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Development of Analytical Method for Simultaneous Estimation of Hydroquinone and Avobenzone in Pharmaceutical Cream Formulation by RP – HPLC

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ABSTRACT

An accurate, simple, and reproducible reversed-phase liquid chromatographic method was developed for the determination of Hydroquinone and Avobenzone in a cream formulation. The chromatographic separation was carried out at Analytical Technology HPLC instrument (Software: HPLC Work Station) equipped with Deuterium lamp as a detector, HPLC pump and manual injecting facility programmed at 20µL capacity per injection were used. The stationary phase was Qualisil Column C18 (250 mm x 4.6 mm, 5µm) at ambient temperature. The mobile phase was Acetonitrile: Methanol (80:20). Detection was carried out at 226nm using UV Detector. The flow rate was 1.0mL/min and retention time was about 4.9mins and 5.8mins for Hydroquinone and Avobenzone. The linearity was obtained in the concentration range of 24-64µg/mL and 30-70µg/mL for Hydroquinone and Avobenzone respectively. Mean percentage recoveries were 99.43% for Hydroquinone and 99.47% for Avobenzone. The LOD of Hydroquinone and Avobenzone was found to be 2µg/mL and 1.0µg/mL whereas the LOQ was 10µg/mL and 5µg/mL respectively. The assay values of both the analytes were found to be well within the limits that are 99.50% and 98.40% for Hydroquinone and Avobenzone respectively. Percentage relative standard deviation of percent assay values for replicate sample preparation was 0.31% for Hydroquinone and 0.38% for Avobenzone. The method was robust with respect to the change in flow rate, and composition of the mobile phase.

INTRODUCTION

Hydroquinone, a dihydroxylated benzene derivative is used therapeutically as a topical agent for the treatment of certain skin conditions [1]. Hydroquinone is a compound mainly used as an antioxidant in the photography industry as well as a depigmenting agent in cosmetic products such as skin- toning creams. Hydroquinone competitively inhibits melanin synthesis by inhibiting sulfhydryl groups and acting as a substrate for *tyrosinase*. Melanosomes and melanocytes are damaged by the semiquinone free radicals released during the above reaction [2–3]. Hydroquinone is considered the gold standard for the treatment of hyperpigmentation. It is commonly used at concentrations of 2-4%. It has shown that Hydroquinone and some of its derivatives were present in analyzed skin-toning creams [4]. Thus, the analytical determination of Hydroquinone and its derivatives in cosmetics is very important for the human health protection and consumers safeguarding.

Fig.1: Molecular structure of Hydroquinone

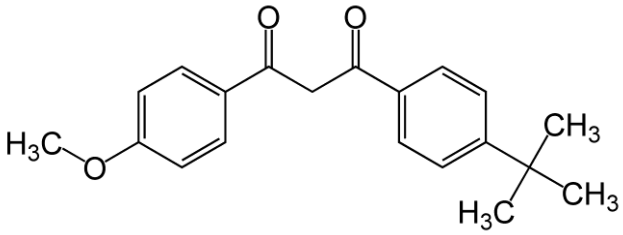
Chemical Name- Hydroquinone is Benzene-1,4-diol. Its molecular formula is $C_6H_4(OH)_2$ and the molecular weight is 110.11 g/mol.

Hydroquinone works by inhibiting the activity of *tyrosinase* (the enzyme that controls the synthesis of melanin) and by increasing, the cytotoxicity of melanocytes (cells that produce melanin). This makes it very effective at treating sunspots, melasma, freckles, post-inflammatory hyperpigmentation and other forms of skin discoloration. In the US, hydroquinone can be used only in concentrations of up to 2% in OTC products, and up to 4% in prescription products. If you decide to use one, make sure it is packaged in an airtight, opaque tube or bottle. When exposed to light and air, it oxidizes (you can see this happening, because hydroquinone warns you by turning brown) and loses its efficacy [5].

Avobenzone is whitish to yellowish crystalline powder [6]. Avobenzone having a phenyl ketone group and sterically hindered group in a molecule. It is one of the most effective sunscreen ingredients. Avobenzone reduces the recurrence rate of black spots due to sunlight or ultraviolet light exposure. Avobenzone inhibits the discoloration process by interfering with melanocyte physiology [7]. The analytical control of the sunscreen cosmetics is

necessary since the content of UV filters in the final product is related to its sun protection efficacy that is usually claimed by the labeled sun protection factor (SPF) [8].

Molecular structure of Avobenzone



Chemical Name- Avobenzone is chemically 4-tertbutyl-4'-methoxy-dibenzoyl methane. Its molecular formula is $C_{20}H_{22}O_3$ and molecular weight is 310.39g/mol.

Avobenzone has been shown to degrade significantly in light, resulting in less protection over time [9, 10, 11]. The UV-A light in a day of sunlight in a temperate climate is sufficient to breakdown most of the compound [12]. The photochemical behavior of Avobenzone, when dissolved in analytical grade organic solvents (such as methanol, acetonitrile, and hexane), has been extensively documented. In addition, it has been found that the Avobenzone photostability is highly dependent on the polarity and periodicity of the solvent [13, 14].

Hydroquinone is the most conventional skin-whitening agent. However, it has numerous unfavorable effects with the long-term application, including irritative dermatitis, melanocyte destruction, and contact dermatitis. High-performance liquid chromatography is a widely used technique for analysis of drug product and drug substances [15-16]. An extensive literature survey revealed Spectrophometric [17-19], TLC [20,21] and HPLC [21-24] for the determination of Hydroquinone and the reported analytical procedure for the estimation of Avobenzone in bulk sample and unit dosage forms include HPLC [25-27], TLC [28,29]. However, there is no method which describes the simultaneous determination of Hydroquinone and Avobenzone from cream dosage form meant for external application. The objective of this investigation was to develop simple accurate and economical procedures for simultaneous estimation of Hydroquinone and Avobenzone from a cream dosage form.

EXPERIMENTAL WORK

Simultaneous estimation of Hydroquinone and Avobenzone

Reagents and chemicals:

Hydroquinone RS, Avobenzone RS, Acetonitrile (HPLC grade), Methanol (HPLC grade).

Instrumentation and chromatographic condition:

Chromatographic separation was performed on an Analytical technology HPLC instrument (Software: HPLC Work Station) equipped with Deuterium lamp as the detector, HPLC pump and manual injecting facility programmed at 20 µL capacity per injection were used. Detection was carried out at 226nm using UV Detector. The separation was achieved on the ODS Qualisil Column C_{18} (250 mm x 4.6 mm, 5µm) at ambient temperature. The elution was carried out isocratic ally at the flow rate of 1ml/min using Acetonitrile: Methanol (80:20) as the mobile phase.

Preparation of Mobile Phase: Acetonitrile: Methanol (80:20)

STANDARD STOCK SOLUTION PREPARATION:

1) Stock solution for Hydroquinone

Weigh accurately about 10.0 mg Hydroquinone RS in 25 mL volumetric flask. Dilute with mobile phase to mark.

2) Stock solution for Avobenzone

Weigh accurately about 10.0 mg Avobenzone RS in 20 mL volumetric flask. Dilute with mobile phase to mark

3) Combined standard stock solution

Mix 5 mL each of above standard stock solution into 50 mL volumetric flask and dilute to mark with mobile phase.

4) Sample Solution:

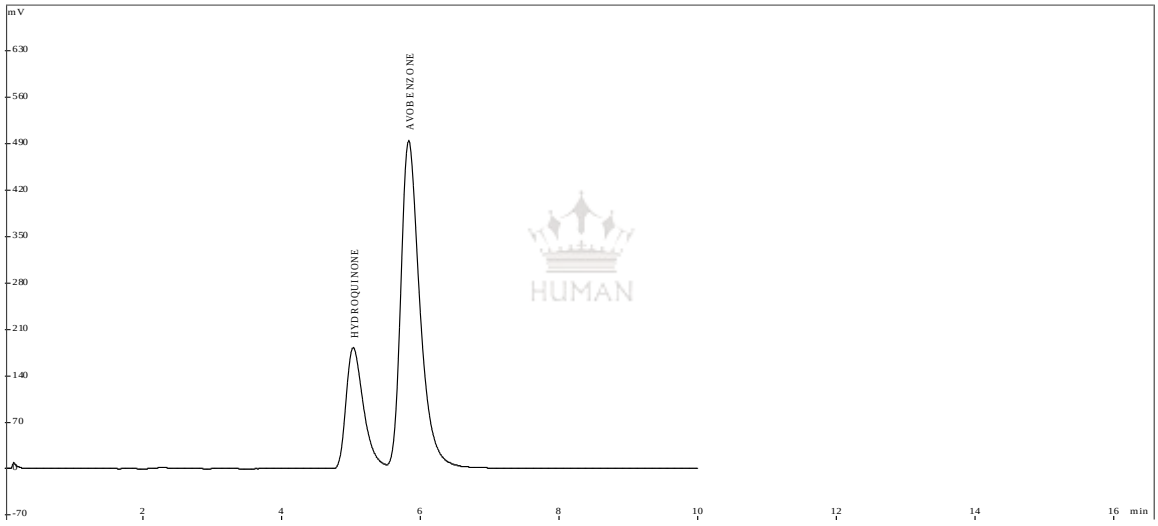
Weigh accurately cream about 1.0g into a 100.0 mL volumetric flask, and add 50.0 mL of the mobile phase, sonicate to dissolve, dilute to volume with mobile phase.

Take 5 mL of above solution in 50 mL volumetric flask and dilute to mark with mobile phase

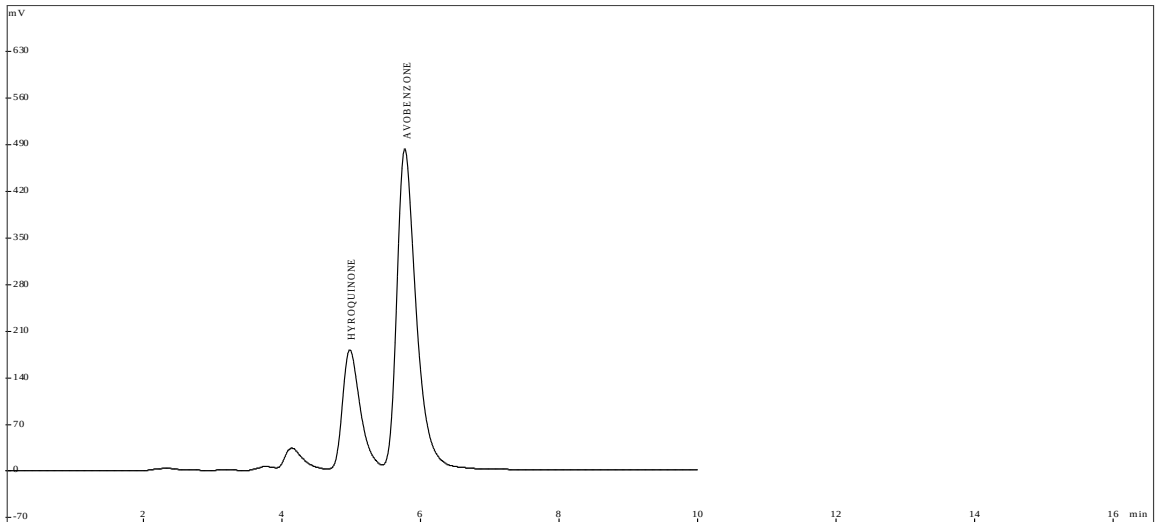
Procedure: Filter both Sample and Standard Solution with 0.2-micron filter paper and inject 20 µl.

Calculate the result by comparing peak area ratio from the sample with that from the standard preparation

Chromatogram of standard



Chromatogram of sample



METHOD VALIDATION:

As per ICH guideline, the method was validated and following parameters were evaluated, along with Ruggedness [31-35].

Analysis of sample was carried out using the above method and the result is tabulated in table 1.

Table 1: Analysis of sample

Contents	Label claim	Found %w/w	Assay % of label amount
Hydroquinone	4.0%w/w	3.98%	99.50%
Avobenzone	5.0%w/w	4.92%	98.40%

Sample in-house production batch

SYSTEM SUITABILITY STUDIES:

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. In that, the column efficiency, resolution and peak-Tailing factor were calculated for the standard solutions Table 2. The values obtained demonstrated the suitability of the system for the analysis of this drug combination.

Table 2: System Suitability Parameter

Parameter	Hydroquinone	Avobenzone
Precision of the method (n =6)	0.59%	0.91%
Theoretical Plates	3566	4240
Resolution Factors	4.695	7.658
Tailing factor	1.000	1.000
Retention time	4.91	5.87

LINEARITY:

The linearity of the method was established by analysis of combined standard solution. The range of an analytical procedure 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.

The linearity of the proposed method was established by using series of standard solutions of Hydroquinone and Avobenzone these studies are repeated in triplicate with different stock solutions. The curve obtained by concentration on X-axis and peak area on Y-axis against showed linearity in the concentration range of 24 to 64µg/mL for Hydroquinone and 30 to 70µg/mL for Avobenzone and its correlation coefficient is 0.995 and 0.999, and linearity graph is shown in Graph No 1.

Table No. 3 Linearity and Statistical analysis data for Hydroquinone

S. No	**Concentration (µg/mL)**	**Area**
1.	24µg/mL	1989659
2.	32µg/mL	2653584
3.	40µg/mL	3314549
4.	48µg/mL	3894948
5.	64µg/mL	4941648
Correlative Coefficient(r^2)		0.995

Graph No 1: Linearity Graph of Hydroquinone

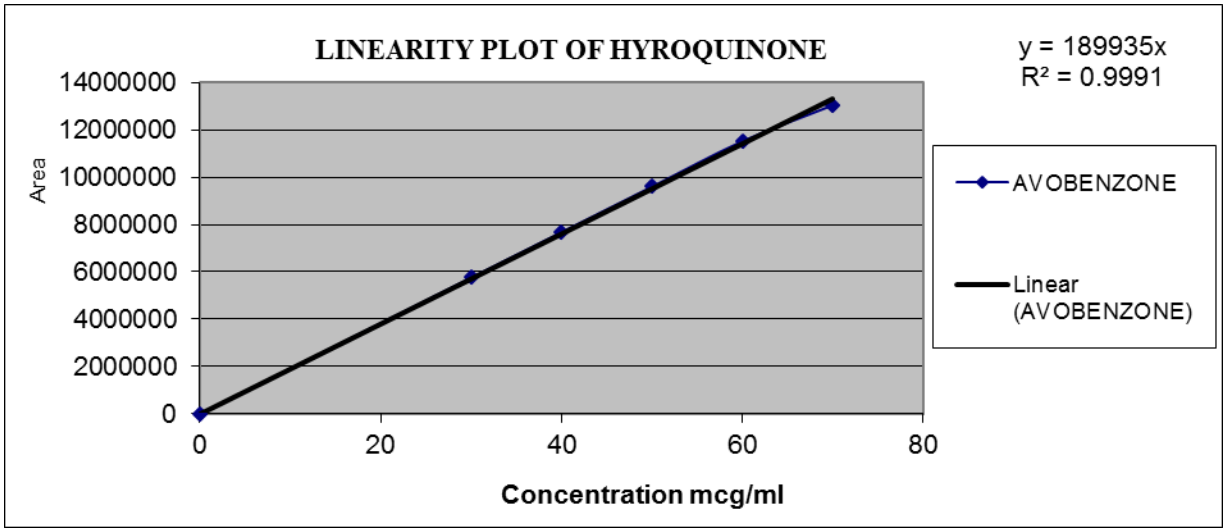
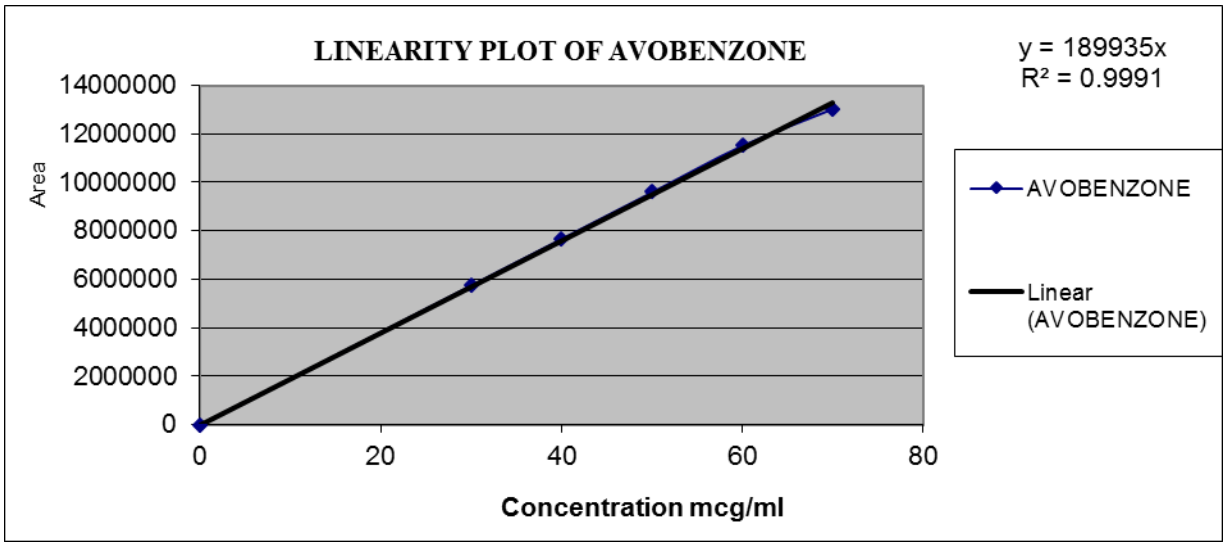


Table No. 4 Linearity and Statistical analysis data for Avobenzone

S. No.	Concentration (µg/mL)	Area
1.	30µg/mL	5760178
2.	40µg/mL	7680205
3.	50µg/mL	9600239
4.	60µg/mL	11520309
5.	70µg/mL	13440912
Correlative Coefficient(r^2)		0.999

Graph No 2: Linearity Graph of Avobenzone



RECOVERY STUDIES:

To study the accuracy and reproducibility of the proposed method recovery experiments were carried out. A fixed amount of pre-analyzed sample was taken and the standard drug was added at 80%, 100%, and 120% levels. Each level was repeated three times. The contents of Hydroquinone and Avobenzone found by the proposed method is shown in Table 5. The mean recoveries of Hydroquinone and Avobenzone were 99.08% and 98.94% respectively, which shows there is no interference from excipient.

Table No. 5 Accuracy (by Recovery) data for the proposed RP-HPLC method for Hydroquinone

Accuracy Level %	Inj. no.	Amount Added (mg)	Amount Recovery (mg)	Area	%Recovery	Mean%
80%	1	32.0mg	31.59mg	2654827	98.71%	99.60%
	2	32.0mg	31.94mg	2645835	99.81%	
	3	32.0mg	32.09mg	2654385	100.28%	
100%	1	40.0mg	39.68mg	3312458	99.20%	99.09%
	2	40.0mg	39.43mg	3314586	98.57%	
	3	40.0mg	39.81mg	3294589	99.52%	
120%	1	48.0mg	48.11mg	3977548	100.11%	99.61%
	2	48.0mg	47.63mg	3986542	99.22%	
	3	48.0mg	47.77mg	3974561	99.52%	

Table No. 6 Accuracy (by Recovery) data for the proposed RP-HPLC method for Avobenzone

Accuracy Level %	Inj. no.	Amount Added (mg)	Amount Recovery (mg)	Area	%Recovery	Mean%
80%	1	40.0mg	40.15mg	7689542	100.37%	99.56%
	2	40.0mg	39.78mg	7635488	99.45%	
	3	40.0mg	39.55mg	7656845	98.87%	
100%	1	50.0mg	49.68mg	9625401	99.36%	99.52%
	2	50.0mg	49.88mg	9612456	99.76%	
	3	50.0mg	49.73mg	9618458	99.46%	
120%	1	60.0mg	59.39mg	11542586	98.98%	99.35%
	2	60.0mg	59.60mg	11534581	99.33%	
	3	60.0mg	59.83mg	11539854	99.71%	

PRECISION STUDIES:

The precision of the method was studied by analysis of multiple sampling of a homogeneous sample. The precision of analytical procedure expresses the closeness of agreement (degree

of scattering) between a series of measurements obtained from multiple sampling of the same homogeneous Sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision, and reproducibility. Precision should be investigated using the homogeneous authenticated sample. Precision Expressed as % RSD is given in Table-7 which should be less than 2%.

Table No. 7 system precision result of the proposed RP-HPLC Method

No. of Sample No.	Hydroquinone	Avobenzone
Sample 1	**99.45%**	**99.21%**
Sample 2	**99.17%**	**99.28%**
Sample 3	**99.12%**	**98.39%**
Sample 4	**99.52%**	**99.59%**
Sample 5	**98.72%**	**98.97%**
%RSD	**0.31%**	**0.38%**

ROBUSTNESS AND RUGGEDNESS OF THE METHOD:

Robustness of the method:

Robustness is a measure of its capacity to remain unaffected by small but deliberate variations in the chromatographic method parameters and provides an indication of its reliability. This was done by small deliberate changes in the chromatographic conditions at 3 different levels and retention time of Hydroquinone and Avobenzone was noted. The factor selected were flow rate, column temperature and % Acetonitrile in the mobile phase. It was observed that there were no deliberate changes in the chromatogram, which demonstrated that the RP-HPLC method developed, are robust. Results described in Table 8.

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 Laboratory, different analyst, different instrument etc. Here this was done by changing the instrument and analyst. Results, presented in Table 9 that indicates the selected factors are remained unaffected by small variations of this parameter.

Table 8: Robustness of the method

Factor	Level	Retention time	
		Hydroquinone	Avobenzone
Flow rate ml/min			
0.8	-0.1	5.5	6.2
1.0	1	4.9	5.8
1.2	+0.1	4.2	5.1
Column Temperature			
23	-0.1	5.2	6.4
25	1	4.9	5.8
27	+0.1	4.7	5.5
% Acetonitrile in the mobile phase			
75	-0.1	5.6	6.4
80	1	4.9	5.8
85	+0.1	4.2	5.1

Table 9: Ruggedness of the method

	Hydroquinone	Avobenzone
Between instrument I and II		
Instrument	% Content	% Content
I	99.49%	100.19%
II	98.87%	99.36%
% Error	0.62%	0.83%
Between Analyst I and II		
Analyst	% Content	% Content
I	100.07%	99.70%
II	99.23%	99.09%
% Error	0.84%	0.61%

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-HPLC method. The LOD of Hydroquinone and Avobenzone was found to be 2.0µg/ml and 1.0µg/ml respectively. The LOQ is the smaller concentration of the analyte response that can be quantified accurately the LOQ was 10.0µg/ml and 5.0µg/ml respectively.

CONCLUSION

Based on the results, it is concluded isocratic RP-HPLC method was successfully developed for the assay of Hydroquinone and Avobenzone in the topical pharmaceutical formulation. The developed method is selective, precise, accurate, linear and robust. The forced degradation data proved that the method is specific for the analytes and free from the interference of the placebo and degradation products. Moreover, it may be applied to the individual and simultaneous determination of Hydroquinone and Avobenzone compounds in a pharmaceutical drug product and substance. It can be utilized for the determination of assay, blend uniformity, and content uniformity of pharmaceutical products. The developed methods were validated based on ICH guidelines and gave comparable results.

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