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Stability indicating method development and validation of Beclomethasone dipropionate and Neomycin sulphate Ointment using HPLC with microbial assay

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Abstract

A simple, economic, selective, precise and accurate stability-indicating RP-HPLC method for the analysis of "Beclomenthasone Dipropionate (BD) an anti inflammatory in its formulation was developed and validated in the present study. Here Neomycin Sulphate (NS) in the ointment is one of a class of aminoglycoside antibiotic that lack a good UV absorbing chromophore, therefore difficult to determine using RP-HPLC with absorbance detection. So Microbiological assay is the best option for their determination. RP-HPLC for Beclomethasone Dipropionate was performed on a Kromosil C18, 5µ, 15cm x 4.6mm column. The mobile phase consist a mixture of ACN: WFI: Methanol: Glacial Acetic Acid: Triethylamine (55: 40: 5: 1: 0.1 v/v). This was found to give sharp peak of Beclomethasone Dipropionate at a wavelength of 235nm with a flow rate of 1.2ml/min. the linear regression analysis data for the calibration curve showed a good linear relationship with a regression coefficient 0.999.

The linear regression equation was Y= 64760x – 1875. Linearity was found in the range of 5-15 µg/ml. the accuracy (%recovery) was found to be in the range of 99.07 – 99.85%. NS was performed on the culture medium of the (Recoiorery) linearity was found in the range of 5-15 µg/ml. the accuracy (% recovery) was found to be in the range of 99.07 - 99.85%. NS was performed on the culture medium of agar was composed of 20 Ml Grove-Randall's 1 culture medium (Difco) that was poured into a 100 x 20 mm Petri dish used for inhibit the strain of Staphylococcus aureus. The linear regression analysis data for the calibration curve showed a good linear relationship with a regression coefficient 0.998. The linear regression equation was y = 2.401x – 0.001. Linearity was found in the range of 2.5 – 15 µg/ml. The accuracy (% revcovery) was found to be in the range of 98.49 – 99.03 %. BD and NS were subjected to stress conditi0on0s including acidic, alkaline, oxidation, photolysis and thermal degradation. BD and NS were more sensitive towards acidic degradation. The method was validated as per ICH guideline. The method was validated for accuracy, precsion, robustness, specificity. The proposed methods can be used successfully for routine analysis of Beclomethasone Dipropionate (BD) by HPLC and Neomycin Sulphate (NS) by Microbiological assay in ointment form.

Keywords: Beclomethasone Dipropionate (BD), Neomycin Sulphate, RP-HPLC Method, Microbiological Assay, Stress Study, Degradation.

Introduction

Both medications are included in pharmacopoeias as independent drugs. In the market, there is a new formulation that combines Beclomethasone dipropionate and Neomycin Sulphate. However, the use of Beclomethasone dipropionate and Neomycin Sulphate in combination is not recommended.

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Material and Methods

Identification of API

Identification by Melting Point

Beclomethasone Dipropionate-117- 120°C
Neomycin sulphate-250-260°C

Determination of wavelength for maximum absorbance

In mobile phase, 10 g/ml solutions of Beclomethasone Dipropionate were prepared separately. In a Double beam UV-visible spectrophotometer, each solution was scanned between 200 to 400 nm (Shimadzu, model 1800). Wavelength was chosen from the Beclomethasone Dipropionate overlay spectra. At 235 nm, the components have a good response.

Experimental work of Development and validation of stability indicating RP-HPLC Method for Beclomethasone Dipropionate

Development and Optimization of RP - HPLC Method

Selection of Detection Wavelength

The wavelength chosen for detection determines the sensitivity of an HPLC system that uses UV detection. The optimal wavelength is the one that gives the best reaction to the substances being detected. The maximal wavelength of beclomethasone dipropionate is 235 nm. After a series of tests with various wave lengths, 235 nm was chosen as the drug's wavelength. At 235 nm, the drug exhibits an excellent peak height and form. All of the degradation products were plainly distinguishable as well. As a result, for simultaneous estimation of Beclomethasone Dipropionate in ointment form, a wavelength of 235 nm was selected.

Selection of Chromatographic Conditions

The HPLC method selection is influenced by the kind of sample (ionic, ionisable, or neutral molecule), its molecular weight, pKa, and solubility. RP-HPLC was chosen for the initial separation based on a literature assessment and its simplicity and appropriateness. In order to optimise chromatographic settings, the impacts of chromatographic factors such as mobile phase, pH, flow rate, and solvent ratio were investigated. Chromatographic metrics such as capacity factor, asymmetry factor, resolution, and column efficiency were also measured. Finally, for calculating the selected medicine, the condition with the best resolution, symmetry, and capacity factor was chosen.

Selection of Proper Column

Various columns are available for the RP-HPLC method, however a literature analysis led to the selection of the C-18 column above the others. Finally, a cromosil C18 column (150mm 4.6 mm, 5 m particle size) was chosen for method development.

Selection of Ratio of Mobile Phase

In comparison to other solutions, a standard solution containing 250 g/mL of Beclometasone Dipropionate was chromatographed with a varied ratio of Methanol.

Table 7.1 shows different trails with different ratios of Water : Methanol, Water : ACN, and Buffer : Methanol. For the following mobile composition, a standard solution containing 10 ppm Beclomethasone Dipropionate was used:

Preparation of solutions of Beclomethasone Dipropionate

Preparation of BD std. Stock solution:

Transferring 25.0 mg of Beclomethasone Dipropionate into a 100 ml volumetric flask yielded the Beclomethasone Dipropionate standard stock solution. After adding 80 mL of Methanol and shaking the flask, the volume was made up to label with Methanol. 2 mL of the aforementioned solution in a 50 mL volumetric flask, diluted with Methanol to the mark, and filtered through a 0.45 filter to create a Beclomethasone Dipropionate solution with a final concentration of 100 g/ml.

Preparation of BD Sample stock solution

The sample solution was made by accurately weighing 2gm of ointment and transferring it to a 100 ml volumetric flask, where 80 ml of Methanol was applied. This solution was sonicated for 15 minutes, then filtered through a 0.5µm Whatman filter paper No. 41 filter, and the final volume was made up to the mark with Methanol. 1 ml of the filtrate was transferred to a 10 ml volumetric flask and diluted to the desired concentration with Methanol, to obtain BD (100µg/ml). The percent Assay was measured after the concentration was calculated using a regression equation.

Specificity:

The ability of a system to calculate the analyte response in the presence of potential impurities is known as specificity. In the Assay study, a peak

purity test for the Beclomethasone Dipropionate peak was performed using a PDA detector.

System suitability test parameter

System suitability checks are used to ensure that the system's resolution and repeatability are sufficient for the analysis. The chromatographic peak resolution, theoretical plate number, and tailing factor were used in this test. The repeatability of these parameters was checked by injecting a Beclomethasone Dipropionate solution six times.

Linearity and Range:

Aliquots of the combined working standard solution (1.0, 1.5, 2.0, 2.5, and 3.0mL) were transferred into a series of 10 mL volumetric flasks and diluted with methanol to the desired concentration. This resulted in a Beclomethasone Dipropionate solution containing 5, 7.5,10,12.5,15 µg/mL. Under operating chromatographic conditions, an aliquot of 10µl of each solution was injected. Calculate the correlation coefficient and regression line equation for Beclomethasone Dipropionate by plotting the calibration curve of Area versus respective concentration. Each response was based on a five determinations.

Precision

Repeatability:

Analyzing a Beclomethasone Dipropionate test solution with a concentration of 10 µg/mL. was used to determine repeatability. Six times a day, take a measurement. Calculate Beclomethasone Dipropionate for % RSD.

4b Intraday Precision:

Beclomethasone Dipropionate standard solutions in the range 5, 10, 15 µg/mL of Beclomethasone Dipropionate were analysed in triplicate to determine intraday precision. Calculate Beclomethasone Dipropionate for % RSD.

Inter day Precision:

The precision of Beclomethasone Dipropionate standard solutions in the range of 5, 10, and 15 µg/mL of Beclomethasone Dipropionate was determined by analysing them on different days. Calculate Beclomethasone Dipropionate for % RSD.

Accuracy:

The recovery of Beclomethasone Dipropionate was determined by using the standard addition method to assess accuracy. Working standard solutions of Beclomethasone Dipropionate (10 µg/mL) in known quantities (1, 2 and 3 mL) were applied to a 2 mL sample solution of Beclomethasone Dipropionate (10 µg/mL) in a 10 mL volumetric flask and diluted up to the mark with methanol. Each solution was injected in triplicate, and recovery was calculated by calculating peak areas using the regression equation of the calibration curve.

Limit of Detection and Limit of Quantitation

LOD and LOQ of the drug were calculated using following equations according to ICH guideline. LOD = 3.3 σ/s LOQ = 10 σ/s Where σ is the SD of the response and S is the slope of the calibration curve.

Robustness:

The robustness study was performed to evaluate the influence of small but deliberate variation in the chromatographic condition. The robustness was checked by changing three small changes.

1) Mobile phase flow rate (±0.1mL/min)
2) Mobile phase composition (Buffer + Methanol, 75+25 and 85+15 v/v)
3) wavelength

Experimental Work for Neomycin Sulphate

Preparation of Standard Solution for Microbial Assay of Neomycin Sulphate

Neomycin Sulphate, 141.3 mg (working std.) Make a final volume of 100ml with Neomycin sulphate and Buffer solution (pH 8). Take 10 ml and make a final volume of (35 ppm) with buffer solution (pH 8) termed sH. Make a final volume of 100 ml with 20 ml of buffer solution (pH 8) (7 ppm) called sL.

Preparation of Sample Solution for Microbial Assay of Neomycin Sulphate

In a clean beaker, take a 7.0gm sample. In a beaker, combine 40 mL chloroform and 30 mL pH8 buffer solution, then pour into a 250 mL Separating funnel. After that, mix thoroughly for 10 minutes. Place it on a tripoid stand to separate the buffer and solvent phases. Fill a beaker with the solvent phase and a 100ml volumetric flask with the buffer phase. In a separating funnel, combine 30ml pH 8 buffer solution with the prior solvent phase and mix thoroughly for 10 minutes. Then place it on a tripoid stand to separate the buffer and solvent phases. Fill a beaker with the solvent phase and a clean 100ml volumetric flask with the buffer phase. Add 30ml buffer pH 8 and the preceding solvent phase to the separating

funnel, mix well for 10 minutes (2nd layer), then place it on a tripoid stand to layer the buffer and solvent. Place the solvent phase in a beaker and the buffer phase in a clean 100ml volumetric flask, with a final volume of 100ml of pH 8 buffer solution. Fill a 100ml volumetric flask halfway with the aforesaid solution and dilute it with the buffer pH 8 solution (35ppm) named TH. Fill a 100ml volumetric flask halfway with the aforesaid solution and dilute it with the buffer pH 8 solution (7ppm) named TL.

Microorganism and Inoculum Standardization

Because of its susceptibility to NS and ability to generate highly defined inhibition of growth zones, the strain of Staphylococcus aureus proves to be the best test microorganism for precision measurements. S. aureus cultures were grown and maintained on Casoy culture media (Difco, Brazil). The microorganisms were standardised using the procedures outlined in the Brazilian and United States Pharmacopeias. Prior to use, the microorganism was grown in a BHI broth in a conical flask, which was incubated during 24 h at 35 ± 2 °C. Using a spectrophotometer with the wavelength set at 580 nm and a 10 mm absorption cell, the broth containing the microorganism was diluted to give a suspension with 25 ± 2 percent turbidity (transmittance) with the some broth sterile solution as the blank. From this standardised suspension, aliquots of 1.0 mL were added to each 100 mL of Grove–Randall's 11 culture medium (Difco, Brazil) at 48 °C, and used as the inoculated layer in the plate

Agar Diffusion Bioassay

20 mL Grove–1 Randall's culture media (Difco) was put onto a 100 x 20 mm Petri plate to make the base layer agar. After the foundation layer had solidified, sections of 5 mL infected Grove–11 Randall's medium were poured onto it. Six stainless steel cylinders of the same size (8 6 10 mm) were placed on the surface of the infected medium in each plate. Three alternating cylinders were filled with 200 litres of reference solutions (S1, S2, and S3), while the remaining three cylinders were filled with sample solution concentrations (T1, T2, and T3; Figure 2). For each assay, six plates were used. The plates were incubated aerobically for 18 hours at 35 °C. A digital calliper was used to measure the widths of the growth inhibition zones (mm).

Preparation of solution for stress study:

Hydrolytic conditions: acid, base induced degradation:

At 70oC, solutions containing 10 L of BD and NS were treated with various concentrations of HCl and NaOH (1 N) over the relevant time intervals. As needed, the solutions were neutralised.

Oxidative condition: hydrogen peroxide-induced degradation

10 mL of BD and NS solutions were treated with 3 mL of 3 percent w/v H2O2 in the dark at 70oC at the appropriate time intervals.

Thermal degradation

Solutions containing 10 l of BD and NS were thinly spread on a petri plate and exposed to the conditions stated at 60°C for appropriate time intervals to assess the drugs' stability under thermal stress conditions.

Results and Discussion

The wavelength chosen for detection determines the sensitivity of an HPLC procedure that uses UV detection. The optimal wavelength is the one that gives the best reaction to the substances being detected. The maximal wavelength of beclomethasone dipropionate is 235 nm. After a series of tests with various wave lengths, 235 nm was chosen for this medication. At 235 nm, the drug has a respectable peak height. All of the breakdown products could be seen plainly. A wavelength of 235 nm was chosen for simultaneous estimation of Beclomethasone Dipropionate in an ointment form.

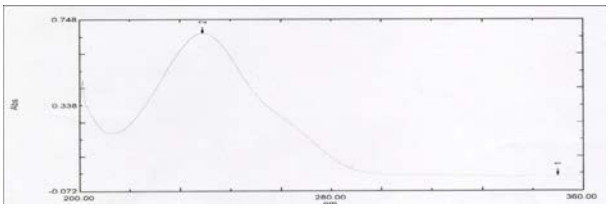

Fig 1: Determination of wavelength for maximum absorbance

Selection of Ratio of Mobile Phase

In comparison to other solutions, a standard solution containing 250 g/mL of Beclometasone Dipropionate was chromatographed with a varied ratio of Methanol. Table 7.1 shows different trails with varying ratios of Water : Methanol, Water : ACN, and Buffer : Methanol. For the following mobile composition, a standard solution

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containing 10 ppm Beclomethasone Dipropionate was used:

Trial – 1

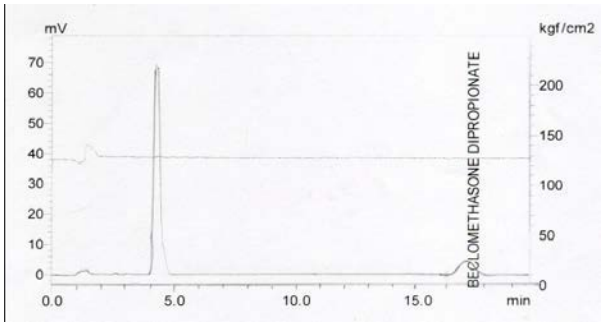

Fig 2: Chromatogram of Beclomethasone Dipropionate (10 µg/ml) with Water and Acetonitrile as mobile phase (50 : 50 v/v)

Trial – 2

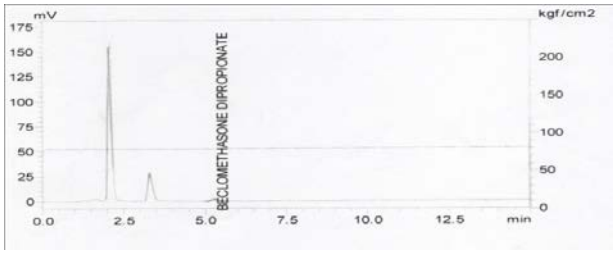

Fig 3 : Chromatogram of Beclomethasone Dipropionate (10 µg/ml) with Acetonitrile And Water as mobile phase (75 : 25 v/v)

Trial - 3

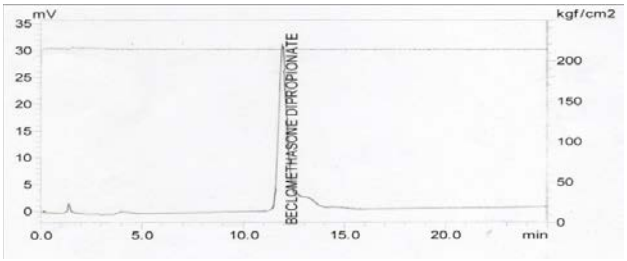

Fig 4 : Chromatogram of Beclomethasone Dipropionate (10 µg/ml) with Water and Methanol as mobile phase (50 : 50 v/v)

Trial – 4

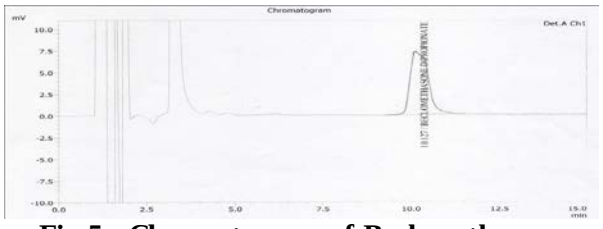

Fig 5 : Chromatogram of Beclomethasone Dipropionate (10 µg/ml) with Acetonitrile, WFI And Methanol as mobile phase (50 : 40 : 10 v/v)

Trial – 5

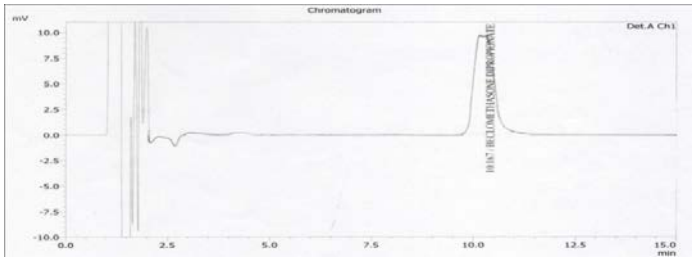

Fig 6 : Chromatogram of Beclomethasone Dipropionate (10 µg/ml) with Acetonitrile : WFI: Methanol : Glacial acetic Acid as mobile phase (55 : 40 : 5 : 1 v/v)

Trial – 6

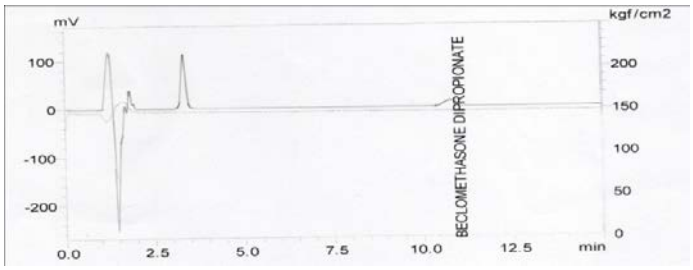

Fig 7 : Chromatogram of Beclomethasone Dipropionate (10 µg/ml) with Acetonitrile : WFI: Methanol : Glacial acetic : Triethylamine Acid as mobile phase (55 : 40 : 5 : 1 : 0.1 v/v)

Trial – 7

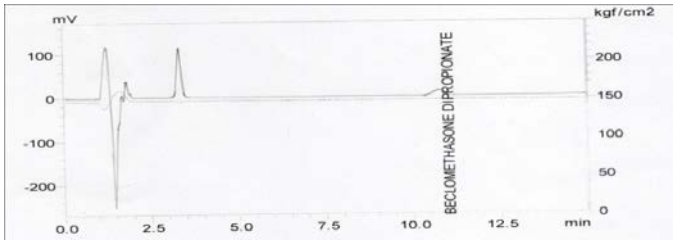

Fig 8 : Chromatogram of Beclomethasone Dipropionate (10 µg/ml) with Acetonitrile : WFI: Methanol : Glacial acetic : Triethylamine Acid as mobile phase (55 : 40 : 5 : 1 : 0.1 v/v)

Table 1: Selection of mobile phase

Sr. No.	Mobile Phase	Inference
1	ACN : WFI (50 : 50 v/v)	Drug elute very let after 15 min
2	ACN : WFI (75 : 25 v/v)	Drug elute very fast
3	Methanol : WFI (50 : 50 v/v)	Peak is merged with other peak
4	ACN : WFI : Methanol (50 :40 :10 v/v)	Not good peak
5	ACN : WFI : Methanol	No good peak

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	: Glacial Acetic Acid (55 :40 : 5 : 1 v/v)	occur
6	ACN : WFI : Methanol : Glacial Acetic Acid : Triethylamine (55 :40 : 5 : 1 : 0.1 v/v)	Good Peak with optimum retention time
7	ACN : WFI : Methanol : Glacial Acetic Acid : Triethylamine (55 :40 : 5 : 1 : 0.1 v/v)	Repeat for conformation of mobile phase ration

Optimized Chromatographic conditions:

Table 2: System Suitability Parameters of chromatogram

System Suitability Parameters	Beclomethasone Dipropionate (n = 5)
Retention Time (min)	10.589 ± 0.0068
Tailing factor	1.304 ± 0.0090
Theoretical plate	4117.044 ± 57.1664
Resolution	16.409 ± 0.0474

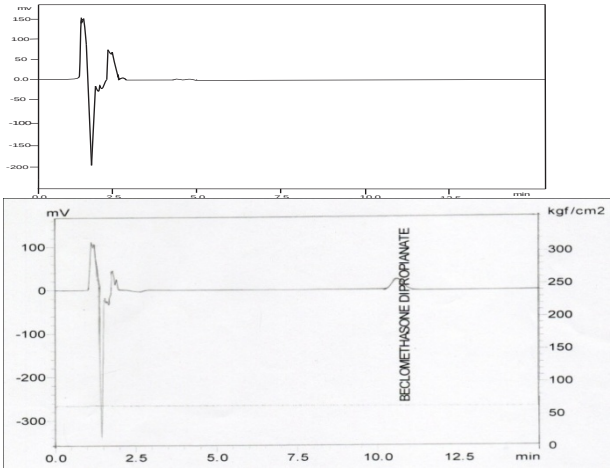

Fig 9: Chromatogram of Mobile Phase

Fig 10: Chromatogram of standard BD

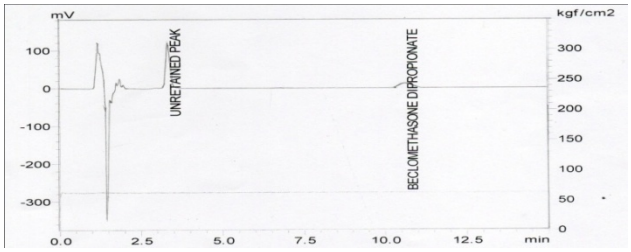

Fig. 11: Chromatogram of sample Beclomethasone Dipropionate

Table 3: Specificity study

Standard		Sample	
Beclomethasone Dipropionate		**Beclomethasone Dipropionate**	
Mean ± SD (n = 3)	**% RSD**	**Mean ± SD (n = 3)**	**% RSD**
646845.3 ± 50.3620	0.0077	632475.7 ± 7.6570	0.0091

The calibration curves for Beclomethasone Dipropionate, presented in Figure and respectively, reveal that the response is linear over the concentration range, with a correlation coefficient (r) value of 0.999.

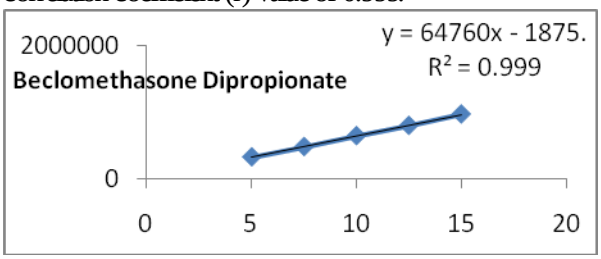

Fig 12 : Calibration curve of Beclomethasone Dipropionate

Table 4 : Repeatability Study

Concentration of Beclomethasone Dipropionate (µg/ml)	Beclomethasone Dipropionate	
	Mean ± SD (n = 6)	**% RSD**
10	646888.67 ± 72.6654	0.0112

Table 5: Intraday & Interday Precision study of Beclomethasone Dipropionate

Drug	Conc. (µg/ml)	Intra-day precision		Inter-day precision	
		Mean ± S.D (n = 3)	**% RSD**	**Mean ± S.D (n = 3)**	**% RSD**
Beclomethasone Dipropionate	5	324909.7 ±35.0047	0.010107	324889.3 ± 81.56184	0.02511
	10	646845.3 ±50.	0.0077	646852 ± 80.1	0,0123

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		3620		3114	
	15	974127 ± 98.5342	0.010101	974248.3 ± 22.2785 4	0.00222

:

Table 6 : Recovery study

Drug	% of Level	Amount Taken (µg/ml)	Amount added (µg/ml)	Total Amount found (µg/ml)	Recovery ± S.D (n=3)
Beclomethasone Dipropionate	80 %	5	2.5	7.5	99.95 ± 0.01
	100 %	5	5	10	99.73 ± 0.02
	120 %	5	7.5	12.5	99.08 ± 0.01

Table 7 : Robustness

Parameter	Value	Area
		Beclomethasone Dipropionate
Wave length	235	646852
	238	536192.7
	240	330736.7
	Mean ± SD	504593.8 ±2627.139
	% RSD	0.7882
Flow rate (± 0.02)	1	646852
	1.5	536192.7
	2	330736.7
	Mean ± SD	504593.8 ±2627.139
	% RSD	0.7882
	ACN:WFI(50:50)	284221.3
	Me:WFI (50:50)	1033847
Mobile phase (± 2.0)	ACN:WFI:Me:GLA:TRI (55:40:1:5:0.1)	292411.7
	Mean ± SD	536826.8 ±596.1547
	% RSD	0.1969

Table 8 : Optical Regression characteristics and validation parameters

Parameter	Beclomethasone Dipropionate
Calibration Range(µg/ml)	5 – 15 µg/ml
Regression Equation	y = 64760x – 1875
Slope (m)	64760
Intercept (c)	1875
Correlation co-efficient(r)	0.9997
Intraday (% RSD, n = 5)	1.07 – 1.37
Interday (% RSD, n = 5)	1.26 – 1.79
Detection limit (µg/ml)	0.0037
Quantitation limit (µg/ml)	0.0113

microbial assay of Neomycin Sulphate

Linearity

The calibration curves for Neomycin Sulphate in Figure and show that the response is linear over the concentration range, with a correlation coefficient (r) of 0.999.

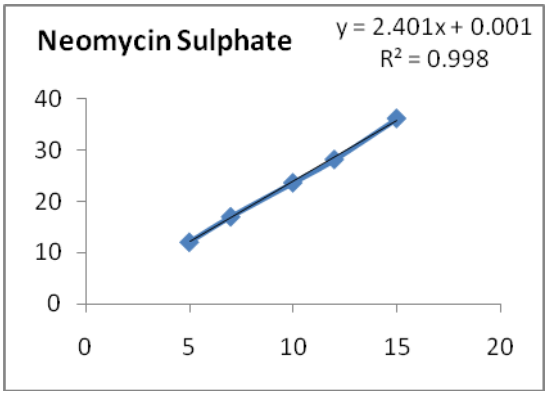

Fig 13 : Calibration curve of NS

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Table 9: Repeatability Study of neomycin sulphate

Concentration of Neomycin Sulphate (µg/ml)	Neomycin Sulphate	
	Mean ± SD (n = 6)	% RSD
10	23.70 ± 0.12	0.51

Table 10: Intraday & Inter day Precision study of Neomycin Sulphate

Drug	Conc. (µg/ml)	Intra-day precision		Inter-day precision	
		Mean ± S.D (n = 3)	% RSD	Mean ± S.D (n = 3)	% RSD
Neomycin Sulphate	5	12.16 ± 0.04	0.37	12.38 ± 0.20	1.68
	10	23.75 ± 0.12	0.5	23.66 ± 0.12	0.52
	15	36.17 ± 0.72	2.01	36.20 ± 1.06	2.94

Table 11: Recovery Study

Drug	% of Level	Amount Taken (µg/ml)	Amount added (µg/ml)	Total Amount found (µg/ml)	Recovery ± S.D (n=3)
Neomycin Sulphate	80 %	5	2.5	7.5	99.03 ± 0.53
	100 %	5	5	10	98.89 ± 0.57
	120 %	5	7.5	12.5	98.49 ± 0.36

Optical Regression characteristics and validation parameters

Table 12 : Optical Regression characteristics and validation parameters

Parameter	Neomycin Sulphate
Calibration Range(µg/ml)	5 – 15 µg/ml
Regression Equation	y = 2.401x – 0.001
Slope (m)	2.401
Intercept (c)	0.001
Correlation co-efficient(r)	0.998
Intraday (% RSD, n = 5)	24.02 - 0.29
Interday (% RSD, n = 5)	24.08 – 0.46
Detection limit (µg/ml)	0.33
Quantitation limit (µg/ml)	1.02

Assay of Pharmaceutical dosage form

Applicability of proposed method was tested by analyzing ointment formulation (Betsonir-N ointment). The results are shown in Table.

Table 13: Analysis of Pharmaceutical Dosage form

Formulation	Beclomethasone Dipropionate		
BETSONIR-N OINTMENT	Amount Labeled (mg)	Amount Found (mg)	% Amount found ± SD (n = 6)
	100	97.6	97.6± 0.01

Force Degradation of Beclomethasone Dipropionate and Neomycin Sulphate

Under stress conditions the BD peak reduced over time with appearance of different unknown degradation products peaks.

Table 14: Percentage of Degradation of Beclomethason Dipropionate

Stress condition	Time				
	1 h	24 h	96 h	168 h	240 h
Acidic	-	19.633	-	-	-
Alkaline	17.064	-	-	-	-
Oxidation	0.32	0.75	24.811	-	-
Thermal	0.91	11.633	-	-	-
Light	-	0.04	0.38	0.61	0.83

The results showed that this medicine degrades when exposed to the stress conditions examined at the following times: 1 hour in an alkaline

environment, 24 hours in an acidic environment with thermal stress and 96 hours in an oxidative environment. The medicine was found to be stable for 240 hours in the presence of light, and these conditions did not enhance the development of degradation products. In any of the settings studied, there was no degradation of placebo.

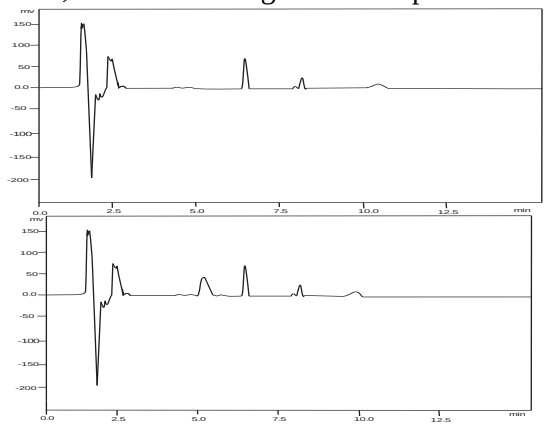

Fig. 14: HPLC chromatograms of BD solution

Fig6.15: HPLC chromatograms of BD solution Degraded In NaOH at 1 h Degraded in HCL at 24 h

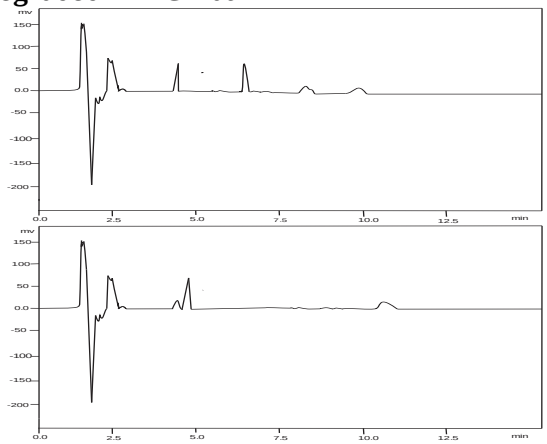

Fig. 16: HPLC chromatograms of BD solution

Fig 6.17: HPLC chromatograms of BD solution degraded in thermal at 24 h degraded in H_2O_2 at 96 h

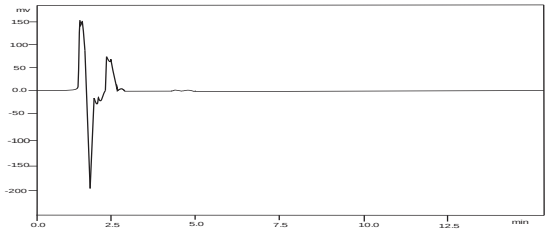

Fig. 18: HPLC chromatograms of Blank solution

The peak purity and retention time (tR) of BD are shown in Table 2 under all forced degradation settings. All of the peak purity values were greater than 980, indicating the lack of any other co-eluting chemicals.

Table 15. Peak purity and retention time for BD under stress degradation studies

Stress condition	Time (hours)	t_R	Peak purity
Alkaline	1	9.539	999.314
Acidic	24	10.710	999.975
Oxidation	96	10.451	999.983
Thermal	24	10.618	999.987
Light	240	10.533	999.978

According to the results obtained, it can be noticed that solutions were stable for 72 h, as during this time the results does not decrease below the minimum percentage (98 %).

Table 6.16 Percentage of degradation of Neomycin Sulphate

Stress condition	Time				
	1 h	24 h	96 h	168 h	240 h
Acidic	-	0.23	0.71	23.53	-
Alkaline	-	19.57	-	-	-
Oxidation	21.69	-	-	-	-
Thermal	0.63	0.98	20.53	-	-
Light	-	0.04	0.38	0.61	15.70

The results demonstrated that this medicine degrades after being exposed to the stress conditions for 1 hour, 24 hours, 96 hours, 168 hours, and 240 hours: 1 hour to oxidation, 24 hours to alkaline, 96 hours to thermal, 168 hours to acidic, and 240 hours to light.

Table 17. Diameters of growth inhibition zones for Neomycin Sulphate reference substance solutions obtained for standard curve.

Concentration µg/mL	Range of zone size, mm[a]	Mean diameters of growth inhibition zones,	RSD %

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		b mm	
5	12.11 – 12.17	12.16	0.37
10	23.63 - 23.87	23.69	0.38
15	35.78 - 37.01	36.17	2.01

Table 18. Accuracy of the microbiological assay of Neomycin Sulphate

Run	Amt. of std Added	Amt. of sam Added	Final Conc. µg/ml	Recovery, a%	RSD %
R1	2.5	5	7.5	99.03	0.53
R2	5	5	10	98.89	
R3	7.5	5	12.5	98.49	

Observe the Zones after incubation. The test is only valid if the zones are not fused and are regular. Zone diameters should be measured and added independently for each of the four dilutions utilised by the zone reader.

Table 6.19: Zone reading

Conc.	Plate No.				Sum of diameter
	1diameter	2diameter	3diameter	4 diameter	
SH	25.3	25.9	26.3	25.8	25.825
SL	22.3	22.7	22.1	22.5	22.40
TH	26.0	25.5	26.5	26.1	26.025
TL	22.1	22.8	22.3	22.7	22.475

Beclomethasone Dipropionate is a corticosteroid that causes a structural alteration in the steroid receptor complex by penetrating and binding to the cytoplasmic receptor protein. This structural alteration allows it to enter the nucleus and bind to certain DNA locations, leading in the transcription of specific m-RNA and, as a result, protein synthesis regulation. It acts as a highly selective glucocorticoid. It increases the activity of enzymes that help to reduce the inflammatory response. In a "irreversible" manner, aminoglycosides such as neomycin Sulphate bind to certain 30S-subunit proteins and 16S rRNA. Neomycin binds specifically to four nucleotides of 16S rRNA and one amino acid of protein S12. This blocks the decoding site in the 16S rRNA of the 30S subunit near nucleotide 1400. This area interacts with the wobble base in the anticodon of tRNA. This results in non-functional or poisonous peptides, as well as polysomes breaking up into non-functional monosomes, due to interference with the initiation complex, mRNA misreading, and erroneous amino acids being added to the polypeptide. Neomycin Sulphate in combination with Beclomethasone Dipropionate has been demonstrated to be more effective than Neomycin Sulphate alone in the treatment of skin disease. The RP-HPLC technology was used to develop simultaneous estimation of Beclomethasone Dipropionate. For Neomycin Sulphate, a microbiological assay is the best option. Because they lack an adequate UV absorbing chromophore, they are difficult to analyse using reversed-phase HPLC with absorbance detection. Using the RP-HPLC technology, good resolution and drug separation were achieved. The retention time of Beclomethasone Dipropionate was determined to be 10.036 minutes at a flow rate of 1 ml/min. The proposed approach was exact and accurate. As a result, the proposed method can be utilised on a regular basis to analyse Beclomethasone Dipropionate and Neomycin Sulphate in ointment dosage form. Using the Microbial Assay method, a high killing zone capacity of bacteria was attained. The NS was performed on agar culture media, which consisted of 20 mL Grove–1 Randall's culture medium (Difco) placed into a 100 x 20 mm Petri dish used to inhibit the Staphylococcus aureus strain. The calibration curve's linear regression analysis data revealed a solid linear relationship with a regression coefficient of 0.998. $y = 2.401x – 0.001$ was the linear regression equation. Linearity was found in the 2.5-15 g/ml range. The accuracy (percent recovery) was discovered to be between 98.49 and 99.03 percent

The forced degradation of Beclomethasone Dipropionate was studied using the RP-HPLC method, while the forced degradation of Neomycin Sulphate was studied using the microbiological assay method, which included Acid, Base, Oxidative, and Thermal degradation. The findings of the degradation were found to be within acceptable limits.

Conclusion

For simultaneous estimation of Beclomethasone Dipropionate, an RP-HPLC method was devised. Microbiological assay is the best alternative for Neomycin Sulphate. They are difficult to determine using reversed-phase HPLC with absorbance detection because they lack a suitable UV absorbing chromophore. In RP-HPLC method, good resolution and separation of drug was achieved. Retention time of Beclomethasone Dipropionate was found to be 10.036 min respectively with a flow rate of 1 ml/min. It was performed on a Kromosil C18, 5µ, 15 cm × 4.6 mm column. The mobile phase consist a mixture of ACN : WFI : Methanol : Glacial Acetic Acid : Triethylamine (55 :40 : 5 : 1 : 0.1 v/v). This was found to give sharp peak of BD at a wavelength of 235 nm with a flow rate of 1.2ml/min. The linear regression analysis data for the calibration curve showed a good linear relationship with a regression coefficient 0.999. The linear regression equation was y = 64760x - 1875. Linearity was found in the range of 5-15 µg/ml. The accuracy (% recovery) was found to be in the range of 99.07- 99.85 %. The proposed method was accurate and precise. Therefore proposed method can be used for routine analysis of Beclomethasone Dipropionate in ointment Dosage form. Using the Microbial Assay method, a high killing zone capacity of bacteria was attained. The NS was performed on agar culture media, which consisted of 20 mL Grove–1 Randall's culture medium (Difco) placed into a 100 x 20 mm Petri dish used to inhibit the Staphylococcus aureus strain. The calibration curve's linear regression analysis data revealed a solid linear relationship with a regression coefficient of 0.998. y = 2.401x – 0.001 was the linear regression equation. Linearity was found in the 2.5-15 g/ml range. The accuracy (percent recovery) was discovered to be between 98.49 and 99.03 percent. The RP-HPLC method was used to research the forced degradation of Beclomethasone Dipropionate, and the microbiological assay method was used to study the forced degradation of Neomycin Sulphate. Degradation results were found to be within acceptable bounds. For the detection of BD and NS in suspensions generated under various conditions, a simple and rapid stability-indicating RP-HPLC method and microbiological cylinder plate assay were devised and validated. The degradation of BD was in following order: oxidation > acidic > alkaline > thermal. At light conditions, there was no significant degradation of BD over time, while the degradation of NS was in following order: oxidation > alkaline> thermal>acidic>light. Because the forced deterioration had no effect on the BD peak, the suggested stability-indicating approach is particular, accurate, and exact, and it only requires a simple sample preparation procedure, making it suitable for use in stability studies and routine quality control analysis.

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