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ANALYTICAL METHOD DEVELOPMENT FOR SIMULTANEOUS ESTIMATION OF OXYBENZONE, OCTOCRYLENE, OCTINOXATE AND AVOBENZONE IN SUNSCREEN BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND ITS VALIDATION

Chirag Bhuva[1]*, Rambir Singh[2] and Anil Sharma[1]

[1]* Department of Quality Assurance, Gyan Vihar School of pharmacy, Suresh Gyan Vihar University, Jaipur, 302025, India
[2] Department of Pharmaceutical Chemistry, Gyan Vihar School of pharmacy, Suresh Gyan Vihar University, Jaipur, 302025, India

ABSTRACT

The separation of oxybenzone, octocrylene, octinoxate and avobenzone were carried out on waters C_{18}, 5 µ (250 X 4.6 mm) column. The compounds were eluted using a mobile phase of MeOH : H_2O (90:10 % v/v) at a flow rate of 1.0 mL/min and were monitored using UV detector at 330 nm. The retention times were 4.5, 7.6, 11.2 and 12.4 for octocrylene, octinoxate and avobenzone respectively. The reliability and analytical performance of the proposed RP-HPLC procedure were statistically validated with respect to linearity, ranges, system suitability, precision, accuracy, robustness, ruggedness, detection and quantification limits. The linear correlation coefficient at range of 12-28 µg/mL of assay concentration levels was found to be 0.999 for all drugs. Repeatability, inter day precision, intraday precision, ruggedness and robustness expressed as the percentage relative standard deviation (% RSD) were less than 2, accuracy by recovery study of were found to be between 99.0 % and 101.0 %. LOD values were found to be 0.07, 0.28, 2.12 and 2.54 µg/mL for octocrylene, octinoxate and avobenzone respectively. LOQ values were found to be 0.00, 0.00, 0.03 and 0.04 µg/mL for oxybenzone, octocrylene, octinoxate and avobenzone respectively.

Keywords: Oxybenzone, Octocrylene, Octinoxate, Avobenzone, RP-HPLC, Method development, Method validation, Simultaneous estimation, High Performance Liquid Chromatography.

INTRODUCTION

Analytical methods development and validation play important roles in the discovery, development and manufacture of pharmaceuticals.[1] Pharmaceutical products formulated with more than one drug, typically referred to as combination products are intended to meet previously unmet patients need by combining the therapeutic effects of two or more drugs in one product. These combination products can present daunting challenges to the analytical chemist responsible for the development and validation of analytical methods. This presentation will discuss the development and validation of analytical method

(Spectrophotometric, HPLC) for drug products containing more than one active ingredient. The official test methods that result from these processes are used by quality control laboratories to ensure the identity, purity, potency and performance of drug products.[2]

$$SD = \sqrt{(xi - x)2/N - 1}$$

Xi = Individual measurement in a set

X – Arithmetic mean of the set and

N = Number of replicates taken in the set.

RSD= SD/Mean

% RSD or coefficient of variance (CV) is expressed as

%RSD= CV= (SD/Mean)*100

METHOD DEVELOPMENT

The purpose of method development is to establish the applicability of an analytical method for its intended use on a certain sample. There are many tests that need to be carried out in the pharmaceutical field such as assay, dissolution, identification and related substance. Each of these tests, particularly the quantitative analysis has to be developed for its fit for use before approving it for routine application. Method development is on certain consideration. It exists today a good practical understanding of chromatographic separation. Any approach towards HPLC method development should be based upon knowledge of chromatographic process. A good method development requires more experimental runs as they are necessary to achieve final result.[1]

Accuracy (Recovery)[7]

Accuracy expresses the closeness of agreement between the value found and the value that is accepted as either a conventional true value or an accepted reference value. It may often be expressed as the recovery by the assay of known, added amounts of analyte. Samples (spiked placebos) are prepared normally covering 50% to 150% of the nominal sample preparation concentration. These samples are analyzed and the recoveries of each are calculated. Spiking can be performed as wet (e.g. via solution) or dry.

Precision[7]

- *Repeatability (method precision)*

Repeatability evaluates the variation experienced by a single analyst on a single instrument. Repeatability does not distinguish between variation from the instrument or system alone and from the sample preparation process. Repeatability is performed by analyzing multiple replicates of an assay composite sample using the analytical method. The recovery value is calculated and reported for each value.

- *Intermediate precision*

Intermediate precision refers to variations within a laboratory as with different days, with different instruments, by different analysts and so forth. Intermediate precision was formally known as ruggedness. A second analyst repeats the repeatability analysis on a different

day using different conditions and different instruments. The recovery values are calculated and reported. A statistical comparison is made to the first analysts results.

Specificity and/or Selectivity[7]

Specificity is the ability to assess unequivocally the analyte in the presence of components that may be expected to be present such as impurities, degradation products and excipients. There must be inarguable data for a method to be specific. Specific measure only the desired component without interference from other species which might be present; separation is not necessarily required. Selectivity is the ability of the analytical method to resolve each and every related compound in the mixture. Specificity is required for assay but selectivity is not. Both specificity and selectivity are required for impurities analysis. Specificity and selectivity is determined by analyzing blanks, sample matrix (placebo) and known related impurities to determine whether interferences occur. Specificity and selectivity are also demonstrated during forced degradation studies.

Detection Limit[7]

The detection limit (DL) or Limit of detection (LOD) of an individual procedure is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated as an exact value. In analytical procedures that exhibit baseline noise, the LOD can be based on a signal to noise ratio (3 to 1), which is usually expressed as the concentration (e.g. percentage, parts per billion) of analyte in the sample. There are several ways in which it can be determined, but usually involves injecting samples which generate S/N of 3:1 and estimating the DL. For instrumental methods limit of detection is calculated by using the following equation,

LOD = 3 * SD/slope of calibration curve

Where SD = Standard deviation of blank readings or intercepts of calibration curves

Quantitation Limit[7]

The quantitation limit (QL) or Limit of quantitation (LOQ) of an individual analytical procedure is the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low concentrations of compounds in sample matrices and is used particularly for the determination of impurities and/or degradation products. It is usually expressed as the concentration (e.g. percentage, parts per million etc.) of analyte in the sample. For analytical procedures that exhibit baseline noise the LOQ is generally estimated from a determination of signal-to-noise ratio (10 to 1) and is usually confirmed by injecting standards which give this S/N ratio and have acceptable % RSDs as well. For instrumental method LOQ can be calculated as follows,

LOQ=10 * SD/slope of calibration curve

Where SD = Standard deviation of blank readings or intercepts of calibration curves

Linearity[7]

Linearity evaluates the analytical procedure ability (within a give range) to obtain a response that is directly proportional to the concentration (amount) of analyte standard. If the method is linear, the test results are directly or by well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range. Note that this is different than Range (sometimes referred to as linearity of method) which is evaluated using samples and must encompass the specification range of the component assayed in the drug product. Linearity may be established for all active substances, preservatives and expected impurities. Evaluation is performed on standards.

A calibration curve is prepared by plotting absorbance (Y) as a function of concentration (X) which produces a linear curve with correlation equation [Y= m X + c]

$$m = \frac{N\sum x_i y_i - \sum x_i \sum y_i}{N\sum x_i^2 (\sum x_i)^2}$$

$$c = \overline{y_i} - \overline{x_i}$$

Range[7]

Range is the interval between the upper and lower concentrations (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity. Range is normally expressed in the same units as test results (e.g. percent, parts per million etc.) obtained by the analytical method. Range (sometimes referred to as linearity of method) is evaluated using samples (usually spiked placebos) and must encompass the specification range of the component assayed in the drug product.

Robustness Testing[7]

Description of robustness testing robustness is the measure of the ability of an analytical method to remain unaffected by small but deliberate variations in method parameters (e.g. pH, mobile phase composition, temperature, instrument settings etc.) and provides an indication of its reliability during normal usage. Robustness testing is a systematic process of varying a parameter and measuring the effect on the method by monitoring system suitability and/or the analysis of samples.

Ruggedness[7]

Ruggedness is the degree of reproducibility of test results obtained by the analysis of the same sample under a variety of normal test conditions such as different laboratories, different analysts, different instruments, different lots of reagents, different elapsed assay times and different assay temperature etc.

MATERIALS AND METHODS

Chemicals and Instruments Used

Reagents and chemicals

- HPLC Grade Methanol, Acetonitrile (Merck)
- HPLC Grade Water (Milli-Q)
- Potassium Dihydrogen Phosphate GR Grade (Merck)
- Milli–Q water and 0.2µ Nylon filter were used throughout the experimental work
- All the chemicals used during this project work were of either AR grade or HPLC grade, procured from Rankem chemicals, Ranbaxy, Mumbai and Qualigen chemicals, Qualigen Fine Chemicals, Mumbai

Instruments and equipments

- HPLC LC-2010C HT Shimadzu with LC Solution
- Column: Water C18, 5 µ (250 X 4.6 mm)
- Shimadzu SPD-M20A Prominence Diode array detector
- Perkin Elmer lambda 25 UV/Vis Double beam spectrophotometer
- Sartorius BT 224 S balance
- pH Meter Thermo electron corporation Orion 3 star pH Benchtop
- Calibrated glassware's were used for the study
- Filter used: Pall Life Sciences. Ultipor N66 Nylon 6.6 Membrane 0.2µ 47 mm

Methods

Development and validation of assay method for simultaneous estimation of oxybenzone, octocryline, octinoxate and avobenzone in sunscreen by RP-HPLC

Selection of Chromatographic Method

Oxybenzone, Octinoxate, Avobenzone and Octocrylene in sunscreen are polar in nature so, RP-HPLC technique was selected.

Selection of Detection Wavelength

An accurately weighed quantity of 50.0 mg Oxybenzone, 50.0 mg Octinoxate, 50 mg Avobenzone and 50.0 mg Octocrylene were transferred in 100.0 mL volumetric flask, dissolved with HPLC grade methanol and volume was made up to the mark with HPLC grade methanol, sonicated for 10 min. From the prepared solution, pipette out 10.0 mL in 100.0 mL volumetric flask and volume was made up to the mark with HPLC grade methanol to make final Concentrations: 50.0 ppm Oxybenzone, 50.0 ppm Octinoxate, 50.0 ppm Avobenzone and 50.0 ppm Octocrylene.

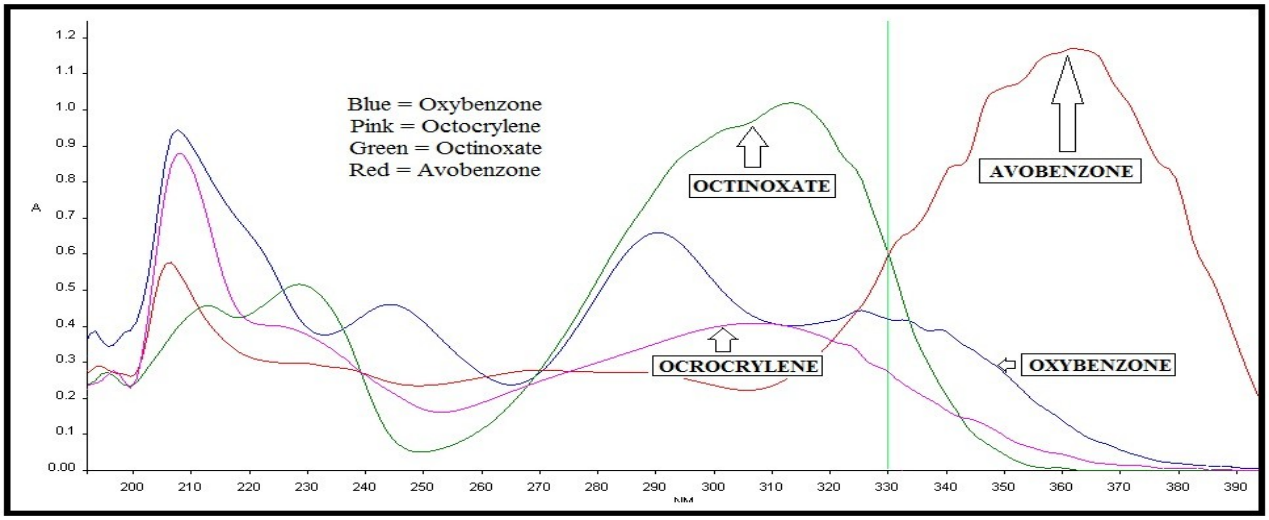


Figure 1: Isobestic point

Optimization of Chromatographic Condition

Preparation of Standard Solution

An accurately weighed quantity of 30.0 mg Oxybenzone, 75.0 mg Octinoxate, 20.6 mg Avobenzone and 30.0 mg Octocrylene were transferred in 100.0 mL volumetric flask, dissolved with HPLC grade methanol and volume was made up to the mark with HPLC grade methanol, sonicated for 10 min.-Stock Solution. From the prepared solution, pipette out 5.0 mL in 50.0 mL volumetric flask and volume was made up to the mark with HPLC grade methanol to make final Concentrations: 30.0 ppm Oxybenzone, 75.0 ppm Octinoxate, 20.6 ppm Avobenzone and 30.0 ppm Octocrylene.

Initial Chromatographic Condition

System : Shimadzu LC-2010 C HT
Column : Chromatopack Peerless Basic C_{18}, 5 µ (250 X 4.6 mm)
Flow rate : 1.0 mL/min
Detection Wavelength : 330 nm
Column Temperature : 25°C
Injection volume : 20 µL
Mobile Phase : The various mobile phases tried are shown in Table 1

Table 1: Trial and error

Sr. No	Mobile Phase	Flow rate (mL/min.)	Observation
1	Methanol: Water (50:50 % v/v)	1	Peaks were not resolved and higher R_t =2 hr.

<table>
<tr><td>2</td><td>Methanol: Water
(60:40 % v/v)</td><td>1</td><td colspan="5">Peaks were not resolved and higher R_t =1.5 hr.</td></tr>
<tr><td>3</td><td>Methanol: Water
(70:30 % v/v)</td><td>1</td><td colspan="5">Peaks were not resolved and higher R_t =1.0 hr.</td></tr>
<tr><td>4</td><td>Methanol: Water
(80:20 % v/v)</td><td>1</td><td colspan="5">Peaks were slightly resolved as compare to above trial and less R_t =0.4 hr.</td></tr>
<tr><td>5</td><td>Methanol: Water
(88:15 % v/v)</td><td>1</td><td colspan="5">Peaks were more resolved as compare to above trial and less R_t =0.3 hr., Intensity is not good.</td></tr>
<tr><td>6</td><td>Methanol: Water
(90:10 % v/v)</td><td>1</td><td colspan="5">Peaks were resolved and good R_t =0.15 hr.
and good resolution, tailing factor <1.5.
<table><tr><td>R_t</td><td>4.660</td><td>7.974</td><td>11.694</td><td>12.916</td></tr></table></td></tr>
<tr><td>7</td><td>Methanol: Water
(95:05 % v/v)</td><td>1</td><td colspan="5">Peaks were merge R_t =0.07 hr.
and good resolution, tailing factor <1.5,
<table><tr><td>R_t</td><td>3.847</td><td>4.923</td><td>6.807</td><td>7.443</td></tr></table></td></tr>
<tr><td>8</td><td>Methanol:Water:ACN:IPA
(85:10:05:0 % v/v)</td><td>1</td><td colspan="5">Intensity good but peak were merge together</td></tr>
<tr><td>9</td><td>Methanol:Water:ACN:IPA
(80:15:05:0 % v/v)</td><td>1</td><td colspan="5">Intensity good but peak were merge slightly and tailing factor > 1.5</td></tr>
<tr><td>10</td><td>Methanol:Water:ACN:IPA
(83:12:05:0 % v/v)</td><td>1</td><td colspan="5">Intensity good but peak were merge slightly and tailing factor > 1.5</td></tr>
<tr><td>11</td><td>Methanol:Water:ACN:IPA
(83:12:05:0 % v/v)</td><td>1</td><td colspan="5">Intensity good but peak were merge slightly and tailing factor > 1.5</td></tr>
<tr><td>12</td><td>Methanol:Water:ACN:IPA
(85:10:0:05 % v/v)</td><td>1</td><td colspan="5">Intensity good but tailing factor > 1.8 and resolution =2</td></tr>
<tr><td>13</td><td>Methanol:Water:ACN:IPA
(80:15:0:05 % v/v)</td><td>1</td><td colspan="5">Intensity good but tailing factor > 1.7 and resolution =2</td></tr>
<tr><td>14</td><td>Methanol: Water
(90:10 % v/v)</td><td>1</td><td>OPTIMIZED</td><td>4.66</td><td>7.97</td><td>11.69</td><td>12.91</td></tr>
</table>

Final optimized chromatographic condition

System : Shimadzu LC-2010 C HT
Column : Chromatopack Peerless Basic C_{18}, 5 µ (250 X 4.6 mm)
Detection Wavelength : 330 nm
Flow rate : 1.0 ml/min
Column Temperature : 25 °C
Injection volume : 20 µL
Mobile Phase : Methanol: Water (90:10 % v/v)
Solvent Ratio : MeOH
Operating pressure : 205 kgf (± 1)

VALIDATION OF RP-HPLC METHOD

Linearity and Range

Preparation of standard solution

An accurately weighed quantity of 30.0 mg Oxybenzone, 30.0 mg Octocrylene 75.0 mg Octinoxate and 20.6 mg Avobenzone were transferred in 100.0 mL volumetric flask, dissolved with HPLC grade methanol and volume was made up to the mark with HPLC grade methanol, sonicated for 10 min. Stock Solution. From that stock solution pipette out 2, 2.7, 3.4, 4, 4.7 mL in six 50.0 mL volumetric flask individually and volume was made up to the mark with HPLC grade water to make final Concentration: 12, 16, 20, 24, 28 µg/ mL.

Procedure

The selected stationary phase was allowed to equilibrate with mobile phase till steady base line was obtained. Then each solution was injected and chromatograms ware recorded. The observations of concentration of the drug and their area under curves are showed in Table: 2.

Table 2: Linearity and range

Sr. No.	Conc. (µg/ mL)	Peak area				Figure
		Oxybenzone	Octocrylene	Octinoxate	Avobenzone	
1	12	953189	552059	2311698	873804	6.3
2	16	1270426	734763	3067800	1165039	6.4
3	20	1587868	921256	3837107	1455813	6.5
4	24	1906100	1106100	4595054	1753868	6.6
5	28	2226005	1288911	5341625	2049834	6.7
	Slope	79533	46126	18967	73522	
	Intercept	1935	1902	37103	10773	
	Correlation	0.999	0.999	0.999	0.999	

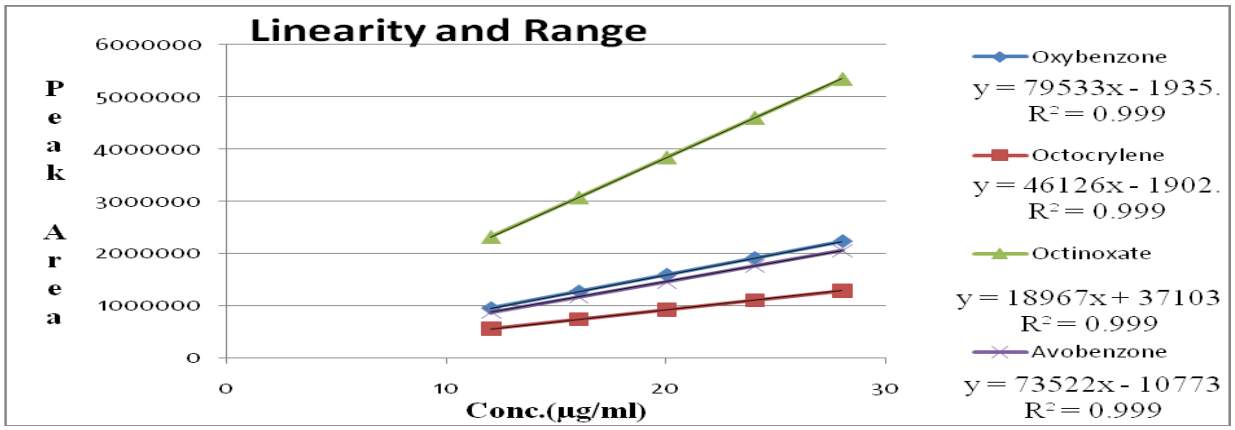


Figure 2: Linearity and range graph

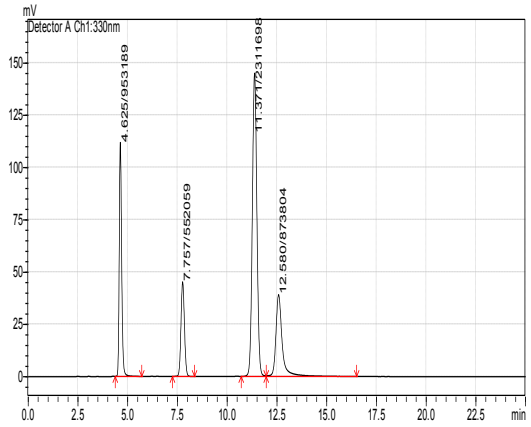


Figure 3: Linearity and range chromatogram 1 (Conc. 12 µg/mL)

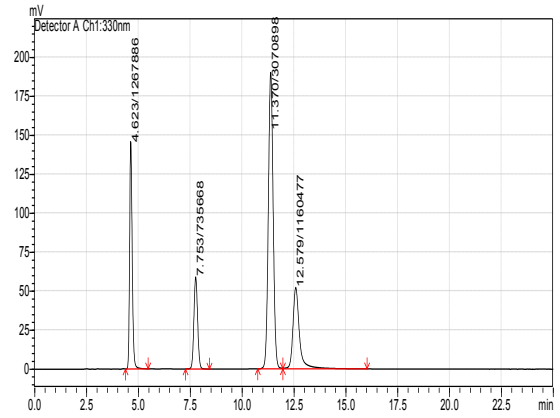


Figure 4: Linearity and range chromatogram 2 (Conc. 16 µg/mL)

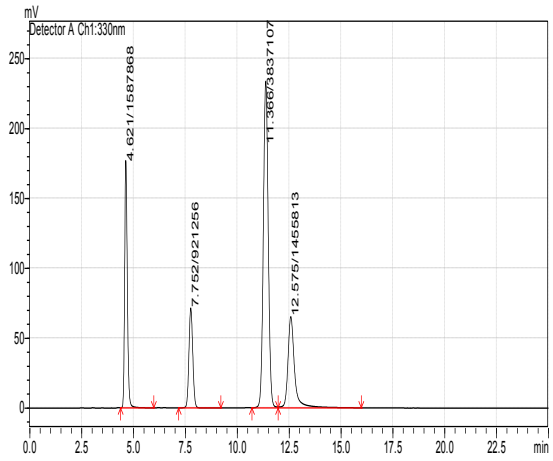


Figure 5: Linearity and range chromatogram 3 (Conc. 20 µg/mL)

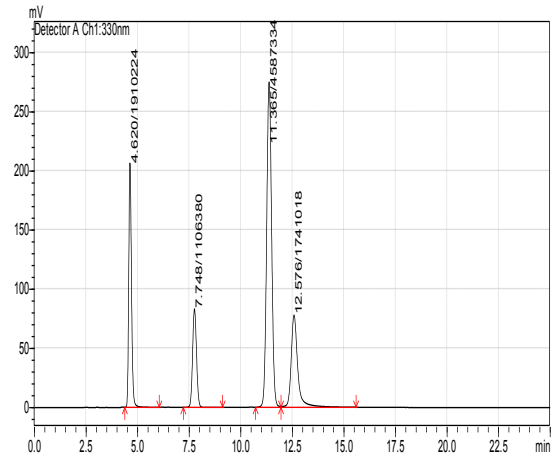


Figure 6: Linearity and range chromatogram 4 (Conc. 24 µg/mL)

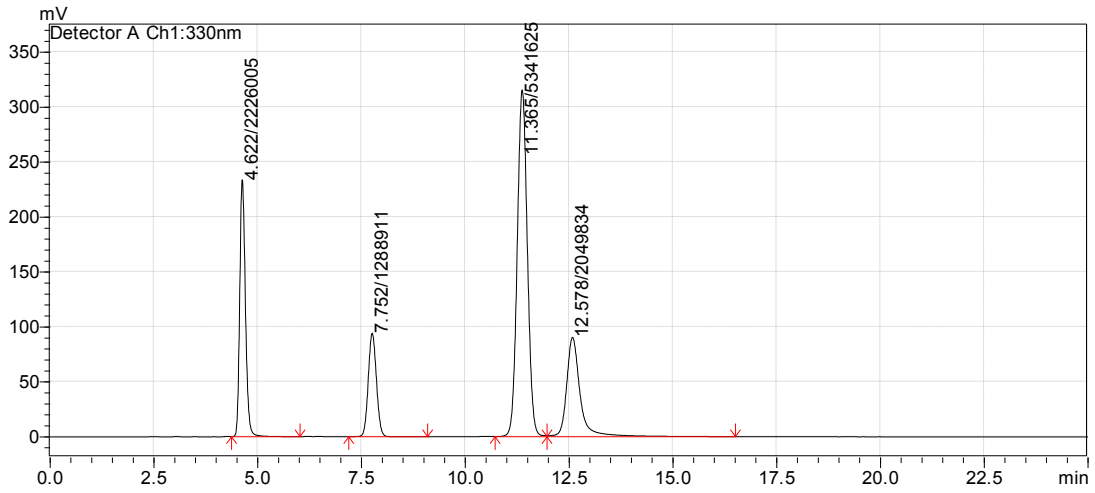


Figure 7: Linearity and range chromatogram 5 (Conc. 28 µg/mL)

Limit of Detection And Limit of Quantification

Based on the Standard Deviation of the Response and the Slope

Limit of detection(LOD) = 3.3 σ/S

Limit of quantification(LOQ) = 10σ/S

Where **σ** = the standard deviation of the response

S = the slope of the calibration curve

Table 2: Limit of Detection and Limit of Quantification

Sr. No.	Slope				Intercept			
	OXY	OCL	OXT	AVO	OXY	OCL	OXT	AVO
1	79533	46126	18967	73522	1935	1902	37103	10773
2	80546	45841	19101	72547	1938	1905	38154	10988
3	78011	46879	18754	72545	1889	2001	36874	99875
4	77266	45257	17586	74112	2001	1897	37541	11012
5	81425	46752	18324	73658	1945	2045	37456	10098
6	75044	44874	17254	73584	2014	2058	37589	10954
SD	2340.99	798.37	760.44	640.28	46.33	68.49	488.14	493.45
LOD (µg/mL)	0.07	0.28	2.12	2.54	-	-	-	-
LOQ (µg/mL)	0.00	0.00	0.03	0.04	-	-	-	-

System Suitability Parameters And Specificity: Blank And Placebo Interference

System Suitability Parameters

For proposed method System suitability parameters like Number of Theoretical plates(N), Peak Asymmetry(As), Resolution(Rs), Tailing Factor included in Table 3.

Table 3: System Suitability Parameters

Parameters	Oxybenzone	Octocrylene	Octinoxate	Avobenzone	Limits required
Number of Theoretical plates	5899.31	7953.53	10672.41	9375.59	More than 2000
Rt	0.093	7.72	11.3	12.49	Less than 2
Resolution(Rs)	0	10.55	9.13	2.48	More than 2
Tailing Factor	1.261	1.09	1.06	1.35	Less than 2

Specificity: Blank and Placebo Interference

Specificity is the ability of the test method to measure an analyte without interference from other samples and the matrix components. In quantitative analysis, a method is called completely selective when it produces correct analytical signal. A method is called completely selective when it produces correct analytical results for mixture without any mutual interaction of the components. Blank (water), Placebo, Standard and Sample solutions were injected and interference was observed.

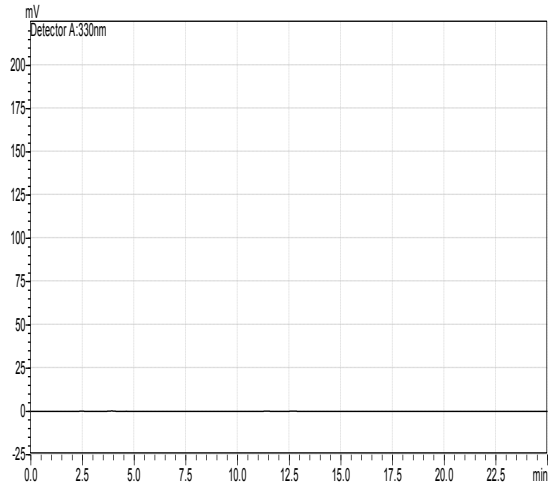


Figure 8: Blank chromatogram

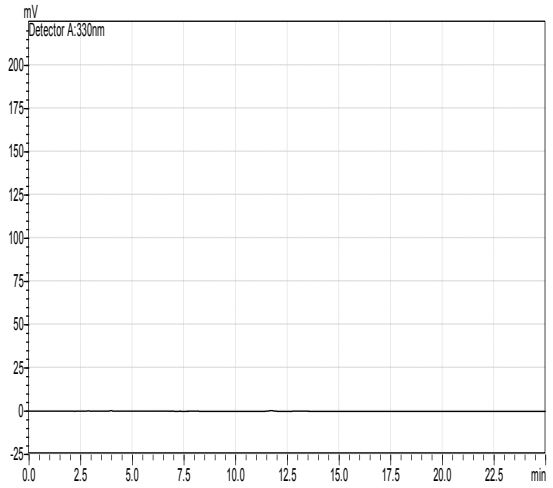


Figure 9: Placebo chromatogram

Precision

Repeatability

Preparation of standard solution

An accurately weighed quantity of 30.0 mg Oxybenzone, 30.0 mg Octocrylene 75.0 mg Octinoxate and 20.6 mg Avobenzone were transferred in 100.0 mL volumetric flask, dissolved with HPLC grade methanol and volume was made up to the mark with HPLC grade methanol, sonicated for 10 min. Stock Solution. From the prepared solution, pipette out 5.0 mL in 50.0 mL volumetric flask and volume was made up to the mark with HPLC grade methanol to make final concentrations: 30.0 ppm Oxybenzone, 75.0 ppm Octinoxate, 20.6 ppm Avobenzone and 30.0 ppm Octocrylene.

Procedure:

The selected stationary phase was allowed to equilibrate with mobile phase till steady base line was obtained. Then 6 injections were injected and chromatograms were recorded. The observations are showed in Table 4.

Table 4: Repeatability

Sr. No.	STD wt taken	Peak area			
		Oxybenzone	Octocrylene	Octinoxate	Avobenzone
1	Oxybenzone = 16.49 mg Octocrylene = 24.17 mg Octinoxate = 60.28 mg Avobenzone = 16.49 mg	1588254	921247	3840044	1473338
2		1589929	922018	3835290	1446470
3		1588344	922213	3836679	1458376
4		1586958	922837	3830643	1459034
5		1590253	933277	3830871	1454340
6		1589602	922440	3831210	1460909
	Mean	1588890	924005.3333	3834122.833	1458744.5
	SD	1255.346167	4572.732866	3850.317879	8808.380958
	% RSD	0.079007745	0.494881653	0.100422392	0.603833019
	LIMIT	**% RSD < 2**	**% RSD < 2**	**% RSD < 2**	**% RSD < 2**

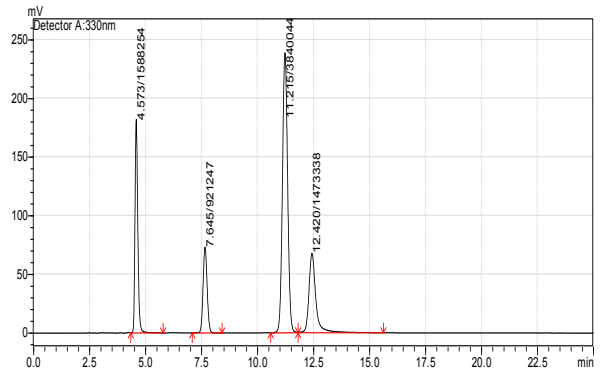


Figure 10: Repeatability chromatogram 1

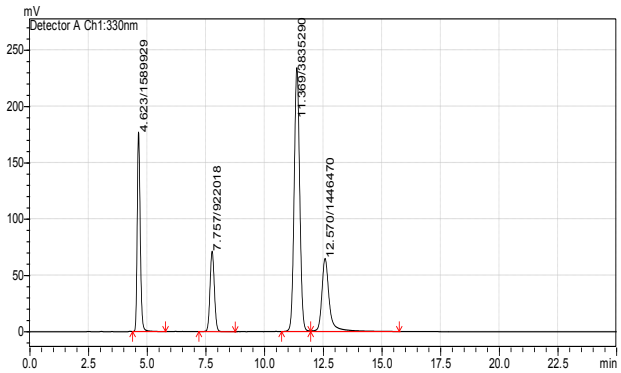


Figure 11: Repeatability chromatogram 2

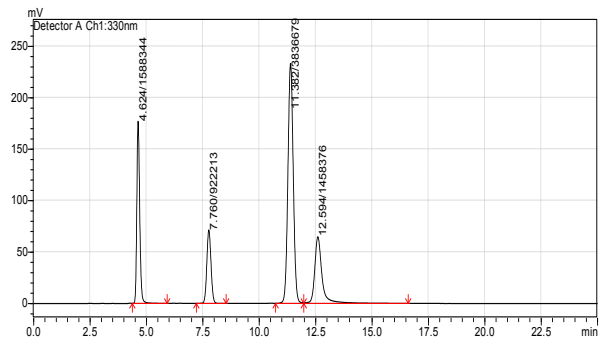


Figure 12: Repeatability chromatogram 3

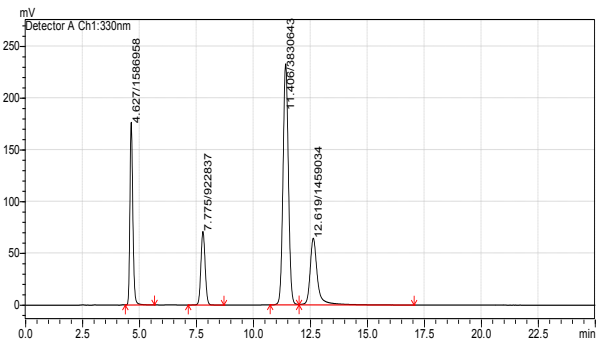


Figure 13: Repeatability chromatogram 4

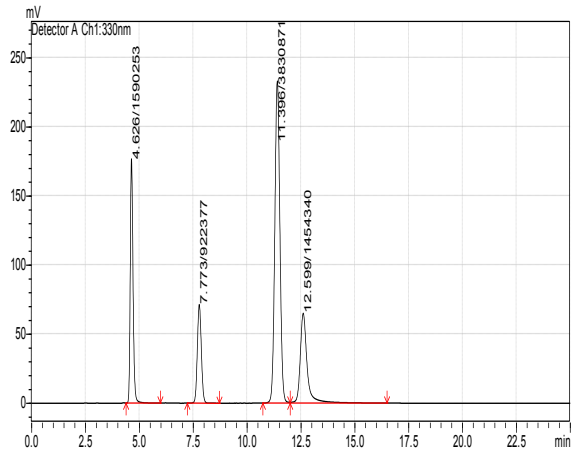


Figure 14: Repeatability chromatogram 5

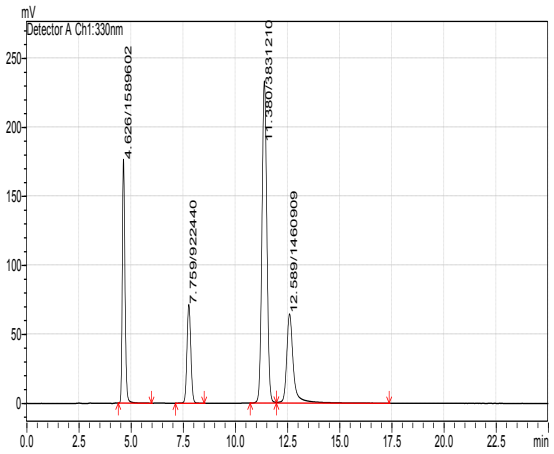


Figure 15: Repeatability chromatogram 6

Inter Day Precision

Inter day precision was done carrying out the analysis of standard solutions at three different concentrations in the linearity range for three different days and % RSD was calculated.

Preparation of standard solutions

An accurately weighed quantity of 30.0 mg Oxybenzone, 30.0 mg Octocrylene 75.0 mg Octinoxate and 20.6 mg Avobenzone were transferred in 100.0 mL volumetric flask, dissolved with HPLC grade methanol and volume was made up to the mark with HPLC grade methanol, sonicated for 10 min.Stock Solution. From that stock solution pipette out 2, 3.4, 4.7 mL in six 50.0 mL volumetric flask individually and volume was made up to the mark with HPLC grade water to make final concentration: 12, 20, 28 µg/ mL.

Procedure

The selected stationary phase was allowed to equilibrate with mobile phase till steady base line was obtained. Then 3 injections of each were injected as per final optimized chromatographic condition at three different 1, 2 & 3 days and chromatograms were recorded. The observations of their area under curves are recorded in Table 5.

Table 5: Inter day precision

Hour	Conc. (µg/mL)	Peak area			
		Oxybenzone	Octocrylene	Octinoxate	Avobenzone
1	12 µg/mL	953189	552059	2311698	873804
2		952402	552824	2307515	867080
3		952796	552442	2309607	870442
	% RSD	0.041299523	0.0692381	0.09055655	0.38624055
1	20 µg/mL	1587868	921256	3837107	1455813
2		1589566	920638	3830272	1448604
3		1588717	920947	3833689.5	1452208.5
	% RSD	0.053439348	0.033552419	0.08914389	0.24820816
1	28 µg/mL	2226005	1288911	5341625	2049834
2		2224937	1290873	5144699	2056336
3		2225471	1289892	5243162	2053085
	% RSD	0.023994921	0.076052879	1.87793168	0.15834707

Intraday precision

Intraday precision was done carrying out the analysis of standard solutions at three different concentrations in the linearity range with in a one day and % RSD was calculated.

Preparation of standard solutions

An accurately weighed quantity of 30.0 mg Oxybenzone, 30.0 mg Octocrylene 75.0 mg Octinoxate and 20.6 mg Avobenzone were transferred in 100.0 mL volumetric flask, dissolved with HPLC grade methanol and volume was made up to the mark with HPLC grade methanol, sonicated for 10 min.-**Stock Solution**. From that stock solution pipette out 2, 3.4, 4.7 mL in six 50.0 mL volumetric flask individually and volume was made up to the mark with HPLC grade water to make final concentration: 12, 20, 28 µg/mL.

Procedure

The selected stationary phase was allowed to equilibrate with mobile phase till steady base line was obtained. Then one each of 12, 20, 28 µg/mL of Oxybenzone, Octocrylene, Octinoxate and Avobenzone combination injections were injected as per final optimized chromatographic condition, then this

procedure was repeated by making new samples, after 3 and 6 hours with in a day, chromatograms were recorded and observations are shown in Table 6.

Table 6: Intraday precision

Hour	Conc. (µg/mL)	Peak area			
		Oxybenzone	Octocrylene	Octinoxate	Avobenzone
0	12 µg/mL	953542	552874	2311874	873542
3		952521	552685	2307857	867985
6		953032	552780	2309866	870764
	% RSD	0.053566	0.017095	0.086953	0.319088
0	20 µg/mL	1589857	929854	3836351	1458744
3		1589123	923214	3839877	1442145
6		1589490	926534	3838114	1450445
	% RSD	0.023089	0.358325	0.045934	0.572204
0	28 µg/mL	2229877	1287857	5345241	2045689
3		2226514	1290041	5148744	2057878
6		2228196	1288949	5246993	2051784
	% RSD	0.075465	0.08472	1.872473	0.297034

Accuracy

Accuracy of the proposed method was ascertained on the basis of recovery studies performed by standard addition method.

Preparation of sample

Accurately weighed quantities of 0.024, 0.030, 0.036 mg of Oxybenzone, 0.024, 0.030, 0.036 mg of Octocrylene, 0.060, 0.075, 0.090 mg of Octinoxate and 0.01648, 0.02060, 0.02472 mg of Avobenzone were homogenized with 10.0 gm cream base individually then 0.1 gm sample of each transfer in to three 100 mL volumetric flask to get spike level of 80 %, 100 % and 120 % of Oxybenzone, Octocrylene, Octinoxate and Avobenzone add 20.0 mL of HPLC grade methanol, sonicated for 10 min and then volume make up to 250.0 mL with HPLC grade water and filtered through ultipor N66 Nylon membrane 0.2 micrometer. A 20 µL volume of each final dilution were injected separately and chromatographs were recorded. The AUC of standard and each sample were recorded and recovery is shown in table 7.

Table 7: Accuracy

STD wt taken	Peak area			
	Oxybenzone	Octocrylene	Octinoxate	Avobenzone
Oxybenzone = 30.2 mg Octocrylene = 30.2 mg Octinoxate = 77.0 mg Avobenzone = 20.7 mg	1594544	865377	3782540	1451587
	1594540	865381	3782548	1451580
	1596110	870672	3807070	1457659
	1601170	868402	3808418	1392678
	1611186	871051	3815559	1398276
	1598258	865148	3806466	1450665
MEAN	**1599301**	**867672**	**3800434**	**1433741**

Wt added in base (mg)	**Peak area of sample**				**% Recovery**			
	OXY	**OCL**	**OXT**	**AVO**	**OXY**	**OCL**	**OXT**	**AVO**
80 %	1350507	719185	3142090	1155075	100.67	100.39	100.12	100.60
	1353928	720842	3145156	1139761				
	1354548	719523	3146857	1139483				
Mean	**1352994**	**719850**	**3144701**	**1144773**				
100 %	1676788	991718	4035490	1445188	99.61	99.52	100.48	100.06
	1676002	991755	4027142	1432195				
	1678167	994063	4038114	1426513				
Mean	**1676986**	**992512**	**4033582**	**1434632**				
120 %	1950821	1059882	4659162	1675425	100.06	100.12	100.13	100.14
	1953767	1060362	4655817	1669327				
	1949839	1057832	4649275	1663578				
Mean	**1951476**	**1059359**	**4654751**	**1669443**	**100.06**	**100.12**	**100.13**	**100.14**
SD	-	-	-	-	0.53	0.44	0.2	0.29

% RSD	-	-	-	-	0.53	0.44	0.2	0.29

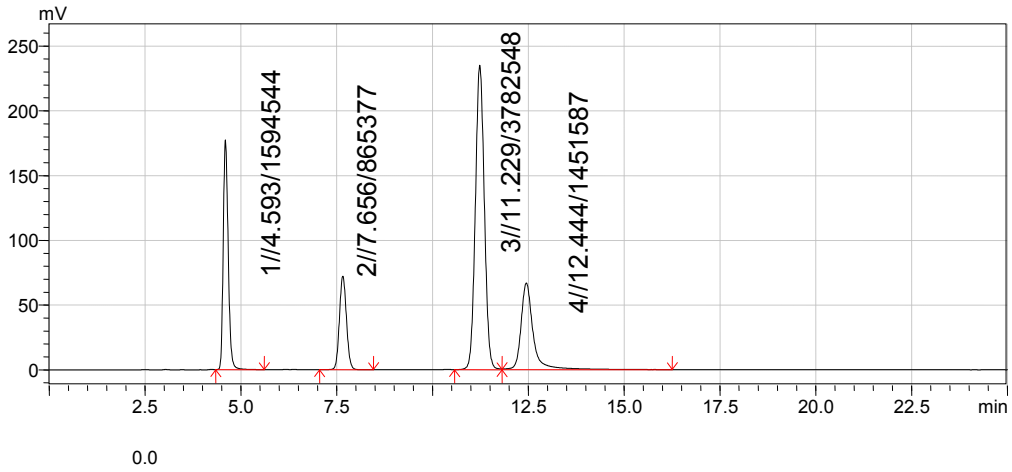


Figure 16: Accuracy chromatogram 1 (STD)

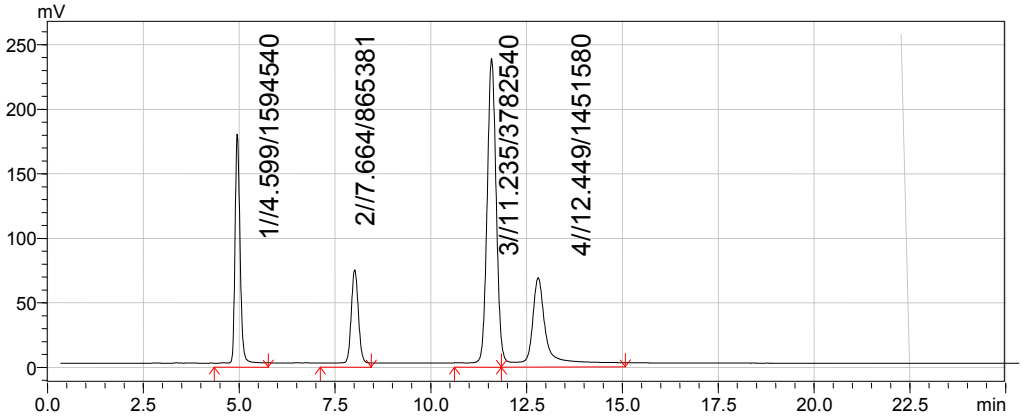


Figure 17: Accuracy chromatogram 2 (STD)

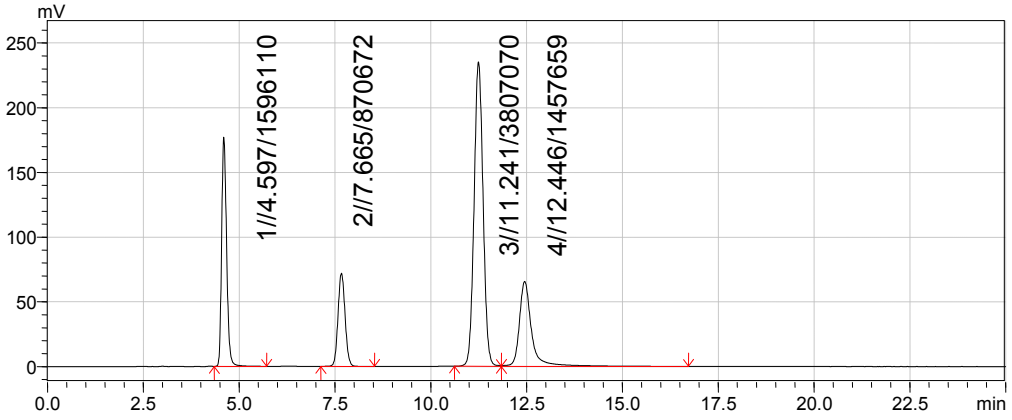


Figure 18: Accuracy chromatogram 3 (STD)

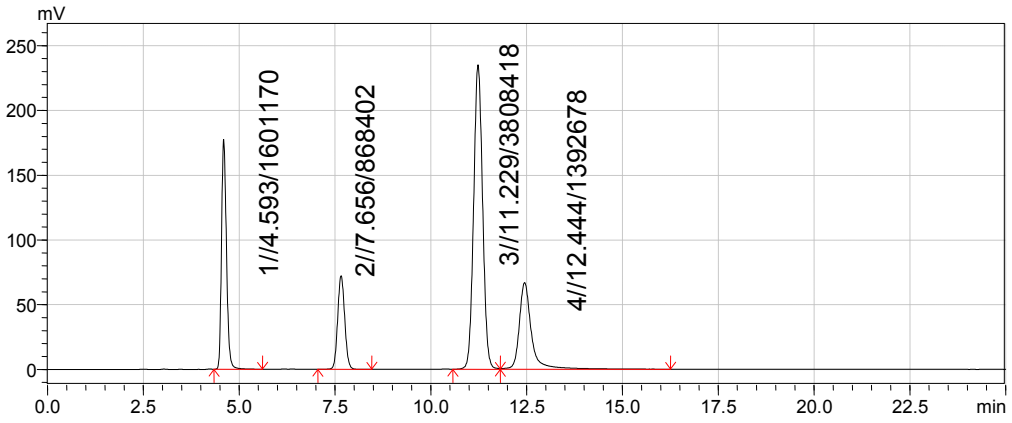


Figure 19: Accuracy chromatogram 4 (STD)

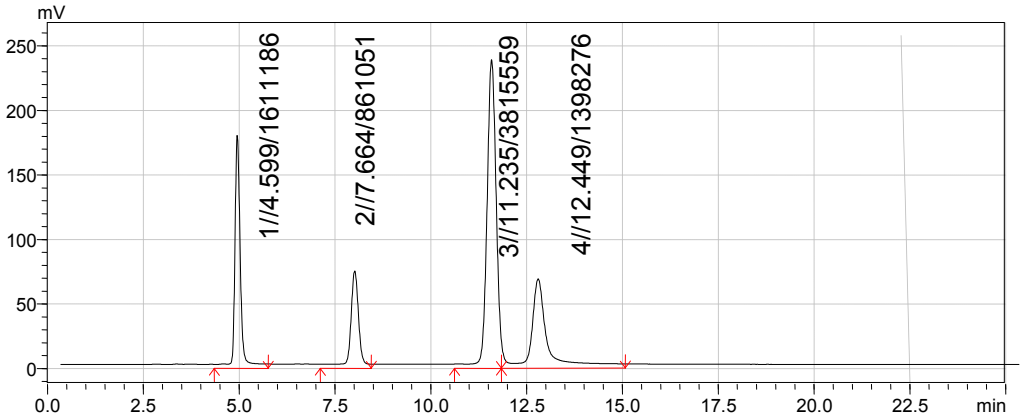
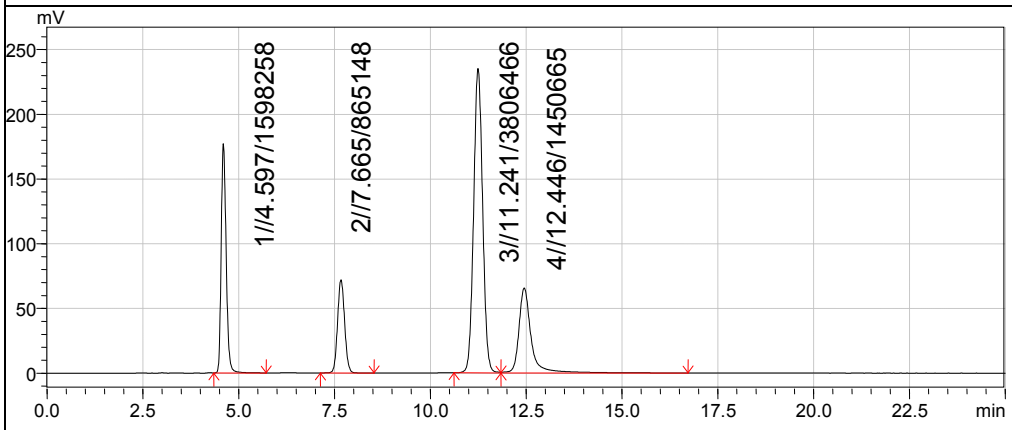


Figure 20: Accuracy chromatogram 5 (STD)



Figue 21: Accuracy chromatogram 6 (STD)

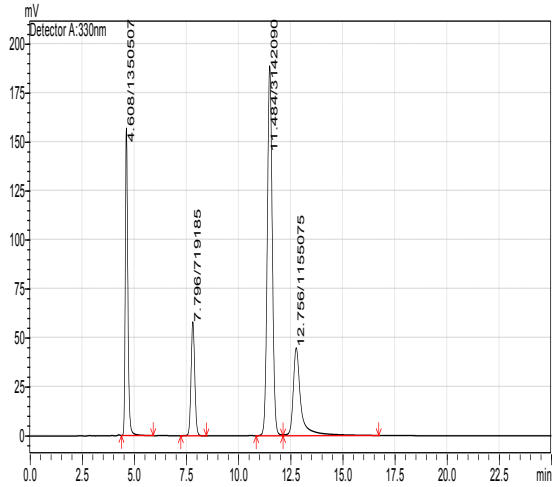


Figure 22: Accuracy chromatogram 7 (80 %)

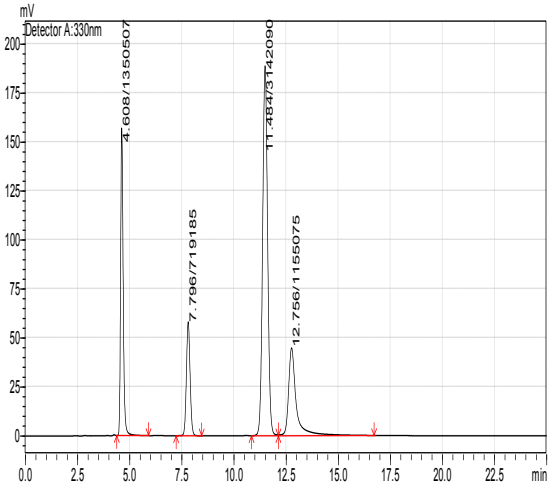


Figure 23: Accuracy chromatogram 8 (80 %)

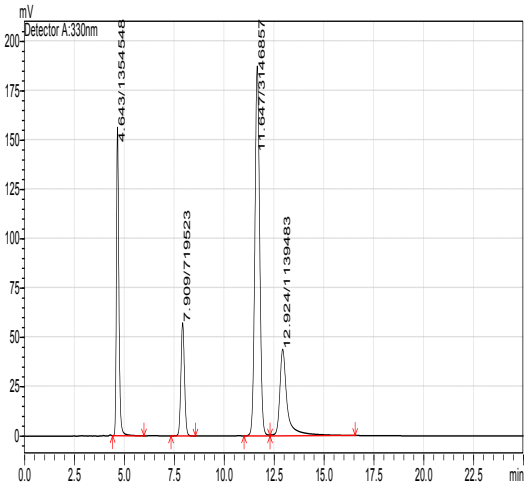


Figure 24: Accuracy chromatogram 9 (80 %)

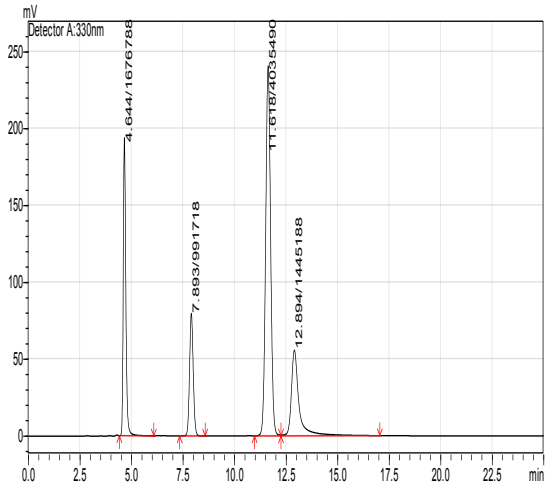


Figure 25: Accuracy chromatogram 10 (100 %)

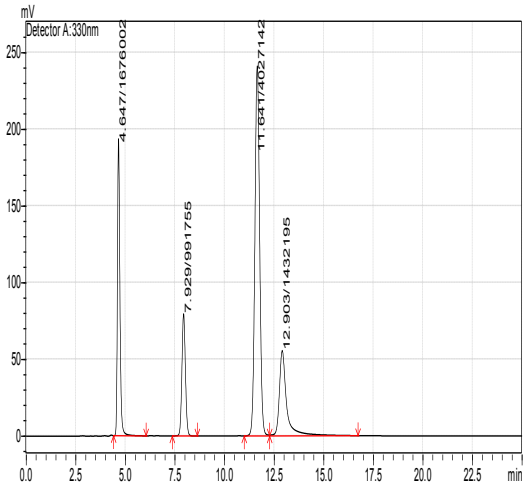


Figure 26: Accuracy chromatogram 11 (100 %)

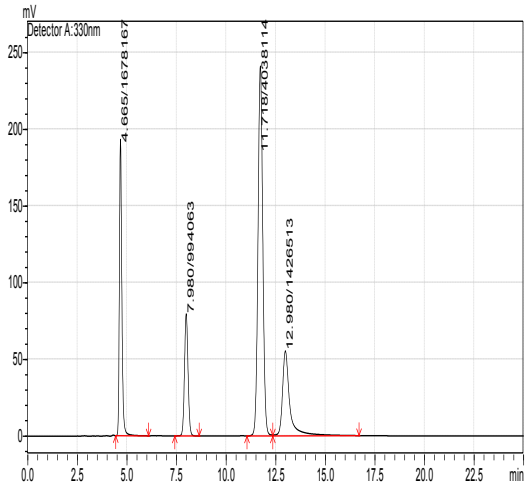


Figure 27: Accuracy chromatogram 12 (100 %)

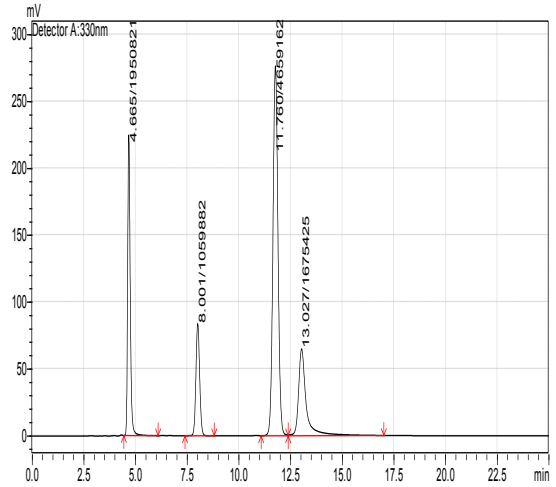


Figure 28: Accuracy chromatogram 13 (120 %)

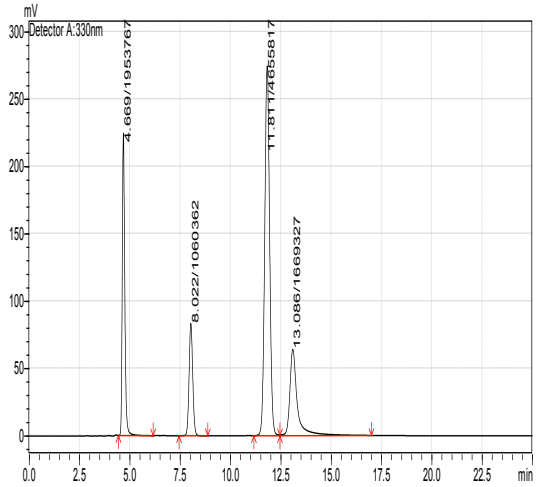


Figure 29: Accuracy chromatogram 14 (120 %)

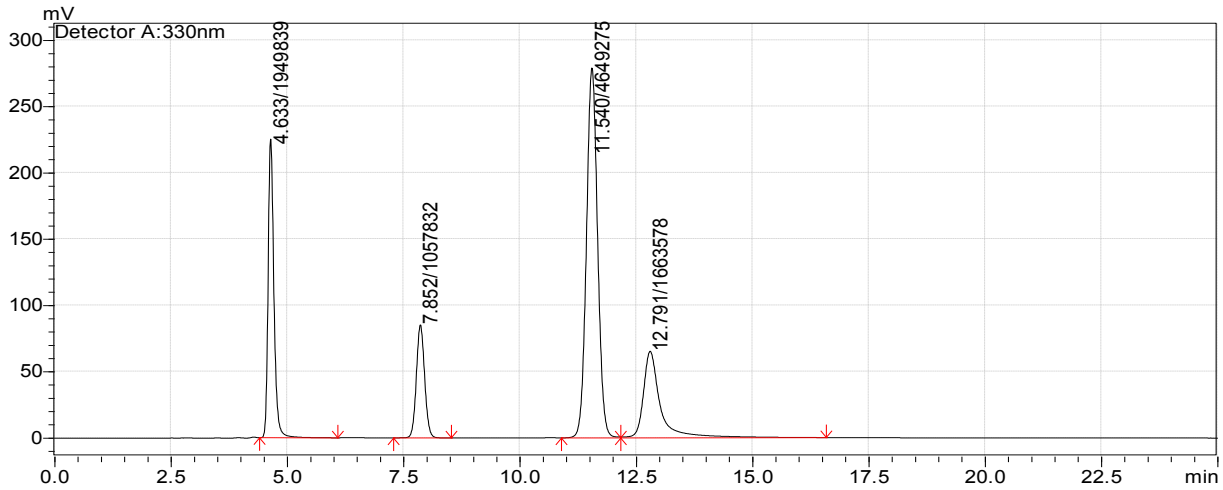


Figure 30: Accuracy chromatogram 15 (120 %)

RUGGEDNESS

Preparation of Sample

An accurately weighed quantity of 30.0 mg Oxybenzone, 30.0 mg Octocrylene, 75.0 mg Octinoxate and 20.6 mg Avobenzone were transferred in 100.0 mL volumetric flask, dissolved with HPLC grade methanol and volume was made up to the mark with HPLC grade methanol, sonicated for 10 min. Stock Solution. From the prepared solution, pipette out 5.0 mL in 50.0 mL volumetric flask and volume was made up to the mark with HPLC grade methanol to make final concentrations: 30.0 ppm Oxybenzone, 75.0 ppm Octinoxate, 20.6 ppm Avobenzone and 30.0 ppm Octocrylene.

Procedure

The selected stationary phase was allowed to equilibrate with mobile phase till steady base line was obtained. The ruggedness of the proposed method has been verified by analyzing the six injections used for method precision by two different analysts, using two different instruments and on different days. The ruggedness results were compared with method precision data. The overall mean, standard deviation (SD) and % RSD of the assay values are shown in table 8 and table 9**.**

Table 8: Ruggedness (Analysis 1 Result)

Analysis 1 Result **Day 1, Analyst 1, Instrument 1**								
Sr. No.	Oxybenzone		Octocrylene		Octinoxate		Avobenzone	
	R_t	Peak area	R_t	Peak area	R_t	Peak area	R_t	Peak area
1	4.62	1608445	7.74	933293	11.34	3877974	12.54	1522755
2	4.62	1607963	7.73	930283	11.33	3892070	12.53	1495022
3	4.62	1604563	7.74	932936	11.34	3886629	12.53	1485779
4	4.63	1608958	7.74	930478	11.33	3878958	12.53	1464883
5	4.63	1607848	7.73	933199	11.32	3877817	12.51	1465169
6	4.63	1607283	7.73	930624	11.32	3876033	12.51	1457748
MEAN	4.62	1607510	7.74	931802.17	11.33	3881580.17	12.52	1481892.6
SD	0.00	1550.92	0.00	1477.07	0.01	6329.93	0.01	24532.38
% RSD	0.10	0.10	0.06	0.16	0.07	0.16	0.09	1.66

Table 9: Ruggedness (Analysis 2 Result)

Analysis 2 Result **Day 2, Analyst 2, Instrument 2**								
Sr. No.	Oxybenzone		Octocrylene		Octinoxate		Avobenzone	
	R_t	Peak area	R_t	Peak area	R_t	Peak area	R_t	Peak area
1	4.63	1611026	7.72	929215	11.31	3865412	12.50	1477528
2	4.67	1605411	7.71	925860	11.29	3875676	12.48	1472493
3	4.63	1599133	7.72	929195	11.30	3874330	12.49	1477786
4	4.62	1604419	7.72	932490	11.33	3874159	12.52	1475444
5	4.63	1599509	7.74	929558	11.34	3876234	12.53	1477377
6	4.63	1603672	7.75	930525	11.34	3865150	12.53	1467737

MEAN	4.63	1603861.67	7.73	929473.83	11.32	3871826.83	12.51	1474727.5
SD	0.02	4367.62	0.02	2163.97	0.02	5131.75	0.02	3964.61
% RSD	0.36	0.27	0.22	0.23	0.19	0.13	0.19	0.27
OVER ALL MEAN	4.63	1605685.83	7.73	930638	11.32	3876703.50	12.52	1478310.1
OVER ALL SD	0.01	2959.27	0.01	1820.52	0.01	5730.84	0.02	14248.49
OVER ALL % RSD	0.23	0.18	0.14	0.20	0.13	0.15	0.14	0.96

Robustness

The robustness of the method was evaluated by deliberately varying the chromatographic conditions viz. composition of organic phase in mobile phase, flow rate, column oven temperature and change in wavelength of detection. For that STD (normal chromatographic condition) and after change (alteration in chromatography) solutions were injected. The amount of sunscreen lotion was calculated from STD (normal chromatographic condition) and After Change (alteration in chromatography). The results were compared and results are tabulated indicated that the method is robust under varied conditions.

Table 10: Set 1 - Change in column temperature by + 5.0 °C (30 ºC)

Sr. No	**Results**	**Peak area**				**Retention time**				**% Obser-vation**
		OXY	OCL	OXT	AVO	OXY	OCL	OXT	AVO	
1	**STD**	1597651	926458	3871377	1460465	4.62	7.74	11.33	12.52	**100.0 %**
2	**After**	1597244	926541	3871654	1460545	4.6	7.75	11.29	12.46	-
3	**Change**	1595841	926874	3865445	1460874	4.61	7.74	11.3	12.44	

Table 11: Set 2 - Change in column temperature by - 5.0 °C (20 ºC)

Sr. No	Results	Peak area				Retention time				% Obser-vation
		OXY	OCL	OXT	AVO	OXY	OCL	OXT	AVO	
1	**STD**	1597651	926458	3871377	1460465	4.62	7.74	11.33	12.52	**100.0 %**

2	**After**	1597548	926548	3871987	1460254	4.66	7.77	11.34	12.57	-
3	**Change**	1597548	926874	3871258	1460123	4.65	7.76	11.36	12.59	

Table 12: Set 3 - Change in flow rate by +0.1 mL/min (1.1 mL/min)

Sr. No	Results	Peak area				Retention time				% Obser-vation
		OXY	OCL	OXT	AVO	OXY	OCL	OXT	AVO	
1	**STD**	1597651	926458	3871377	1460465	4.62	7.74	11.33	12.52	**100.0 %**
2	**After**	1596658	926154	3870654	1460258	4.6	7.71	11.28	12.5	-
3	**Change**	1596745	926254	3870325	1459874	4.59	7.72	11.3	12.49	

Table 13: Set 4 - Change in flow rate by - 0.1 mL/min (0.9 mL/min)

Sr. No	Results	Peak area				Retention time				% Obser-vation
		OXY	OCL	OXT	AVO	OXY	OCL	OXT	AVO	
1	**STD**	1597651	926458	3871377	1460465	4.62	7.74	11.33	12.52	**100.0 %**
2	**After**	1598456	924578	3870632	1458478	4.65	7.76	11.29	12.48	-
3	**Change**	1598471	924562	3870654	1458689	4.64	7.77	11.31	12.49	

Table 14: Set 5 - Change in wavelength by + 2.0 nm (332 nm)

Sr. No	Results	Peak area				Retention time				% Obser-vation
		OXY	OCL	OXT	AVO	OXY	OCL	OXT	AVO	
1	**STD**	1597651	926458	3871377	1460465	4.62	7.74	11.33	12.52	**100.0 %**
2	**After**	1596547	925874	3869548	1459548	4.62	7.74	11.33	12.52	-
3	**Change**	1596987	925148	3869487	1459423	4.62	7.74	11.33	12.52	

Table 15: Set 6 - Change in wavelength by - 2.0 nm (228 nm)

Sr. No	**Results**	**Peak area**				**Retention time**				% Obser-vation
		OXY	**OCL**	**OXT**	**AVO**	**OXY**	**OCL**	**OXT**	**AVO**	
1	**STD**	1597651	926458	3871377	1460465	4.62	7.74	11.33	12.52	**100.0 %**

2	**After**	1596541	925874	3869541	1459874	4.62	7.74	11.33	12.52	-
3	**Change**	1596548	925841	3869523	1459587	4.62	7.74	11.33	12.52	

Table 16: Set 7 - Change in organic phase composition in mobile Phase by MeOH: H2O (92:08)

Sr. No	Results	Peak area				Retention time				% Obser-vation
		OXY	OCL	OXT	AVO	OXY	OCL	OXT	AVO	
1	**STD**	1597651	926458	3871377	1460465	4.62	7.74	11.33	12.52	**100.0 %**
2	**After**	1599874	927586	3872548	1464265	4.65	7.75	11.36	12.54	-
3	**Change***	1599632	927584	3872548	1464875	4.65	7.77	11.38	11.53	

Table 17: Set 8 - Change in organic phase composition in mobile Phase by MeOH: H2O (88:12)

Sr. No	Results	Peak area				Retention time				% Obser-vation
		OXY	OCL	OXT	AVO	OXY	OCL	OXT	AVO	
1	**STD**	1597651	926458	3871377	1460465	4.62	7.74	11.33	12.52	**100.0 %**
2	**After**	1596487	927485	3870648	1459875	4.59	7.71	11.28	12.48	-
3	**Change**	1596325	927412	3870951	1459254	4.58	7.72	11.3	11.49	

Application of the Proposed Method in Marketed Formulation

Preparation of sample

An accurately weighed quantity of sunscreen lotion equivalent to 30.0 mg Oxybenzone, 30.0 mg Octocrylene, 75.0 mg Octinoxate and 20.6 mg Avobenzone were transferred in 100.0 mL volumetric flask, dissolved with HPLC grade methanol and volume was made up to the mark with HPLC grade methanol, sonicated for 10 min (50 µg/mL) and filtered through 0.2 µ nylon filter. Six sample solutions were injected, after equilibration of stationary phase, the chromatograms were recorded. The content of sunscreen lotion were calculated by comparing the peak area of sample with that of standard using following formula,

$$\% \text{ Label Claim} = \frac{Au}{As} \times \frac{Wstd}{dilution} \times \frac{dilution}{Wcrm} \times \frac{1}{L.C.} \times P$$

Where,

Au = Peak area of sample

As = Peak area of Standard

Wstd = Wt (mg) of STD.

Wcrm = Wt of cream

P = Potency of Standard (% purity)

L.C. = Label Claim in mg of cream

Table 18: Application of the proposed method in marketed formulation (STD)

Sr. No	Weight of standard	Peak area of standard			
		Oxybenzone	Octocrylene	Octinoxate	Avobenzone
1	**Oxybenzone = 30.0 mg** **Octocrylene = 30.1 mg** **Octinoxate = 77.4 mg** **Avobenzone = 20.7 mg**	1594991	868285	3861902	1395401
2		1588187	864630	3853488	1390401
3		1587105	864809	3846455	1385371
4		1588146	863935	3845529	1371623
5		1588654	865412	3850458	1385426
6		1590245	866254	3850658	1389548
	Mean	1589554.67	865554.167	3851415	1386295
	SD	2852.97043	1549.09566	5916.2487	8089.95676
	% RSD	0.17948237	0.17897154	0.1536123	0.58356676

Table 19: Application of the proposed method in marketed formulation (Sample)

Weight of Sample	Peak area of sample				% Label claim			
	OXY	OCL	OXT	AVO	OXY	OCL	OXT	AVO
0.1005 mg of Sunscreen Lotion	1642169	870523	3883971	1172037	99.87	100.26	100.57	99.95
	1642884	870302	3881997	1172306	100.34	100.66	100.74	100.33
	1649780	872004	3896708	1167818	100.83	100.83	101.31	100.31
	1647376	870623	3889289	1178967	100.62	100.77	101.14	102.29
	1645875	870654	3886854	1178541	100.49	100.61	100.95	101.23
	1644875	870354	3885748	1178548	100.33	100.47	100.91	100.93
Mean	1645493	870743	3887428	1174703	100.41	100.60	100.94	100.84
SD	2839.29	633.51	5180.35	4646.55	0.33	0.21	0.26	0.85
% RSD	0.17	0.07	0.13	0.40	0.32	0.21	0.26	0.84

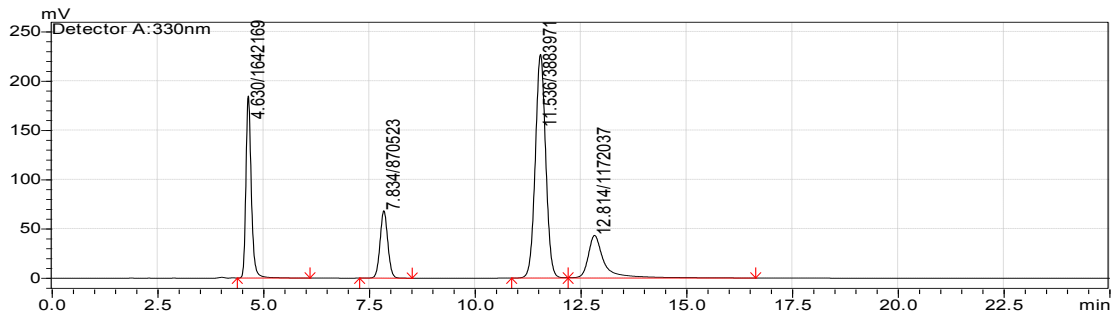


Figure 31: Application of the proposed method in marketed formulation.

RESULTS AND DISCUSSION

Clinically, Oxybenzone, Octocrylene, Octinoxate and Avobenzone used in sun-screen formulation for prevention against UV-A & UV-B rays. No specific method on HPLC is reported for simultaneous estimation of Oxybenzone, Octocrylene, Octinoxate and Avobenzone in sun-screen lotion formulation. Hence, the project was undertaken in order to develop simple, accurate and rapid analytical methods for Simultaneous estimation of Oxybenzone, Octocrylene, Octinoxate and Avobenzone in sun-screen lotion.

The analysis was performed using Waters, 5 µ, C_{18} column (250 X 4.6mm), injection volume 20 µL and Methanol: Water (90:10) with gradient elution used as mobile phase which shows sharp peak when detected at same wavelength 330 nm. The linearity range of Oxybenzone, Octocrylene, Octinoxate and Avobenzone were found to be 12 µg/mL to 28 µg/mL.

Validation

Validation was performed to assure the reliability of the proposed method and was carried out as per ICH guidelines for the following parameters.

Accuracy

Accuracy of the proposed method was ascertained on the basis of recovery studies performed by standard addition method. The recoveries of Oxybenzone, Octocrylene, Octinoxate and Avobenzone were observed to be in the range of 98 to 102 %. (Table 6)

Precision

Replicate estimation of Oxybenzone, Octocrylene, Octinoxate and Avobenzone in formulation analyzed by proposed method has yielded quite acceptable results. SD and RSD of series of measurement were found to satisfactory as shown and hence the proposed method was found to be precise. (Table 4, 5)

Linearity and Range

For Oxybenzone, Octocrylene, Octinoxate and Avobenzone the percent label claim vs. area under curve plot shows a linear relationship with correlation coefficient very close to 1. From linearity Study it can be concluded that sun-screen lotion having Oxybenzone, Octocrylene, Octinoxate and Avobenzone can be measured at concentration range 12 to 28 µg/mL in a formulation. (Table 2)

Ruggedness

The ruggedness of the proposed method has been verified by analyzing the six samples of same batch used for method precision by two different analysts using two different instruments, by different analytes.

The results of estimation for Oxybenzone, Octocrylene, Octinoxate and Avobenzone by different analysts were very much reproducible with overall S.D. & RSD in limit (Table 7, 8)

for Oxybenzone, Octocrylene, Octinoxate and Avobenzone. This indicates the ruggedness of the method in the hands of different analysts.

Robustness

Deliberately varying the chromatographic conditions like flow rate ± 0.2 mL, column oven temperature by 5 ^{O}C units. The results of estimation for Oxybenzone, Octocrylene, Octinoxate and Avobenzone were very much reproducible with overall % label claim and overall S.D shown in (Table 9 to 16).

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