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Original Research Article

Development and validation of RP-HPLC method for simultaneous estimation of three components in cream formulation used in hemorrhoids disease

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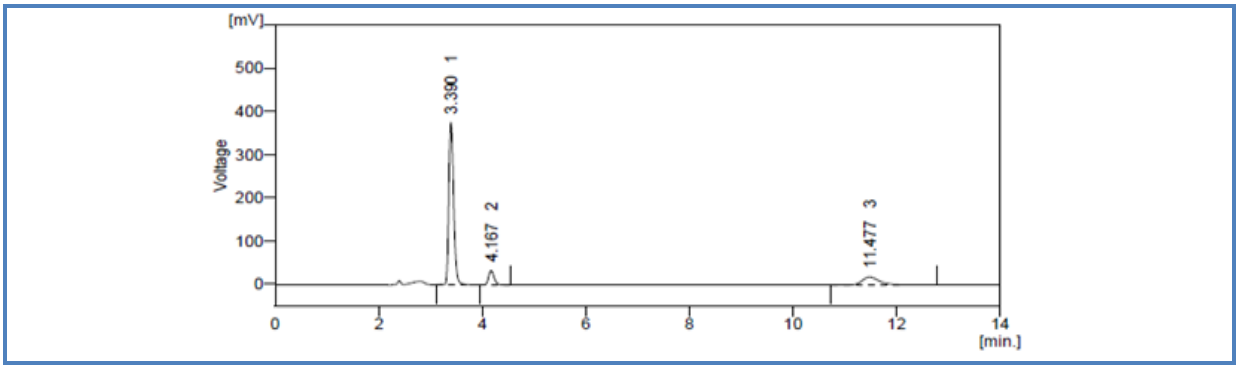
ABSTRACT

Developing a single analytical method for the determination of individual drug from a multidrug composition is a very difficult task. The present work describes a simple, rapid, precise reverse phase chromatographic method that has been developed and validated for simultaneous estimation of multicomponent cream formulation containing Lignocaine HCl, Beclomethasone dipropionate and Phenylepherine HCl. The estimation was carried out on Hypersil BDS C_{18} (5 µm × 25 cm × 4.6 mm i.d.) column using a mixture of ammonium acetate buffer pH 5.0 and methanol in the ratio 60:40 (v/v) as a mobile phase, at a flow rate of 1.0 mL/min and detection was performed at 222 nm. Three drugs, Lignocaine HCl, Beclomethasone dipropionate and Phenylepherine HCl, were eluted at the retention times of 3.3 min, 4.1 min and 11.49 min, respectively. The method was validated for accuracy, precision, linearity, specificity and sensitivity as per ICH guideline. The validated method is a rapid and cost effective and successfully applied to the commercially available pharmaceutical dosage form, yielding a very good and reproducible result.

Graphical Abstract



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Introduction

The skin Hemorrhoids are swollen veins located around the anus or in the lower rectum. About 50 percent of adults experience the symptoms of hemorrhoids by the age of 50. Hemorrhoids can either be internal or external. Internal hemorrhoids develop within the anus or rectum. External hemorrhoids develop outside of the anus. Hemorrhoids are also known as piles. External hemorrhoids are the most common and most troublesome. Hemorrhoids cause pain, severe itching, and difficulty in sitting. Fortunately, they are treatable [1, 2]. Combination of Lignocaine HCl, Beclomethasone dipropionate and Phenylepherine HCl cream were used in Hemorrhoids. Lignocaine Hydrochloride (LH) is chemically 2-(diethylamino)-*N*-(2, 6-dimethylphenyl) acetamide hydrochloride. This drug belongs to the widest used local anesthetic agents applied in regional management of major pain, administered spinally and epidurally or peripherally [3–5]. Beclomethasone dipropionate (BEC) chemically is 9 α-chloro-11 β-hydroxy-16 β-methyl-3, 20-dioxopregna-1, 4-diene-17, 21-diyl dipropionate [6]. Phenylepherine HCl (PEH) is chemically R)-1-(3-hydroxyphenyl)-2-methylamino-ethanol hydrochloride [8]. According to the detailed survey of analytical literature, various HPLC methods have been reported for the estimation of LH [9–12], BEC [13–15] and PHE [16, 17] individually or with other drugs but none of the reported analytical methods are available for simultaneous estimation of LH, BEC and PEH in their combined dosage form. None of the reported analytical procedures describes a simple and satisfactory RP-HPLC method for simultaneous determination of LH, BEC and PEH in their combined dosage forms. So, the objective of this work was to develop simple, precise, and rapid RP-HPLC methods for the combination of drug cream formulation containing LH, BEC and PEH.

Results and Discussion

Optimization of mobile phase

Optimization of mobile phase was performed based on resolution of drugs, asymmetric factor, theoretical plates obtained for LH, BEC and PHE. The mobile phase consisting of ammonium acetate buffer pH 5 and methanol (60:40 v/v) which was selected gave sharp, well resolved peaks of LH,BEC and PHE (Figure 1).

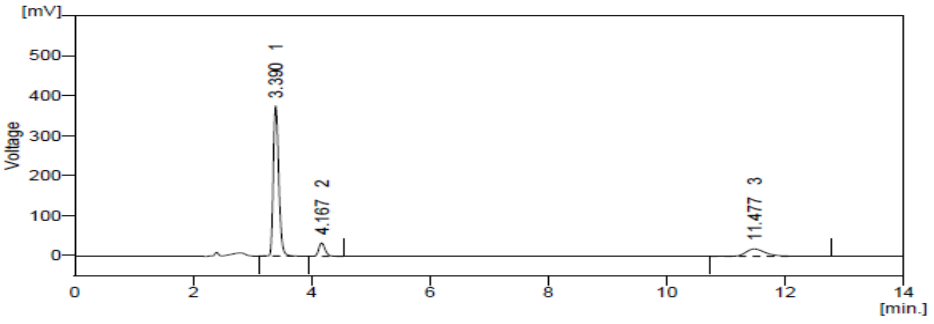

Figure 1: Optimized HPLC chromatogram of standard solution of LH (100 µg/mL), BEC (1 µg/mL) and PHE (4 µg/mL)

Optimized chromatographic condition

The chromatographic condition parameters had optimized for RP-HPLC and were shown in Table 1.

Table 1. Optimized chromatographic conditions for RP-HPLC

Parameters	Chromatographic Condition
Mode of elution	Isocratic
Mobile Phase	Buffer(ammonium acetate pH5) : Methanol (60:40)
Column	C18 (25cm x 0.46 cm) Hypersil BDS
Flow rate	1mL/min
Runtime	13min
Injection volume	20 µL
Detection wavelength	222 nm

The retention time for LH, BEC and PHE were 3.3, 4.1 and 11.4 min, respectively. The asymmetric factors for LH, BEC and PHE were 1.43, 1.37 and 1.54, respectively. UV overlain spectra of LH, BEC and PHE showed (Figure 2) that all the drugs absorbed appreciably at 222 nm, so the same was selected as the detection wavelength during the studies.

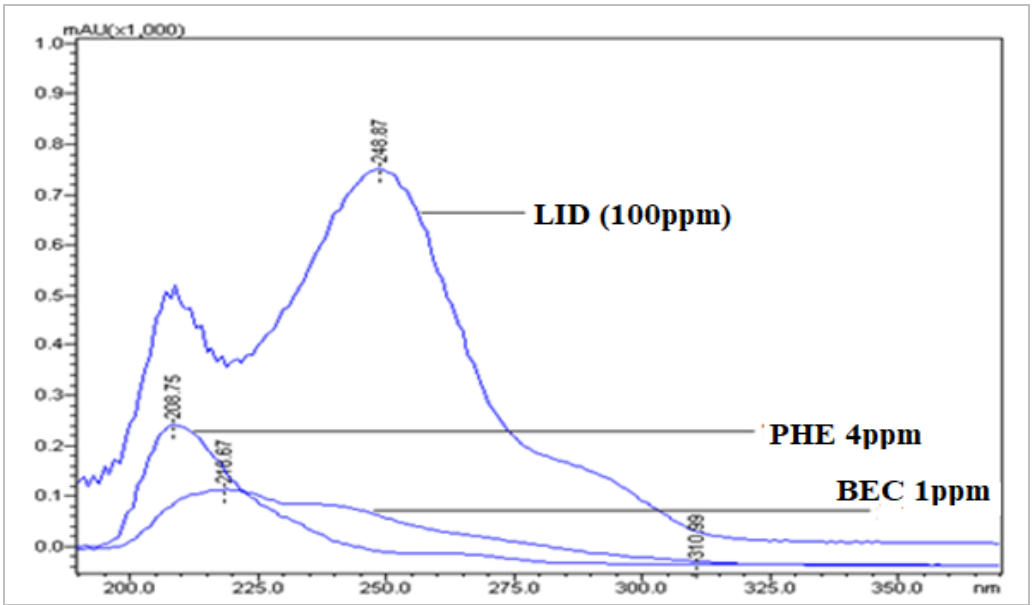

Figure 2. Overlain UV Spectra of LH, BEC and PHE (222nm Selected)

Validation

Calibration curve

The linearity of the method was determined at five concentration levels ranging from 50 to 150 µg/mL for LH, 0.5-1.5 µg/mL for BEC and 2 to 6 µg/mL for PHE (Figure 3). The calibration curve was constructed by plotting response factor against concentration of drugs. The results show that an excellent correlation exists between response factor and concentration of drugs within the concentrated range indicated above. The data of regression analysis of the calibration curve are shown in Table 2.

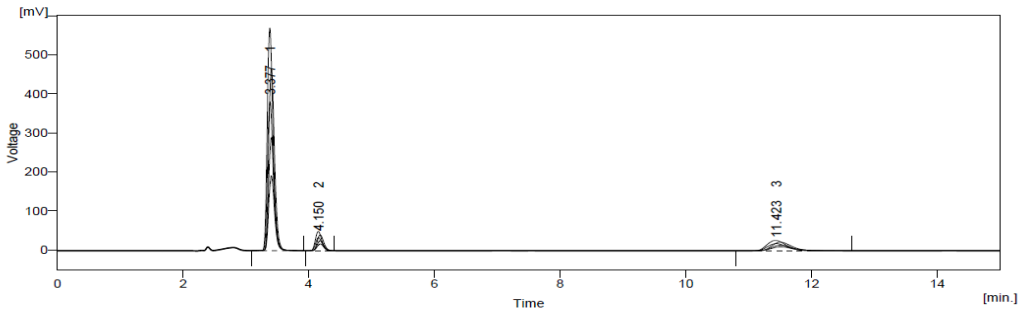

Figure 3. Overlain chromatogram of different concentrations of mixtures of LH, BEC and PHE

Table 2. Regression analysis of calibration curve

Parameter	LH	BEC	PHE
Range	50-150 µg/mL	0.5-1.5 µg/mL	2-6 µg/mL
Regression equation	y = 23.88x + 60.54	y = 227.64x + 13.676	y = 98.163x + 20.295
Correlation co-efficient(R^2)	0.999	0.997	0.997

Accuracy

The accuracy of the proposed method was evaluated by calculating the recovery studies of the test drug at three different concentration levels (80%, 100%, and 120%) by standard addition method. A known amount of LH, BEC and PHE was added to prequantified sample solution and three replicates of each concentration were injected in developed chromatographic conditions. The % recovery results were shown in (Table 3).

Table 3. % Recovery results of LH, BEC and PHE

Spiked Level	**%Recovery**			**%RSD**		
	LH	**BEC**	**PHE**	**LH**	**BEC**	**PHE**
80%	101.126 101.577 99.989	100.519 98.324 100.769	101.761 99.720 100.652	0.81	1.34	1.022
100 %	100.883 100.480 98.434	101.533 100.160 99.722	101.132 99.863 100.341	1.31	0.94	0.641
120 %	100.819 99.015 98.599	101.406 99.573 98.475	101.291 100.259 99.157	1.18	1.48	1.067

Precision

The values of %RSD for intraday and interday variation were found very well and within 2% limit, indicating that the current method is repeatable (Table 4, 5).

Table 4. Repeatability data for LH, BEC and PHE

Conc (µg/mL)	Mean ± S.D (n=6)	% R.S.D
LH (100)	2451.654±20.689	0.844
BEC (1)	244.765 ±2.143	0.875
PHE (4)	418.823 ±2.112	0.504

Table 5. Intraday and interday precision data for LH, BEC and PHE

Drug	Concentration (µg/mL)	Intraday Precision		Interday Precision	
		Mean Area±SD	%RSD	Mean Area±SD	%RSD
LH	50	1227.763 ± 3.658	0.298	1222.3.15 ± 11.51	0.942
	100	2417.821± 14.232	0.588	2430.983± 18.703	0.769
	150	3649.414± 9.853	0.269	3674.560± 26.192	0.713
BEC	0.5	122.316 ± 0.568	0.464	121.894 ± 1.154	0.946
	1	241.042± 2.196	0.911	242.902± 1.771	0.729
	1.5	364.244 ± 1.228	0.337	366.667 ± 3.303	0.901
PHE	2	207.008 ± 1.732	0.836	204.668 ± 3.889	1.900
	4	413.565± 2.353	0.569	413.011± 7.480	1.811
	6	621.323 ± 3.967	0.638	626.649 ± 6.029	0.962

System suitability

The system suitability should be based on the criteria and parameters collected as a group that was able to define the performance of the system (Table 6).

Table 6. System suitability parameters

Parameters	LH	BEC	PHE
Retention Time	3.393	4.170	11.497
Theoretical Plates	5974	7078	5977
Asymmetry	1.435	1.370	1.544
Resolution	-	4.155	18.477

Specificity

The chromatograms of blank, placebo, test sample and standard were used to justify the specificity of target analyte. The method was specific since excipients in the formulation did not interfere in the estimation of LH, BEC and PHE (Figure 4 (a), (b), (c) and (d)).

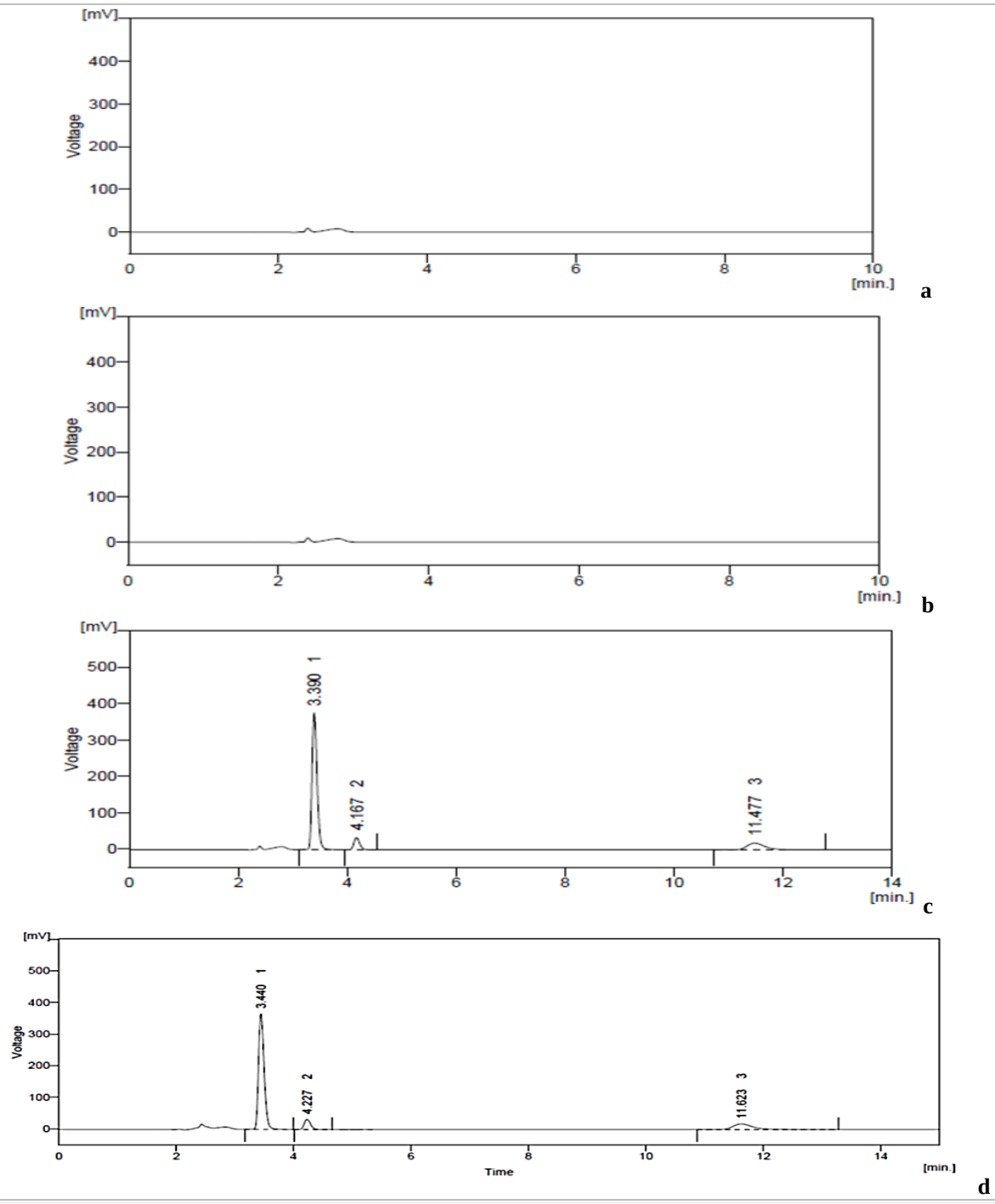

Figure 4. HPLC chromatogram of LH,BEC and PHE (a) Blank, (b) Placebo, (c) Standard and (d) Sample

Sample

LOD and LOQ

The Data for the LOD and LOQ for LH, BEC and PHE are shown in Table 7.

Table 7. LOD and LOQ data for LH, BEC and PHE		
Limit of Detection (LOD)		
LH	BEC	PHE
LOD = 3.3 x (SD / Slope) = 3.3 x (22.854/23.88) = 3.158 µg/mL	LOD = 3.3 x (SD / Slope) = 3.3 x (4.921/227.64) = 0.0713 µg/mL	LOD = 3.3 x (SD / Slope) = 3.3 x (8.317/98.163) = 0.280 µg/mL
Limit of Quantitation (LOQ)		
LOQ = 10 x (SD / Slope) = 10 x (22.854/23.88) = 10.448 µg/mL	LOQ = 10 x (SD / Slope) = 10 x (4.921/227.64) = 0.216 µg/mL	LOQ = 10 x (SD / Slope) = 10 x (8.317/98.163) = 0.848 µg/mL

Robustness

The effects of robustness study under different altered conditions of this proposed method are satisfactory (Table 8). The mean recovery and % RSD of analyzed sample indicate that the current method is robust.

Table 8. Robustness data for LH, BEC and PHE						
		At Normal Range (LH) Flow rate 1 mL/min Mobile phase (60:40) pH 5				Peak Area ± %RSD 2451.25 ±0.45
Sr. No.	Flow rate +0.1	Flow rate -0.1	M.P + 2	M.P - 2	pH + 0.2	pH - 0.2
1	2680.590	2195.333	2278.577	2598.571	2634.485	2244.109
2	2650.124	2212.550	2303.676	2601.503	2663.532	2266.586
3	2683.523	2201.830	2303.604	2604.458	2644.869	2290.562
%RSD	0.692	0.395	0.630	0.113	0.556	1.025
		At Normal Range (BEC) Flow rate 1mL/min Mobile phase (60:40) pH 5				Peak Area ± %RSD 2451.25 ±0.45
Sr. No.	Flow rate +0.1	Flow rate -0.1	M.P + 2	M.P - 2	pH + 0.2	pH - 0.2
1	2680.590	2195.333	2278.577	2598.571	2634.485	2244.109
2	2650.124	2212.550	2303.676	2601.503	2663.532	2266.586
3	2683.523	2201.830	2303.604	2604.458	2644.869	2290.562
%RSD	0.692	0.395	0.630	0.113	0.556	1.025
		At Normal Range (PHE) Flow rate 1mL/min Mobile phase (60:40) pH 5				Peak Area ± %RSD 244.25±0.11
1	457.516	375.650	389.719	443.558	449.678	383.973
2	448.749	378.703	382.958	445.451	443.180	387.816
3	458.339	376.820	394.049	440.967	451.440	392.041
%RSD	1.168	0.408	1.437	0.507	0.971	1.040

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Assay of Marketed Formulation

The % assay of the marketed formulation was found to be 98.67 % for LH, 98.51 % BEC and 98.66 % for PHE (Table 9)

Table 9. Analysis on marketed formulation (n=6)

Cream Formulation			
Label claim (%w/w)	LH (2.5% w/w)	BEC (0.025% w/w)	PHE (0.1% w/w)
Assay (% of label claim*) Mean ± S. D.	98.678±0.412	98.516±0.302	98.668±0.319

Conclusions

The reported RP-HPLC method was proved to be simple, rapid, and reproducible. The validation data indicate good precision, accuracy, and reliability of the method. The developed method offers several advantages in terms of simplicity in mobile phase, isocratic mode of elution, easy sample preparation steps, and comparative short run time making the method specific and reliable for its intended use in simultaneous determination of LH, BEC and PHE in cream formulation.

Experimental Work

Material And methods

Chemical and reagents

The Active pharmaceutical ingredient of LH, BEC and PEH were obtained as gift samples from Yash Pharma. The used solvent and reagent were of HPLC grade. Acetonitrile, water, potassium dihydrogen phosphate and ammonium acetate were obtained from Merck Pvt.ltd. The marketed formulation (cream) was obtained from local market.

Instrumentation

Chromatographic separation was performed on a Thermo separation product with UV 2000 liquid chromatographic system. A Hypersil BDS C_{18}column (25 cm × 4.6 mm i.d., 5 µ) was used for the separation.

Preparation of mobile phase:

The prepared mobile phase is a mixture of ammonium acetate buffer (pH 5) and methanol (60:40 v/v). It was filtered through a 0.2 µ membrane filter and degassed.

Preparation of standard stock solution

A 100 mg of the standard LH, 10 mf BEC and 40 mg PHE were accurately weighed and transferred to each of 100 mL volumetric flask and dissolved in 50 mL methanol. The flask was sonicated for 10 min. The flask was shaken and volume was made up to the mark with methanol to give solutions containing 1000 µg/mL LH, 100 µg/mL BEC and 400 µg/mL PHE.

Preparation of standard working solution:

Preparation of the standard working solution of LH, BEC and PHE was made by accurately mixing 1 mL of from the stock solutions to get the concentration as described in the marketed formulations. Hence, dilutions were accordingly made to prepare a calibration graph.

Chromatographic Conditions

A reversed phase BDS C_{18} column equilibrated with mobile phase comprising of Ammonium acetate buffer pH 5: methanol

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(60: 40; v/v) was used. Mobile phase flow rate was maintained at 1 mL/ min, and the eluent was monitored at 222 nm. A 20 µL of the sample was injected using a fixed loop, and the total run time was 13 min. All the chromatographic separations were carried out at controlled room temperature (25 ± 2°C)

Calibration Curves for LH, BEC and PHE

Appropriate aliquots of LH, BEC and PHE working standard solution were taken in different 10 mL volumetric flasks. The volumes were made up to the mark with mobile phase to obtain final concentrations of 50-150 µg/mL for LH, 0.5-1.5 µg/mL for BEC and 2-6 µg/mL for PHE . The solutions were injected using a 20 µL fixed loop system, and chromatograms were recorded. Calibration curves were constructed by plotting peak area versus concentrations of the drug and regression equations were computed for LH, BEC and PHE.

Analysis of marketed formulations

Take Cream equivalent to 100 mg of LH, 1mg BEC and 4 mg of PHE was transferred to a 100 mL volumetric flask, Put this solution on water bath till the cream get miscible with Solvent, Cool this solution and Add 60 mL Mobile phase, then, Shake for 15 min and make up volume with Mobile phase. The solution was filtered through Whatman filter paper no. 42. Take 1 mL from this and transferred to 10 mL volumetric flask and made up volume up to the mark with mobile phase. It was injected as per the above chromatographic conditions, and peak area was recorded. The quantifications were carried out by keeping these values to the linear equation of calibration curve.

Validation

The method was validated as per ICH for accuracy, precision, specificity, detection limit, quantitation limit, and robustness.

Accuracy

The accuracy of the method was determined by calculating recoveries of LH, BEC and PHE by method of standard additions. Known amounts of LH (40, 50, 60 µg/mL), BEC (0.4, 0.5 and 0.6 µg/mL) and PHE (1.6, 2 and 2.4 µg/mL) were added to a prequantified sample solutions, and the amounts of LH, BEC and PHE were estimated by measuring the peak area and by fitting these values to the straight-line equation of calibration curve.

Precision

The instrument precision was evaluated by injecting the solution containing LH (100 µg/mL), BEC (1 µg/mL) and PHE (4 µg/mL) six times repeatedly and peak area was measured. The results are reported in terms of relative standard deviation. The intraday and inter day precision study of LH, BEC and PHE was carried out by estimating the corresponding responses 3 times on the same day and on 3 different days (first, second and third day) for 3 different concentrations of LH (50, 100 and 150 µg/mL), BEC (0.5, 1 and 1.5 µg/mL) and PHE (2, 4 and 6 µg/mL), and the results are reported in terms of relative standard deviation (RSD).

Specificity

The specificity was estimated by spiking commonly used excipient (starch, talc, and magnesium stearate) into a pre-weighed quantity of drug. The chromatogram was taken by appropriate dilutions, and the quantities of drugs were determined.

System suitability

System suitability should be based on the criteria and parameters collected as a group that will be able to define the performance of the system.

Limit of Detection and Quantification

The detection limit is defined as the lowest concentration of an analyte that can reliably be differentiated from background levels. Limit of quantification of an individual analytical procedure is the lowest amount of analyte that can be quantitatively determined with suitable precision and accuracy. LOD and LOQ were calculated using the following equation as per ICH guidelines.

$LOD = 3.3 \times \sigma/S$ and

$LOQ = 10 \times \sigma/S$, where σ is the standard deviation of y-intercepts of regression lines and S is the slope of the calibration curve.

Robustness

Robustness of the method was studied by deliberately changing the experimental conditions like flow rate, percentage of mobile phase, pH of mobile phase.

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Disclosure statement

No potential conflict of interest was reported by the authors

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