable good response at this wavelength.

Optimized chromatographic conditions

Column-Analytical® Hyperchrome ODS C18, 5 µm, 250 mm X 4.6 mm

Mobile Phase-Acetonitrile and KH_2PO_4 buffer, pH 5.5 (60:40 v/v)

Detection Wavelength-210 nm

Flow rate-1.0 ml/min

Temperature: Ambient-28-30 °C

Injection volume-20 ml

Preparation of solutions

Standard stock solution of bepotastine besilate

A Standard stock solution of bepotastine Besilate (0.5 mg/ml) was prepared by dissolving 12.5 mg of bepotastine besilate in 25 ml mobile phase.

Standard stock solution of benzalkonium chloride

A Standard stock solution of benzalkonium chloride (1 mg/ml) was prepared by dissolving 25 mg of benzalkonium chloride in 25 ml mobile phase.

Pharmaceutical dosage form solution

2.5 ml of pharmaceutical dosage form was transferred to 50 ml volumetric flask spiked with 5 ml of benzalkonium chloride (1 mg/ml) about 30 ml of mobile phase was added and sonicated for 20 min. The volume was completed to the mark with mobile phase to obtain a sample stock solution of 1500 µg/ml bepotastine Besilate and 210 µg/ml of benzalkonium chloride.

Concentrations ranging from 50-200 µg/ml for bepotastine Besilate and 10-100 µg/ml for benzalkonium chloride were prepared from stock solution and different validation parameters were performed.

Buffer preparation

Phosphate buffer was prepared by dissolving 1.36 gm of potassium dihydrogen phosphate in 400 ml double distilled water 0.5 ml triethylamine was added to it and pH was make up to 5.5 using dilute orthophosphoric acid, and final volume was made up to 500 ml with double distilled water.

Validation procedure

Validation of the newly developed method was studied in terms of accuracy, precision, linearity and range, robustness, ruggedness, limit of detection (LOD), limit of quantitation (LOQ), specificity and system suitability testing as per ICH guidelines [10].

System suitability study was done to check the performance of the system and the response after replicate injection of the standard solution. The chromatogram of standard bepotastine, std benzalkonium chloride and mix standard are shown in fig. 3-5.

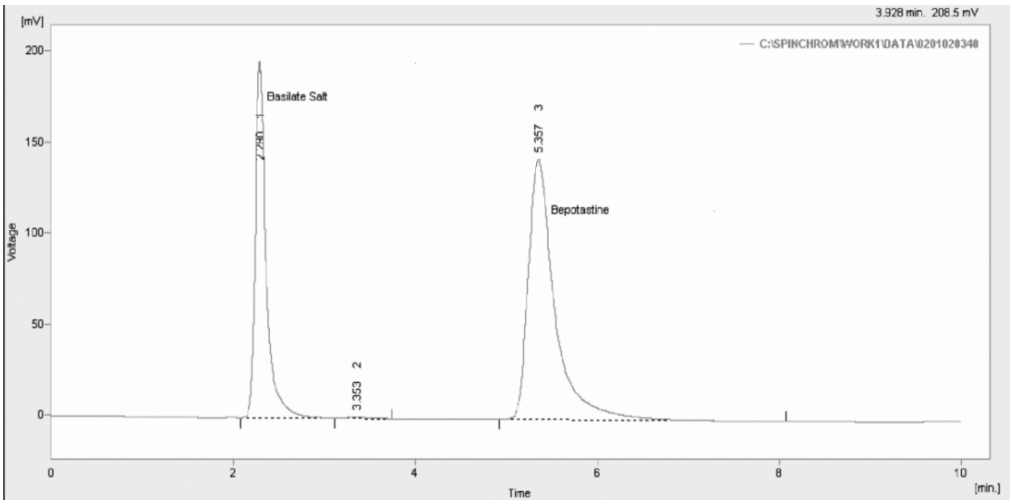

Fig. 3: Chromatogram of STD bepotastine besilate

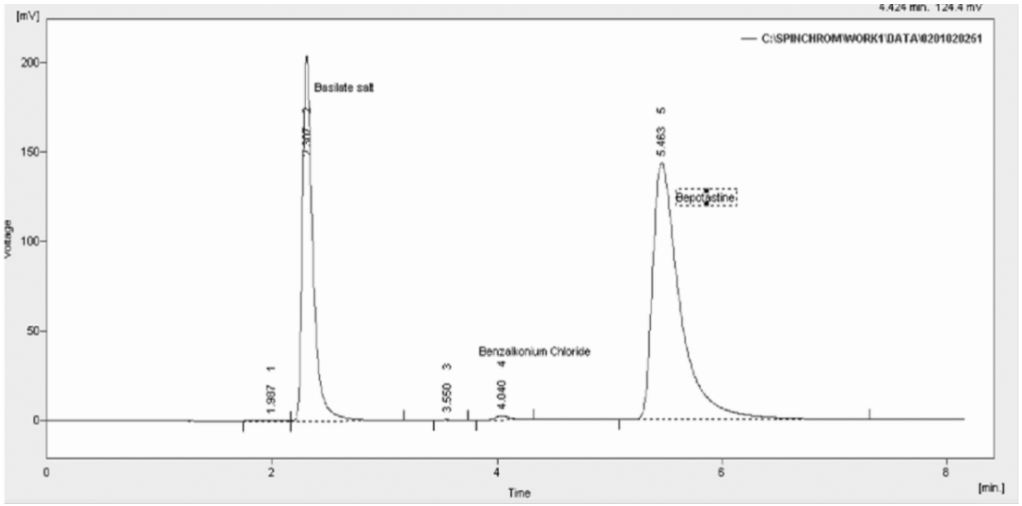

Fig. 4: Chromatogram of mixed standard bepotastine basilate and benzalkonium chloride

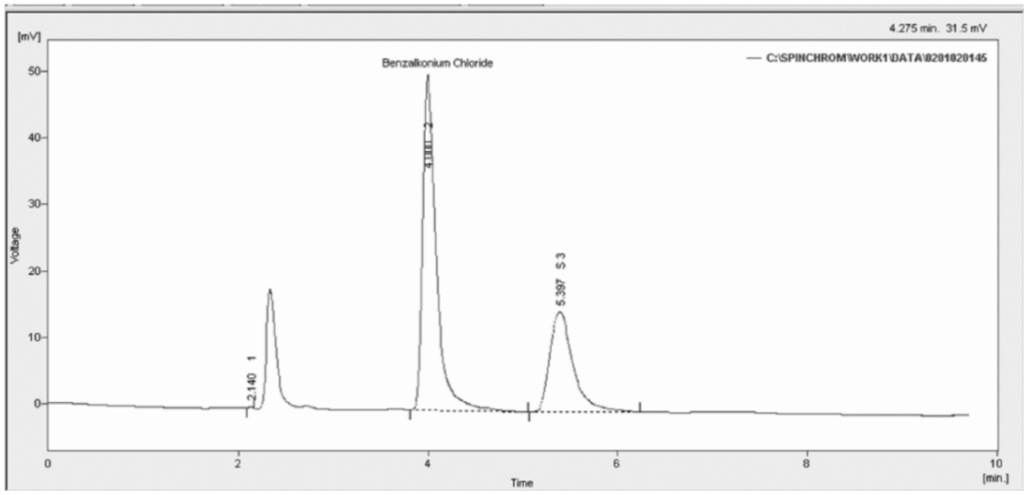

Fig. 5: Chromatogram of standard benzalkonium chloride

The accuracy of the method was determined by calculating percentage recoveries of bepotastine and benzalkonium chloride respectively by standard addition method. A known amount of standard solution of Bepotastine (60, 90 and 120 µg/ml) was added to sample solution of Bepotastine (60µg/ml). Similarly, a known amount of standard solution of benzalkonium chloride (16, 20 and 24 µg/ml) was added to sample solution of benzalkonium chloride (1 µg/ml). The precision of the method was studied by checking repeatability, interday and intraday precision for single concentration thrice. For linearity and range the solutions were prepared from a stock solution of bepotastine Besilate (500 µg/ml) ranging from 50-200 µg/ml and from a stock solution of benzalkonium chloride (1000 µg/ml) ranging from 10-100 µg/ml, the calibration curve was generated, and regression parameters were obtained.

Robustness of the method was studied by changing the flow rate by±0.2 ml/min, change in wavelength by±5 units, change in pH by±0.2 units and change in mobile phase composition. The changes in the response of bepotastine were noted and compared with the normal condition.

Specificity: forced degradation studies

Forced degradation of drug product was carried out under thermolytic, relative humidity, acid/base hydrolytic and oxidative stress conditions. Thermal and relative humidity degradation of the standard drug were carried out in the solid state. After the degradation stock solutions were prepared by dissolving in the mobile phase. From these solutions, aliquots were diluted with mobile phase to achieve a concentration of 150 µg/ml of bepotastine besilate and 21 µg/ml of benzalkonium chloride based on the labeled strength. For thermal stress, samples of drug substances and drug product were placed in a controlled temperature oven at 60 °C for 3 hr. Acid hydrolysis of drug substance and drug product in solution state was conducted with 0.1 N hydrochloric acid at 50 °C for 3 hr. Base hydrolysis of drug product was conducted by 1N sodium hydroxide solution at 50 °C for 3 hr. For oxidative stress, sample solutions of a drug product in 3% hydrogen peroxide were kept at 50 °C for 3 hr. The humidity effect was carried in the solid state, kept at 75% RH at ambient temperature for 48 h.

RESULTS

System suitability study

System suitability parameters recorded during the experimentation are shown in table 1.

Accuracy

The accuracy of the method was determined for bepotastine besilate and benzalkonium chloride by spiking its stock solution in a blank matrix in triplicate at levels 80, 100, 120% of the specified limit. The mean % recovery at the 80, 100 and 120% level for Bepotastine and benzalkonium chloride was found to be 100.09 and 100.81% respectively and % RSD was found to be 0.21 and 0.85% respectively, which meets the established acceptance criteria. Thus, the study proves that the method is accurate in the considered range (table 2 and 3).

Table 1: Observation of system suitability parameter

S. No.	AUC of BKC	AUC of Bepo B
1.	20.086	2451.739
2.	19.983	2450.697
3.	20.134	2452.042
4.	20.010	2451.686
5.	20.045	2450.304
Mean	20.051	2451.2936
±SD	0.0596	0.7496
%RSD	0.30	0.03
Theoretical plate per column	5941	3139
Retention Time	4.040	5.467
Resolution	-	4.665
Asymmetry	1.268	1.628

Table 2: Result of recovery study for bepotastine

Level	Wt. of sample taken	Area response (mV)	Added amount (µg/ml)	Recovered amount (µg/ml)	% recovery	Mean % recovery±SD	% RSD
80%	1 ml~ 60µg/ml	1961.112	60	60.0	100.0	100.1±0.1	0.1%
		1952.739	60	60.07	100.1		
		1970.013	60	60.09	100.2		
100%		2453.101	90	89.60	99.56	99.88±0.2872	0.29%
		2459.139	90	90.03	100.0		
		2460.489	90	90.12	100.1		
120%		2942.106	120	120.40	100.3	100.3±0.3	0.30%
		2930.897	120	120.12	100.1		
		2963.784	120	120.56	100.5		
Mean						100.09	0.21%

Table 3: Result of recovery study for benzalkonium chloride

Level	Wt. of sample taken	Area response (mV)	Added amount (µg/ml)	Recovered amount (µg/ml)	% Recovery	Mean % Recovery±SD	% RSD
80%	2.5 ml~1µg/ml	17.124	16	16.07	100.4	100.4±0.4	0.40%
		17.322	16	16.12	100.8		
		17.017	16	16.01	100.0		
100%		21.105	20	20.32	101.6	101.8±1.5099	1.48%
		21.830	20	20.67	103.4		
		21.009	20	20.08	100.4		
120%		25.197	24	24.16	100.7	100.25±0.3931	0.39%
		24.996	24	23.99	99.96		
		25.268	24	24.27	100.1		
Mean						100.81	0.85%

Specificity

The forced degradation studies were performed to check the possible degradation of an active pharmaceutical ingredient when exposed to various conditions such as acidity, alkalinity, oxidative, neutral and thermal conditions.

The analytical method was used to measure the analyte response in the presence of its degradation products. The degradation results for all stress conditions are discussed (table 4). The overlain chromatograms of various stress conditions are shown in fig. 6a-i respectively.

Assay

Analysis of samples of marketed bepotastine besilate ophthalmic solution containing 1.5% bepotastine besilate was carried out, the chromatogram of formulation recorded is shown in fig. 7. % Assay for Bepo B and BKC was found to be 100.50 and 51.648% respectively. (table 5).

Precision

The precision of the proposed method was determined by carrying out repeatability and intermediate studies.

Table 4: Summary of stability testing

S. No.	Stressed condition	% degraded drug	
		Bepo B Std exposed	Bepo B eye drop exposed
1	1N NaOH	5.640±0.02	33.70±0.11
2	0.5N NaOH	2.22±0.13	27.15±0.34
3	1N HCl	10.56±0.08	21.92±0.21
4	0.5 N HCl	7.60±0.32	20.65±0.36
5	Neutral	2.00±0.11	12.09±0.51
6	3%H_2O_2	0.00	11.27±0.43
7	Humidity (40 °C/75%RH)	7.08±0.19	2.29±0.28
8	UV light	7.86±0.36	10.27±0.53
9	60°C Dry heat	10.44±0.29	0.00

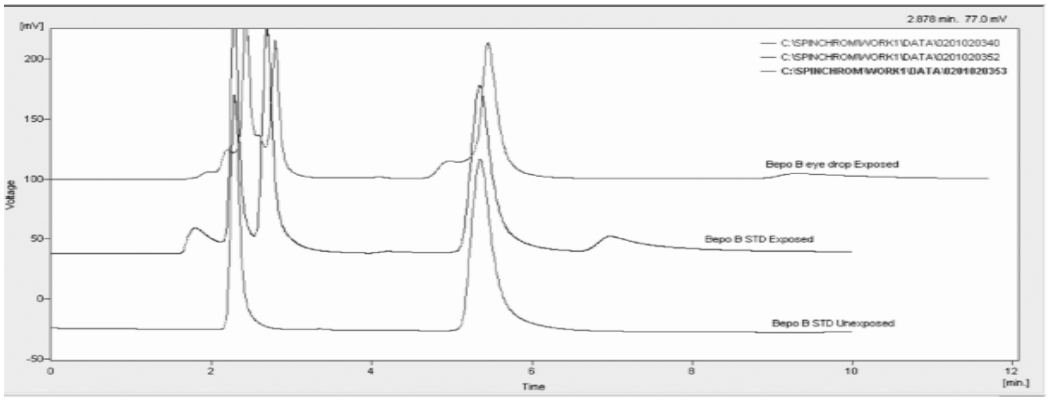

Fig. 6a: 1 N NaOH

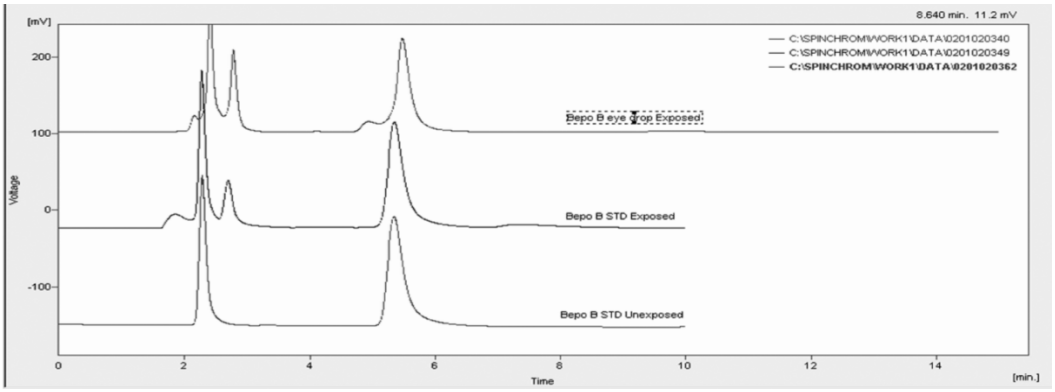

Fig. 6b: 0.5 N NaOH

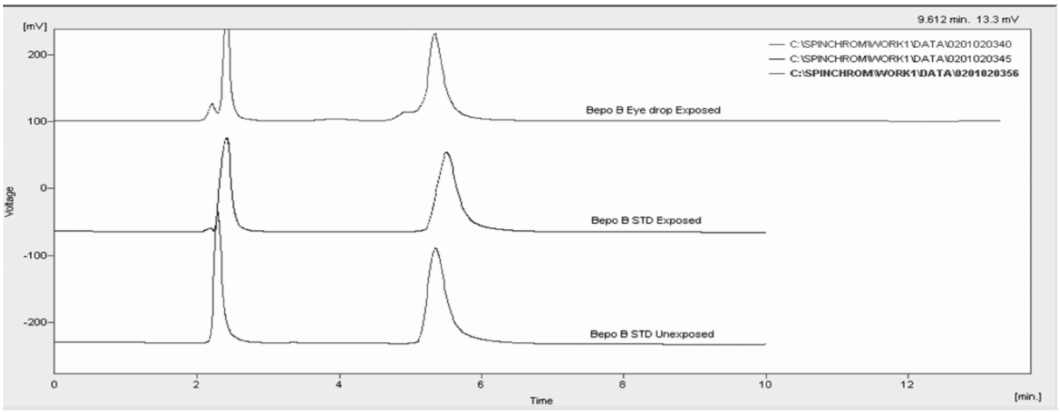

Fig. 6c: 1 N HCl

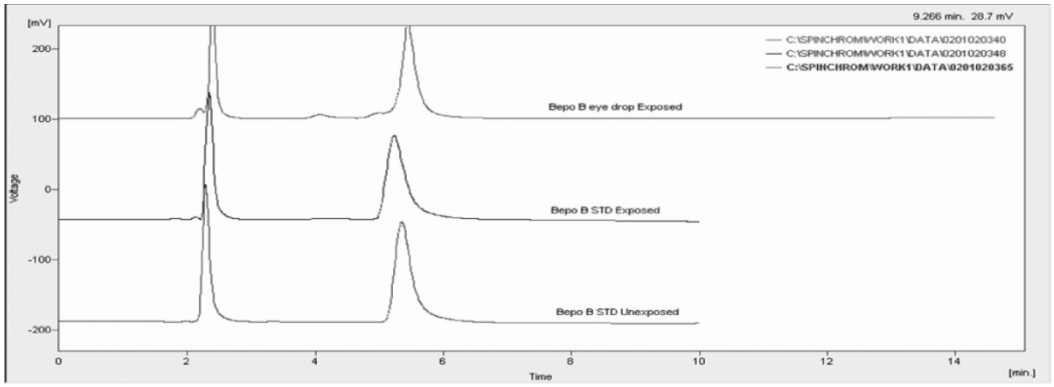

Fig. 6d: 0.5 N HCl

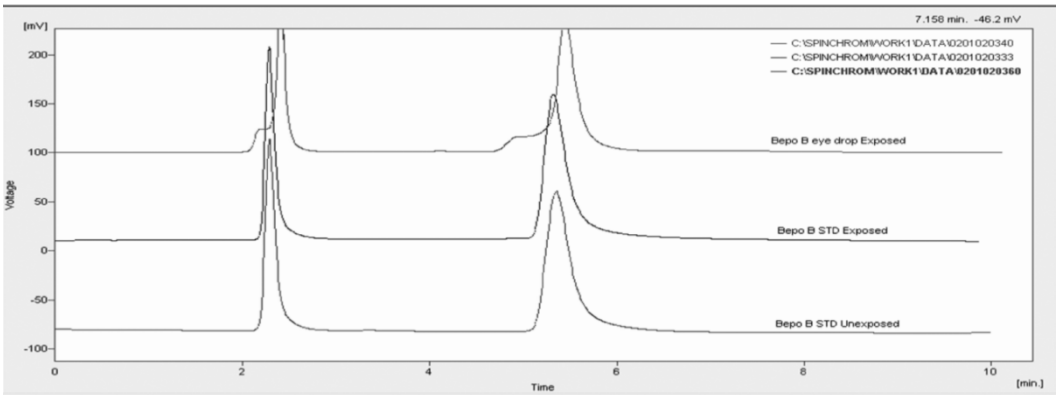

Fig. 6e: Neutral condition

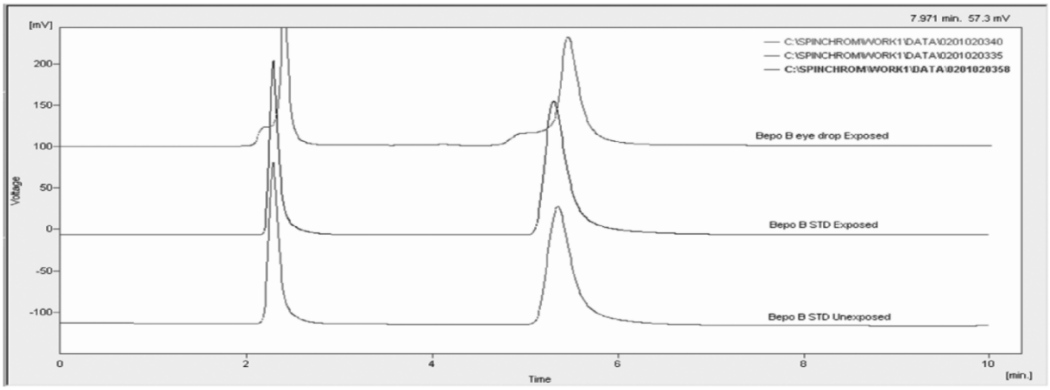

Fig. 6f: 3% H_2O_2

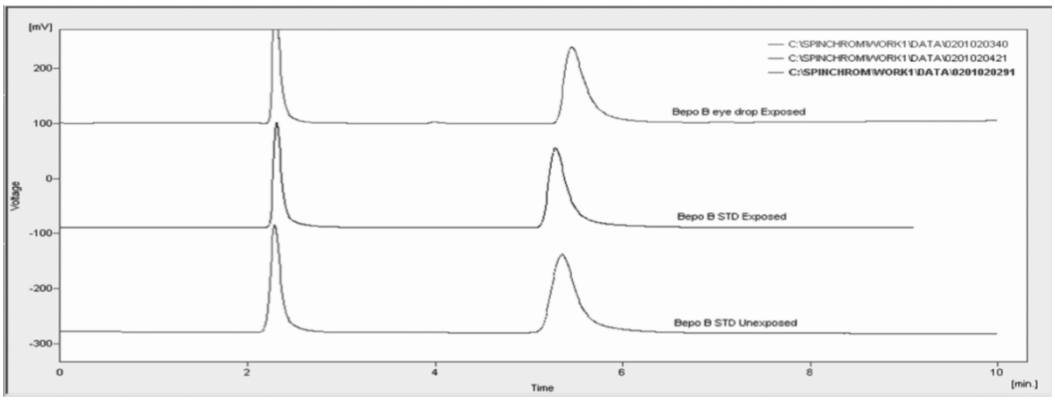

Fig. 6g: Humidity (40 °C/75% RH)

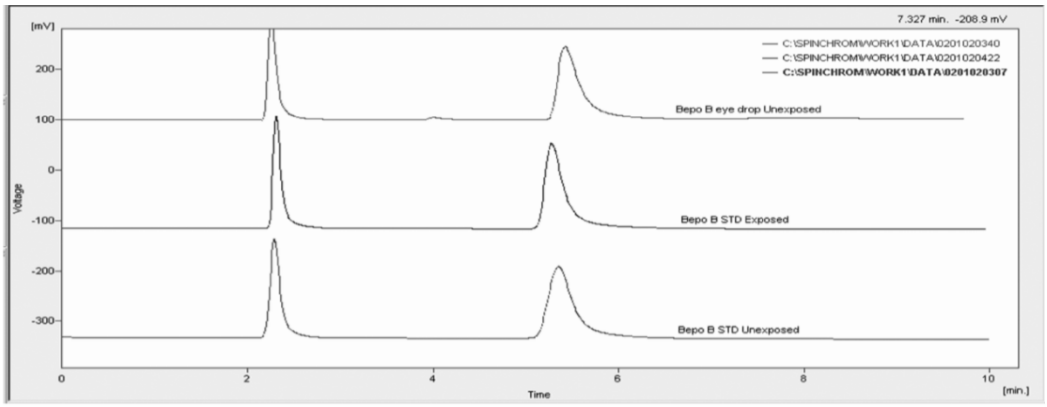

Fig. 6h: UV light

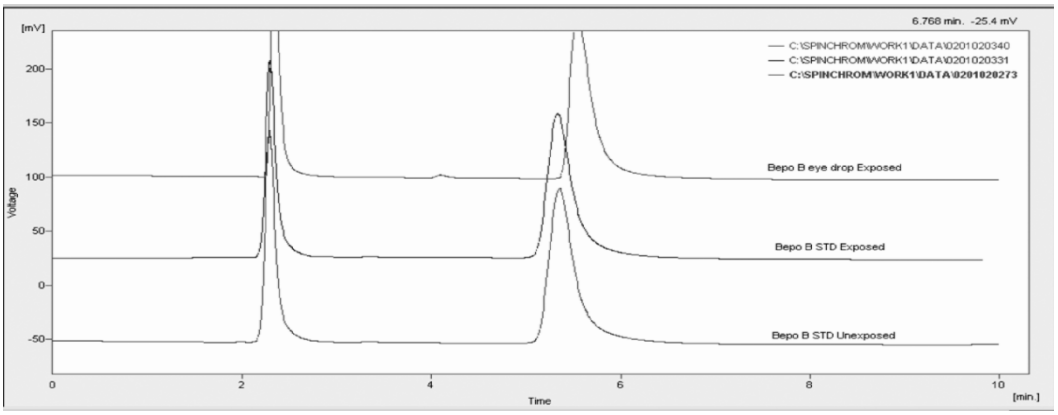

Fig. 6i: 60 °C dry heat

Fig. 6a-i: Overlain chromatogram of standard and sample under Stressed conditions

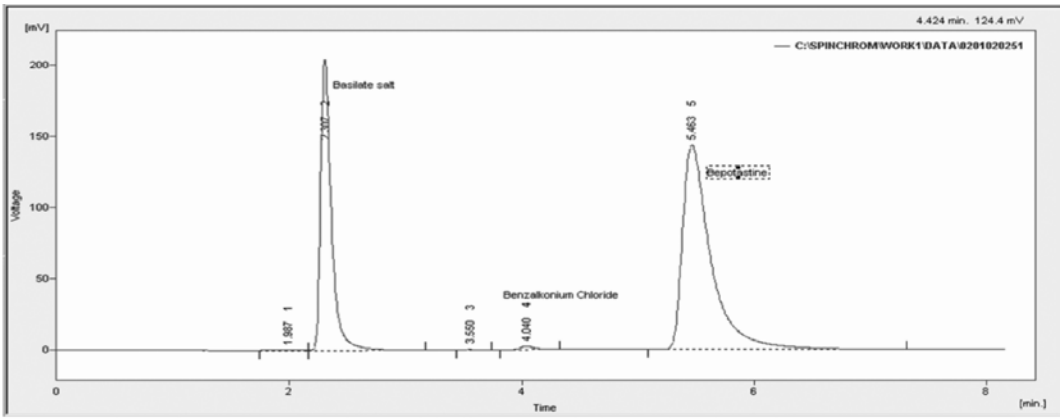

Fig. 7: Chromatogram of Bepotastine Basilate ophthalmic formulation

Table 5: Results of estimation in marketed formulation

S. No.	Amt. of formulation taken (mL)	Detector response (peak area)				% Label claim	
		Bepo B STD	BKC STD	Bepo B Sample	BKC Sample	Bepo B	BKC
1	~2.5 ml			2480.476	21.107	101.2	51.82
2				2490.760	21.113	101.6	51.20
3		2451.739	20.086	2454.562	21.105	100.1	51.82
4				2452.145	21.132	100.0	52.00
5				2443.917	21.120	99.63	51.40
Mean						100.50	51.648
±SD						0.8465	0.333
%RSD						0.84	0.65

The repeatability was checked by repeatedly (n=5) injecting sample solutions and computing the relative standard deviation (%RSD) of the assay results. The % RSD observed was 0.84% for Bepo B and 0.65% for BKC, which was well within the acceptance criteria and the study concludes repeatability of the method. The method precision was repeated on different days, by a different analyst. The SD was calculated and found to be 0.15, 0.85 and 0.99 respectively which was within the limit. Hence, the method is precise and rugged. (table 6).

Table 6: Results of intermediate precision

Parameters	Mean % label claim±SD of bepotastine
Different Analyst (n=3)	100.38±0.15
Intraday variation (n=5)	99.99±0.85
Interday variation (n=5)	98.71±0.99

LOD and LOQ

LOD and LOQ were estimated from the calibration curve for Bepo B was found to be 0.05430 µg/ml and 0.01646µg/ml respectively.

LOD and LOQ were calculated by using formula LOD = 3.3 × N/B and LOQ = 10 × N/B were used, where N is the standard deviation of the response and B is the slope of the corresponding calibration curve.

Robustness

To prove the reliability of the analytical method during the normal usage, some small but deliberate changes were made in the analytical method. The robustness of the method was studied by changing the flow rate (0.8, 1.2 ml/min), wavelength (215 and 205 nm), pH (5.3 and 5.7) and mobile phase composition, the retention time, theoretical plates, asymmetry factor were observed. Theoretical plates and asymmetry values were found well within acceptance criteria. Thus, the study proves the reliability of the test method for minor changes under chromatographic conditions. Hence, the method can be termed as robust. (table VII).

Table 7: Observation and results of robustness study

S. No.	Deliberate changes	RT	Asymmetry	Theoretical Plates
1	Standard condition	5.467	1.628	3139
2	Change in flow rate (0.8 ml/min)	6.563	1.529	3623
3	Change in flow rate (1.2 ml/min)	4.513	1.256	3126
4	Change in pH (5.3)	5.491	1.367	3089
5	Change in pH (5.7)	5.376	1.561	3156
6	Change in wavelength (215 nm)	5.477	1.444	3331
7	Change in wavelength (205 nm)	5.457	1.485	3211
8	Change in mobile phase(55:45)	5.404	1.315	3132
9	Change in mobile phase(65:35)	5.412	1.232	3069
CV		0.0944	0.0915	0.0541
Overall CV		0.0800		

Table 8: Statistical comparison of the results obtained by applying the proposed HPLC method for determination of bepotastine Besilate and benzalkonium chloride simultaneously and the reported HPLC method for determination of bepotastine besilate in bulk drug and ophthalmic formulation

Items	HPLC method for estimation of Bepotastine and Benzalkonium chloride		Reported method (only for estimation of Bepo B)
	Bepo B	BKC	Bepo B
Mean	100.50	51.648	98.70
% RSD	0.84	0.65	ND
N	5	5	5
Students' t-test	4.735 (5.893)*		
F-value	1.244 (4.21)**		

fig. between parenthesis represent the corresponding tabulated value of t at P=0.001, ** fig. between parenthesis represent the corresponding tabulated value of F at P=0.05

Linearity and range

Accurately weighed quantities equivalent to 80, 90, 100, 110 and 120% of label claim (bepotastine besilate) were taken and dilutions were made as described under marketed formulation. Then, each solution was injected and chromatograms were recorded. The correlation coefficient was found to be of Bepotastine 0.9993 and 0.9982 for BKC.

DISCUSSION

The main aim of the chromatographic method was to separate bepotastine Besilate and benzalkonium chloride in a single mobile phase with similar chromatographic conditions. Sharath *et al.*,[8] revealed that separate chromatographic conditions were used for estimation of drug and benzalkonium chloride specifically in the ophthalmic formulation. Previously methanol: buffer system was used as a mobile phase, but benzalkonium chloride cannot be resolved from bepotastine. Similarly, when ACN: Buffer system has used both bepotastine as well as benzalkonium chloride having theoretical plates 3139 and 5941 respectively, with the resolution of 4.665.

Hence, The objective of the study to develop and validate the stability indicating HPLC method for simultaneous estimation of Bepo B and BKC was accomplished by using Analytical® Hyperchrome ODS C18, 5 µm, 250 mm X 4.6 mm i.d. column, flow rate 1 ml/min, mobile phase Acetonitrile: buffer (60:40 v/v) was found to be giving better resolution. % RSD for accuracy study for Bepo B and BKC was found to be 0.21 and 0.85% respectively, which meets the established acceptance criteria. The % RSD for the precision study was found to be 0.84% for Bepo B and 0.65% for BKC. % RSD for Intermediate precision study was found to be 0.66% which is within specified limits hence it can be said that the method is precise. Theoretical plates and asymmetry values were found well within acceptance criteria in robustness. From the specificity study it can be said that standard bepotastine besilate is soft susceptible to degradation as degradation is below 15% and no relevant degradation product is observed and bepotastine besilate eye drop is overdone susceptible to degradation as degradation is above 15% in acidic and alkaline condition.

Unknown impurity was found at RRT 0.9093 in the acidic and alkaline condition which does not match with the reported impurities given by Narasimha R. K. *et al.*, [9] which may be generated because of excipients present in the formulation.

CONCLUSION

The proposed method was found to be simple, precise, accurate and rapid for determination of Bepo B and BKC from pure and its ophthalmic dosage forms. The sample recovery in a formulation was in good agreement with their respective label claims and they suggested non-interference of formulation excipients in the estimation. Hence, this method can be easily and conveniently adopted for routine analysis of Bepo B in pure and its dosage forms.

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CONFLICT OF INTERESTS

Declared none

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