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METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATIONOFHYDROQUINONE, OXYBENZONE AND OCTINOXATE IN SUNSCREEN CREAM BY REVERSED PHASE ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY

Ashok Kumar*, Umme Rab, Vijay Kumar Munipalli, Sujata S. Kaisare, Sayali Warde and Raman Mohan Singh

Central Drugs Testing Laboratory – Mumbai, Zonal FDA Bhawan, GMSD Compound, Belasis Road, Mumbai – 400008.

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***Corresponding Author**
Ashok Kumar
Central Drugs Testing Laboratory – Mumbai, Zonal FDA Bhawan, GMSD Compound, Belasis Road, Mumbai – 400008.

ABSTRACT

A simple, precise, accurate, selective, and economic reversed-phase RP-HPLC method has been developed for simultaneous estimation of Hydroquinone, Oxybenzone & Octinoxate in cream formulation. The separation of the drugs was achieved on ACQUITY UPLC@BEH-C18 Column (100mm x 2.1 mm x 1.7µm) using 0.1% Trifluoro acetic acid: Acetonitrile in the ratio of 60:40% v/v as mobile phase. The flow rate was 0.4ml/min and effluents were monitored at 279nm.The method was successfully applied on formulation and there is no interference of matrix components. The linearity of method was obtained in the range of 10µg/ml to 60µg/ml, 5µg/ml to 60µg/ml and 10µg/ml to 75µg/ml of Hydroquinone, Oxybenzone and Octinoxate respectively. Mean percentage recoveries were 100.00% for Hydroquinone and 100.05% for Oxybenzone and 100.04% for Octinoxate. The LOD of Hydroquinone, Oxybenzone and Octinoxate was found to be 3µg/mL, 2µg/mL and 5µg/mL where as the LOQ was 10µg/mL, 6µg/mL and 15µg/mL respectively. The assay values of all true analytes were found to be 99.50%, 99.33% and 99.40% for Hydroquinone, Oxybenzone and Octinoxate respectively. Percentage relative standard deviation of percent assay values for replicate sample preparation was 0.44% for Hydroquinone, 0.44% for Oxybenzone and 0.34% for Octinoxate. The method was robust with respect to the change in flow rate, and composition of the mobile phase. The validation parameters were done in

accordance with International Conference on Harmonization (ICH) guidelines. The validation results from statistical analysis of the data, demonstrated the method is reliable.

KEYWORDS: RP-UPLC, Hydroquinone, Oxybenzone, Octinoxate, Brite Cream.

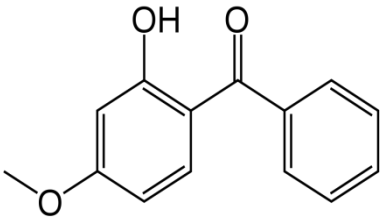
INTRODUCTION

A sunscreen cosmetic could be defined as "any cosmetic product containing UV filters in its formulation in order to protect the skin from the solar deleterious UV- light, avoiding or minimizing the damage that this radiation might cause on human health".[1] Extra terrestrial sunlight includes X-ray, ionizing, ultraviolet (UV), visible and infrared radiation, and radio waves. Hydroquinone, Oxybenzone and Octinoxate are also called as UV filters as they prevent damage to the skin from UV radiation. UV spectrum is divided into three bands. UV-A (200-290nm), UV-B (320-400nm) and UV-C (210-320nm). UV-A radiation is more harmful then other two. UV-B radiation is fully absorbed by the stratum corneum and the top layers of the epidermis, whereas up to 50% of incident UV-A radiation penetrates Caucasian skin deep into the dermis.[2]

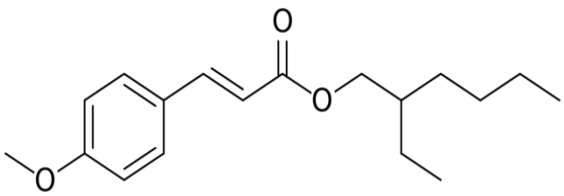
Hydroquinone is chemically Benzene-1,4-diol or Quinol with molecular weight 110.11g/mol. It is a white granular solid which is soluble in water and having a melting point 172^0 C. It is a derivative of Hydroquinones. Hydroquenones absorb UV-A at approximately 290 nm. The photomutagenic properties of these compounds might be a contributing factor to the increased melanoma incidence that has been found in sunscreen users. Other possibilities include consequent over exposure to sun without UV-B protection and vitamin D deficiency from overuse of sunscreen.

Oxybenzone is chemically (2- hydroxy-4-methoxyphenyl)-phenyl- methanone with a molecular weight 228.2g/mol. It is a white crystalline powder which is insoluble in water and having a melting point 49^0C. It is a derivative of benzophenone. Benzophenones

absorb UV-B at approximately 360 nm. The most popular benzophenone and one of the most common sunscreen ingredients is benzophenone-3 or oxybenzophenone. The photo mutagenic properties of these compounds might be a contributing factor to the increased melanoma incidence that has been found in sunscreen users.



Octinoxate is chemically ethyl hexylmethoxy cinnamate. It is frequently used in combination with other UVC absorbers to achieve high SPF values in the final product. Upon exposure to sunlight, octinoxate degrades into a photoproduct with less UV-absorbing ability. Several studies suggest ways to improve the photo stability of cinnamate Encapsulation of ethyl hexyl *p*-methoxy cinnamate into nano particles results in a reduction of the photodegradation.[7] Animal studies indicate that octyl methoxy cinnamate may produce hormonal (estrogen-like) and possibly other adverse effects.



The **sun protection factor** is a measure of the ability of a sunscreen to protect against erythema,[8] which is thus primarily a measure of UVC protection. The SPF is a ratio of the dose of UV radiation required to produce a minimal erythema 24 hours after exposure in sunscreen-protected skin to the dose required to produce the same degree of erythema in unprotected skin. In other words: the SPF number defines how long you can stay in the sun before getting burnt. If it normally takes you 20 minutes in the sun before you get burned, an SPF 15 product will let you stay 15 times longer in the sun : 20min x15(SPF)=300 min (5 hours).

Literature survey reveals that no method has been reported for analysis and separation of Hydroquinone, Oxybenzone and Octinoxate in sun-screen cream; hence trials were made

to develop UPLC[9] method for the separation of these drugs in cream formulation.

MATERIAL & REAGENTS

HPLC grade Acetonitrile, Trifluoro acetic acid AR grade from Merck were used. HPLC grade deionized filtered water was used to prepare buffer. Secondary reference namely Hydroquinone, Oxybenzone and Octinoxate having % purity 98.59, 99.45 and 99.00 respectively were used. The formulation Brite Cream (Wallace Pharmaceuticals ltd) was procured from local market.

Instrumentation and Chromatographic conditions

The LC system of Thermo Scientific UPLC with UV detector was used for this study and Chromatographic separation was achieved on ACQUITY UPLC@BEH-C18 Column (100 x 2.1mm i.d x 1.7µm) equilibrated with mobile phase Trifluoro acetic acid (0.1%): Acetonitrile (60:40) was used. Detection wavelength 279 nm was selected and the flow rate of pump was set 0.4ml/min and 2 µl volumes were injected.

Selection of wave length

Each solution was scanned using double beam UV Visible Spectrophotometer (Perkin-Elmer Lambada) between the range of wavelength 190 nm to 400 nm and overlain spectra was obtained. The wavelength selected was 279nm which is isobestic point of Hydroquinone, Oxybenzone and Octinoxate.

Reference Standard solution preparation

Accurately weighed 40mg of Hydroquinone, 30mg of Oxybenzone, and 50mg of Octinoxate in three volumetric flask and made volume up to 100ml using mobile phase. Sonicated it for 15min and then taken 1ml of each solution and made the volume up to 10ml with mobile phase to get concentration of 40µg/mL Hydroquinone, 30µg/mL of Oxybenzone and 50µg/mL Octinoxate.

Analysis of marketed formulation

Weighed accurately 1.0 gm of cream in to 100ml volumetric flask and added 50.0ml of mobile phase, sonicated to dissolve, diluted to volume with mobile phase. Taken 1ml of above solution in 10ml and diluted up to mark with mobile phase.

Table 1: Analysis of sample was carried out using the above method and the results obtained are tabulated below.

Contents	Label claim	Found %w/w	Assay % of label amount
Hydroquinone	4.0%w/w	3.98%	99.50%
Oxybenzone	3.0%w/w	2.98%	99.33%
Octinoxate	5.0%w/w	4.92%	98.40%

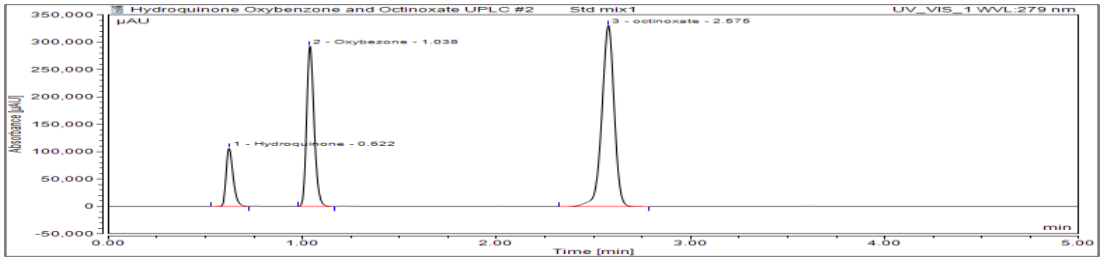


Fig. 1: Chromatogram of Standard Containing Hydroquinone, Oxybenzone, and Octinoxate.

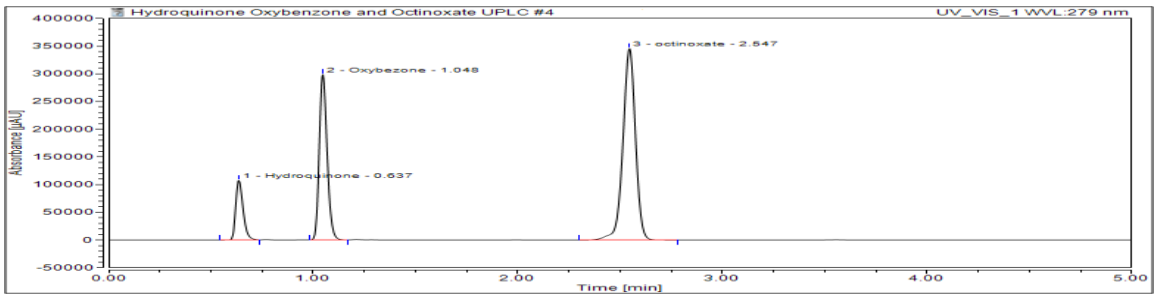


Fig. 2: Chromatogram of Sample Containing Hydroquinone, Oxybenzone, and Octinoxate.

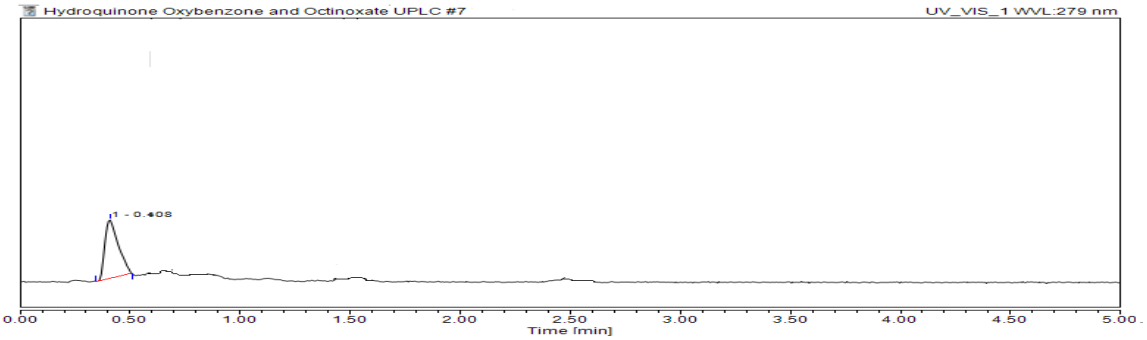


Fig. 3: Chromatogram of Blank.

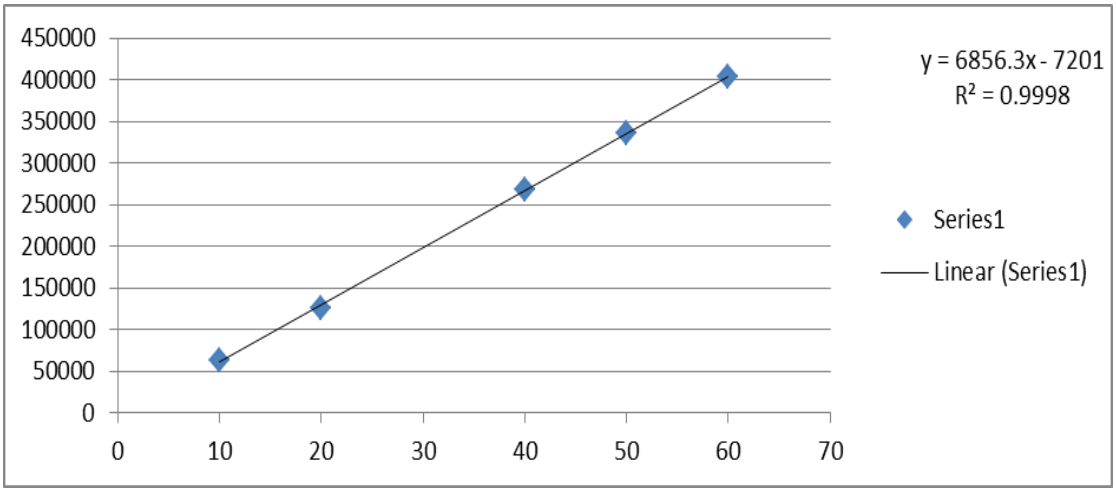
METHOD VALIDATION

The validation of the HPLC method was carried out in accordance with the ICH guidelines. The method was validated for various parameters like linearity, accuracy, precision, and limit of detection, limit of quantification, sensitivity, selectivity and robustness.

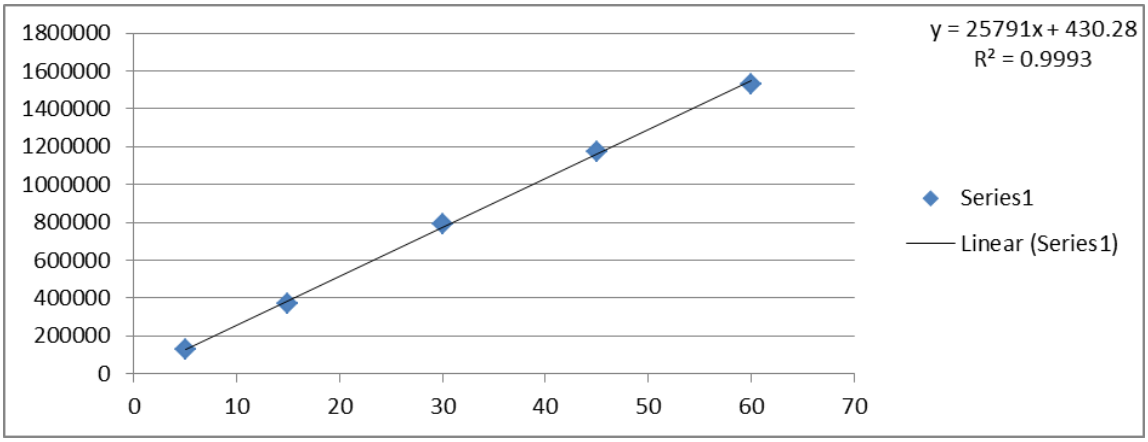
Linearity: The linearity of the proposed method was established by using series of standard solutions of Hydroquinone, Oxybenzone and Octinoxate and the studies were repeated in triplicate and the curve showed linearity in the concentration range 10µg/ml to 60µg/ml for Hydroquinone, 5µg/ml to 60µg/ml of Oxybenzone and 10µg/ml to 75µg/ml for Octinoxate. The r^2 was found 0.999, 0.999 and 0.999 for Hydroquinone, Oxybenzone and Octinoxate respectively. All the linearity data depicted in **Table 2**. and linearity graph is shown in Graph No 1, 2 & 3.

Table No. 2: Linearity and Statistical analysis data for Hydroquinone, Oxybenzone & Octinoxate.

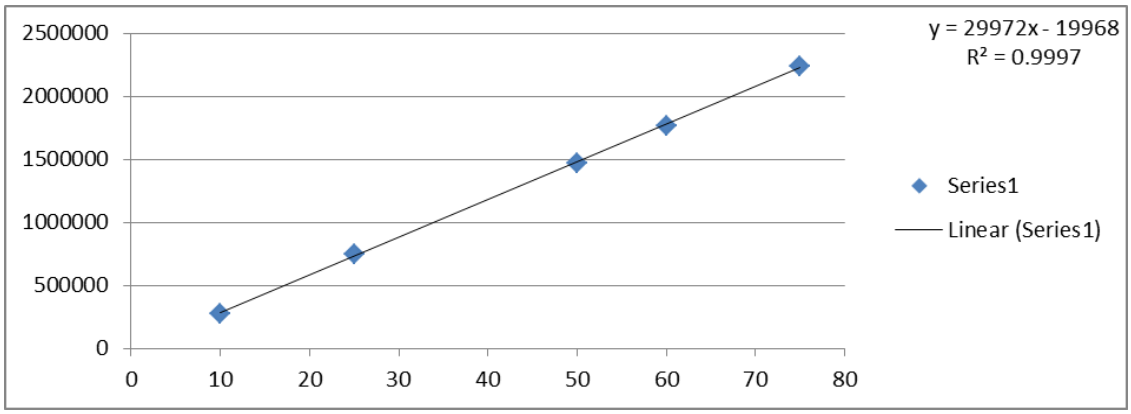
S. No	Hydroquinone		Oxybenzone		Octinoxate	
	Concentration (µg/mL	Average Area	Concentration (µg/mL)	Average Area	Concentration (µg/mL)	Average Area
1.	10µg/mL	63270	5µg/mL	130604	10µg/mL	273259
2.	20µg/mL	126541	15µg/mL	370874	25µg/mL	746584
3.	40µg/mL	268684	30µg/mL	788820	50µg/mL	1468286
4.	50µg/mL	336351	45µg/mL	1176531	60µg/mL	1766351
5.	60µg/mL	403288	60µg/mL	1532955	75µg/mL	2239621
Correlative Coefficient(r^2)		0.999		0.999		0.999
Slope		-7201		+430.2		-19968
Intercept		6856		25791		29972



Graph No. 1 Calibration Curve for Hydroquinone.



Graph No. 2 Calibration Curve for Oxybenzone.



Graph No. 3 Calibration Curve for Octinoxate.

Precision: The precision study was carried out by preparing solutions at a concentration of 40µg/ml for Hydroquinone, 30µg/ml for Oxybenzone and 50µg/ml Of Octinoxate. The % assay for sun-screen lotion was calculated for six replicate injections and % RSD was calculated. Precision was performed by measuring ruggedness and repeatability study. Results of repeatability depicted in **Table3**.

Table 3: Results of Method Precision (repeatability) study.

Sr No.	Peak Area		
	Hydroquinone	Oxybenzone	Octinoxate
1	266869	780900	1456537
2	268079	785789	1461526
3	271105	799772	1486794
4	268759	795347	1473285
5	268862	792770	1469510
6	267968	788734	1457356
Mean	268607	790552	1467501
SD	1417	6813	11563
%RSD	0.53	0.86	0.79

Table No. 4: Results of System Precision (repeatability) study.

No. of Sample	Hydroquinone (%)	Oxybenzone (%)	Octinoxate(%)
Sample 1	100.34	100.11	99.67
Sample 2	99.68	100.06	99.64
Sample 3	99.11	99.86	99.66
Sample 4	99.77	99.97	100.13
Sample 5	99.73	99.04	100.38
Mean	**99.73**	**99.81**	**99.90**
%RSD	**0.44%**	**0.44%**	**0.34%**

Accuracy: To study the accuracy and reproducibility of the proposed method recovery experiments were carried out by standard addition method to the pre-analyzed sample and spiked at 110%, 120%, and 130% levels. The results of % recovery Hydroquinone, Oxybenzone and Octinoxate by the proposed method are shown in Table 5. The mean recoveries of Hydroquinone, Oxybenzone and Octinoxate were 100.00%, 100.05% and 100.04% respectively. The mean recovery was well within the acceptance limit hence the method was accurate, as depicted in **Table4**.

Table No. 5: Accuracy (Standard Addition method) data for the proposed RP-HPLC method for Hydroquinone.

Amount (%)	Area found	Recovery (%)	Mean (%)	Mean (%)	RSD (%)
110	293569	100.00	100.13	100.00	0.18
	295879	100.34			
	298405	100.06			
120	320471	100.07	100.01		0.10
	321376	99.90			
	325544	100.07			
130	346471	99.87	99.86		0.18
	347376	99.68			
	352544	100.03			

Table No. 6: Accuracy (Standard Addition method) data for the proposed RP-HPLC method for Oxybenzone.

Amount (%)	Area found	Recovery (%)	Mean (%)	Mean %	RSD (%)
110	859900	100.11	100.01	100.05	0.09
	863789	99.93			
	879772	100.00			
120	937271	100.02	100.01		0.18
	941276	99.82			
	961544	100.19			
130	1017271	100.21	100.12		0.12
	1021276	99.98			
	1041544	100.18			

Table No. 7: Accuracy (Standard Addition method) data for the proposed RP-HPLC method for Octinoxate.

Amount (%)	Area found	Recovery (%)	Mean (%)	Mean %	RSD (%)
110	1599537	99.83	99.97	100.04	0.12
	1608526	100.05			
	1635794	100.02			
120	1752271	100.25	100.17		0.29
	1761276	100.42			
	1781544	99.85			
130	1892271	99.94	99.98		0.08
	1901276	100.07			
	1931544	99.93			

Robustness of the method

This was done by small deliberate changes in the proposed chromatographic conditions with respect to the factors selected i.e. flow rate, column temperature and % of organic solvents in the mobile phase. It was observed that there were no significant deviations after making deliberate changes which demonstrated that the RP-UPLC method is robust and reliable. Results described in Table 8.

Table 8: Robustness of the method.

Parameter	Change in Parameter	% Hydroquinone Estimation	% Oxybenzone Estimation	% Octinoxate Estimation	% RSD	Limit
Flow	0.38 ml/min	101.79	101.79	101.79	1.046	NMT 2.0%
	0.40 ml/min	100.52	100.52	100.52		
	0.42 ml/min	102.62	102.62	102.62		
Temperature	20˚ C	100.12	100.12	100.12	0.71	
	25˚ C	100.10	100.10	100.10		
	30˚C	101.34	101.34	101.34		
Mobile Phase Ratio (ACN : Buffer)	66 : 34	100.14	100.14	100.14	0.68	
	70 : 30	100.08	100.08	100.08		
	56 : 45	101.29	101.29	101.29		

The ruggedness of the method

The USP guideline defines ruggedness as "the degree of reproducibility" of the test result obtained by the analysis of the same samples under a variety of normal test condition such as; different analyst, different instrument etc. The analysis was carried out by different analysis on different instruments. The results obtained mentioned in Table (10).

Table 9: Ruggedness of the method.

	Hydroquinone	Oxybenzone	Octinoxate
Between instrument I and II			
Instrument	% Content	% Content	% Content
I (Thermo Scientific)	99.49%	100.19%	99.97%
II (Agilent Tech.)	98.87%	99.36%	100.8%
Between Analyst I and II			
Analyst	% Content	% Content	% Content
I	100.07%	100.11%	99.70%
II	99.23%	99.73%	99.09%

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The limit of detection and limit of quantification of the developed method were determined by injecting progressively low concentration of the standard solutions using the developed RP-UPLC method. The LOD of Hydroquinone, Oxybenzone and Octinoxate were found to be 3.0µg/ml, 2.0µg/ml and 5.0µg/ml respectively. The LOQ is the smaller concentration of the analyte response that can be quantified accurately and the LOQ values are 10.0µg/ml, 6.0µg/ml and 15.0µg/ml respectively.

Table 10: Results of System Suitability Study.

Peak Name	Retention Time	Area	Resolution	Tailing Factor	Theoretical Plates
	min	µAU*sec			
Hydroquinone	0.632	266869	6.21	1.33	2551
Oxybezone	1.042	780900	16.88	1.18	3725
Octinoxate	2.532	1456537	n.a.	0.95	8669

RESULTS AND DISCUSSION

Method development optimization (Evaluation of Stationary phase and Mobile Phase)

The main objective of method development was to achieve simple, rapid and efficient separation between true ingredients. The optimizations of the stationary phase were done considering polarity of the molecules to be separated.

Stationary phase such as waters ACQUITY UPLC@BEH-C18 Column (100mm x 2.1 mm x 1.7µm) was selected and screened for separation of gave good separation with good resolution between ingredients. System suitability was performed by preparing standard solution containing 40µgm/ml Hydroquinone, 30µgm/ml Oxybenzone and 50µgm/ml Octinoxate and injecting six replicate and all parameters are found to be within range.

The result of system suitability is depicted in Table5.

Different mobile phase system containing water and organic solvents were tried but close elution of ingredients were observed. Then trials were done with modification of mobile phase using trifluoro acetic acid (0.1%). Finally good separation with acceptable SST parameters found with the mobile phase consisting of 0.1% Trifluoroaetic acid and Acetonitrile in the ratio of 60:40 (%v/v). The flow rate was 0.4 ml/min with UV detection at 279 nm. The retention time of Hydroquinone, Oxybenzone and Octinoxate were 0.62min, 1.03min & 2.57min respectively. The % Relative standard deviation were less than 2%, tailing factor were less than 2.0 & theoretical plates were found more than 2000 for all three components. The calibration curve was found to be linear in the range of 10µg/ml to 60µg/ml for Hydroquinone, 5µg/ml to 60µg/ml Oxybenzone and 10µg/ml to 75µg/ml Octinoxate with correlation coefficient of 0.999 for all three components. The results of percentage recovery data were found 100.00% of Hydroquinone, 100.05% of Oxybenzone and 100.04% of Octinoxate. The LOD and LOQ for Hydroquinone, Oxybenzone and Octinoxate were determined in the basis of peak response and slope of the regression equation. The LOD of Hydroquinone, Oxybenzone and Octinoxate was found to be 3.0µg/ml, 2.0µg/ml and 5.0µg/ml respectively. The LOQ is the smaller concentration of the analyte response that can be quantified accurately the LOQ was 10.0µg/ml, 6.0µg/ml and 15.0µg/ml respectively.

The low % RSD value for intraday and interday precisions revealed that the proposed method is reproducible and robust. No interfering peaks were found in the chromatogram indicating that the excipients used in cream formulations did not interfere with the estimation of drug by the proposed UPLC method. Variation in parameters for robustness studies was observed that there were no marked changes in the chromatograms, which demonstrated that the RP-UPLC method developed was robust. The proposed method was subjected to method validation according to ICH guidelines.

The results of validation parameters of proposed method were presented in table 4.

Parameters	Hydroquinone	Oxybenzone	Octinoxate
RT	0.6 min	1.0 min	2.5 min
Resolution			
Tailing	1.33	1.18	0.95
Theoretical plates Ph Eup	2551	3725	8669
Assay%			

Linearity	10-60 µg/ml	5-60 µg/ml	10-75 µg/ml
Slope	-7201	433.8	-19981
Intercept	6856	25790	29973
Coefficient of correlation	0.999	0.999	0.999
Recovery %			
LOD µg/ml	3 µg/ml	2 µg/ml	5 µg/ml
LOQ µg/ml	10 µg/ml	6 µg/ml	15 µg/ml

CONCLUSION

A new RP-UPLC method was successfully developed for the estimation of Hydroquinone, Oxybenzone and Octinoxate in sunscreen cream formulation. The method was critically validated and statistically generated high quality data proves that the method is linear, sensitive, selective and robust. The method can be applied for the quality control analysis of pharmaceutical formation sunscreen cosmetic products.

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