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DEVELOPMENT OF ANALYTICAL METHOD FOR SIMULTANEOUS ESTIMATION OF HYDROQUINONE AND MONOBENZONE IN TOPICAL FORMULATION BY RP-HPLC

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ABSTRACT

A new specific, precise, accurate and robust RP-HPLC method has been developed for the simultaneous determination of Hydroquinone and Monobenzone in a pharmaceutical cream formulation. The chromatographic separation was carried out at Analytical technology HPLC instrument (Software: HPLC Work Station) equipped with Deuterium lamp as detector, HPLC pump and manual injecting facility programmed at 20 µL capacity per injection was used. The stationary phase was Zodiac Column C_{18} (250 mm x 4.6 mm, 5µm) at ambient temperature. The mobile phase was Acetonitrile: Triethylamaine Buffer (60:40) adjust pH 3.5 by 10% orthophosphoric acid. Detection was carried out at 230nm using UV Detector. The flow rate was 1.0mL/min and retention time was about 3.3mins and 5.1mins for Hydroquinone and Monobenzone. The linearity was obtained in the concentration range of 24-64µg/mL and 30-70µg/mL for Hydroquinone and Monobenzone respectively. Mean percentage recoveries were 99.89% for Hydroquinone and 99.57% for Monobenzone. The LOD of Hydroquinone and Monobenzone was found to be 1 µg/mL and 2.0µg/mL whereas the LOQ was 5µg/mL and 10 µg/mL respectively. The assay values of both the analytes was found to be well within the limits that is 100.05% and 99.55% for Hydroquinone and Monobenzone respectively. Percentage relative standard deviation of percent assay values for replicate sample preparation was 1.09% for Hydroquinone and 0.96% for Monobenzone. The method was robust with respect to change in flow rate, and composition of mobile phase.

KEYWORDS: Cream formulation, Hydroquinone, Monobenzone, Simultaneous Estimation, RP-HPLC analytical method development, Validation.

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 antioxidant in the photography industry as well as depigmenter agent in cosmetic products such as skin- toning creams. The Hydroquinone mechanism of action in the biological process is based on the inhibition of melanin formation and due to the toxicological effects of Hydroquinone, it can cause dermatitis, EU regulations allow its content in cosmetics within the 2% (w/w) level. It has shown that Hydroquinone and some of its derivatives were present in analysed skin-toning creams.[2] Thus the analytical determination of Hydroquinone and its derivatives in cosmetics is very important for the human health protection and consumers safeguarding.

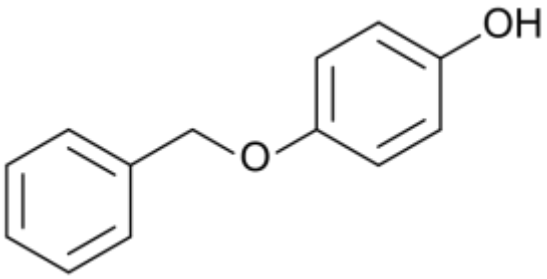
The molecular structure of the drug is given in Fig. 1

Molecular structure of Hydroquinone

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

Monobenzone is used as a topical drug for medical depigmentation.[3-4] It is a colourless solid that is classified as the monobenzyl ether of hydroquinone. Monobenzone is soluble in alcohol, benzene, and diethyl ether, and practically insoluble in water.[5] The topical application of monobenzone in animals increases the excretion of melanin from melanocytes. The same action is thought to be responsible for the depigmenting effect of the drug in humans. Monobenzone may cause destruction of melanocytes and permanent depigmentation. The histology of the skin after depigmentation with topical monobenzone is the same as that seen in vitiligo; the epidermis is normal except for the absence of identifiable melanocytes. Therefore, monobenzone is used as a topical medicine to permanently depigment normal skin surrounding vitiliginous lesions only in patients with disseminated (greater than 50 percent of body surface area) idiopathic vitiligo. Monobenzone is also being considered for the treatment of Melanoma.[6]

The molecular structure of the drug is given in Fig. 2



Molecular structure of Monobenzone

Chemical Name- Monobenzone is 4-(Benzyloxy) phenol. Its molecular formula is $C_{13}H_{12}O_2$ and molecular weight is 200.24 g/mol.

Hydroquinone is the most conventional skin whitening agent. However, it has numerous unfavorable effects with long-term application, including irritative dermatitis, melanocyte destruction, contact dermatitis. High performance liquid chromatography is a widely used technique for analysis of drug product and drug substances.[7-8] An extensive literature survey revealed Spectrophometric[9-11], TLC[12] and HPLC[13-15] for the determination of Hydroquinone and the reported analytical procedure for the estimation of monobenzone in bulk sample and unit dosage forms include HPLC[16-19], TLC[20] and Electrophoresis techniques.[21] But there is no method which describes the simultaneous determination of Hydroquinone and Monobenzone 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 Monobenzone from a cream dosage form.

EXPERIMENTAL WORK

Simultaneous estimation of Hydroquinone and Monobenzone by HPLC.

Reagents and Materials

Hydroquinone RS, Monobenzone RS, Acetonitrile (HPLC grade), Triethylamaine, Distilled water, Orthophosphoric acid (85% AR grade).

Instrumentation and chromatographic condition

Chromatographic separation was performed on a Analytical technology HPLC instrument (Software: HPLC Work Station) equipped with Deuterium lamp as detector, HPLC pump and manual injecting facility programmed at 20 µL capacity per injection was used. Detection was carried out at 230nm using UV Detector. The separation was achieved on the ODS

Zodiac Column C_{18} (250 mm x 4.6 mm, 5µm) at ambient temperature. The elution was carried out isocratically at flow rate of 1ml/min using Acetonitrile: Triethylamaine Buffer (60:40) as mobile phase.

Triethylamaine Buffer: 0.25% Triethylamaine adjust pH 3.5 by 10% Orthophosphoric acid**.**

Preparation of Mobile Phase: Acetonitrile: Triethylamaine Buffer (60:40).

PREPARARTION OF STANDAERD STOCK SOLUTION

1. Hydroquinone

Weigh accurately and transfer about 20.0 mg Hydroquinone RS and transferred in to 25 mL volumetric flask sonicate to dissolve and make up the volume with the mobile phase to mark.

2. Monobenzone

Weigh accurately and transfer about 25.0 mg Monobenzone RS and transferred in to 25 mL volumetric flask sonicate to dissolve and make up the volume with the mobile phase to mark.

3. Combined standard solution

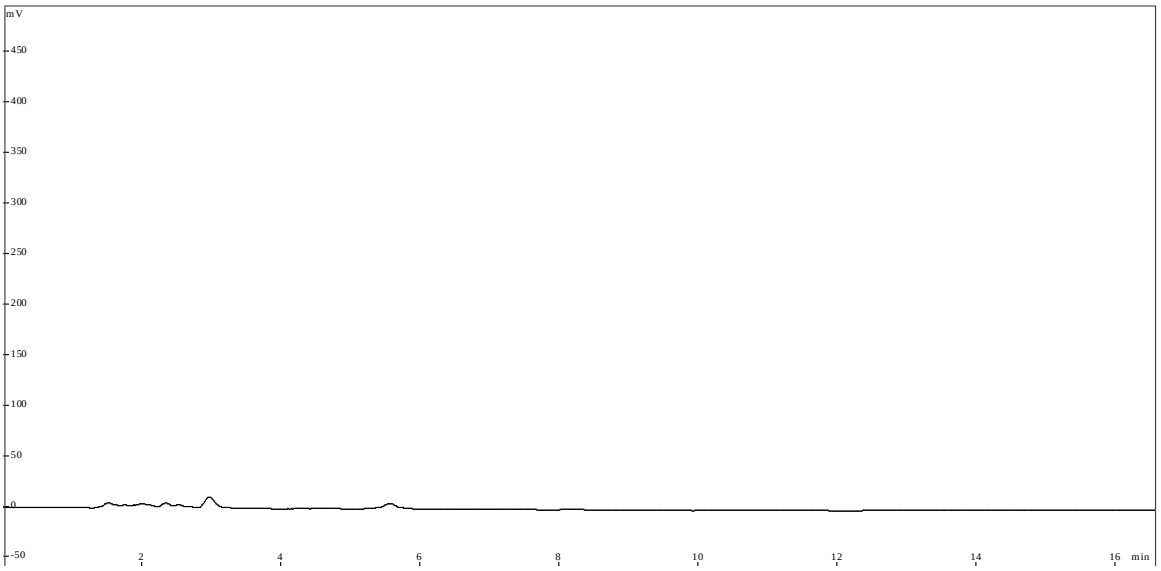
Mix 1 mL each of above standard stock solution in to 20 mL volumetric flask and dilute to mark with mobile phase.

4. Sample Solution

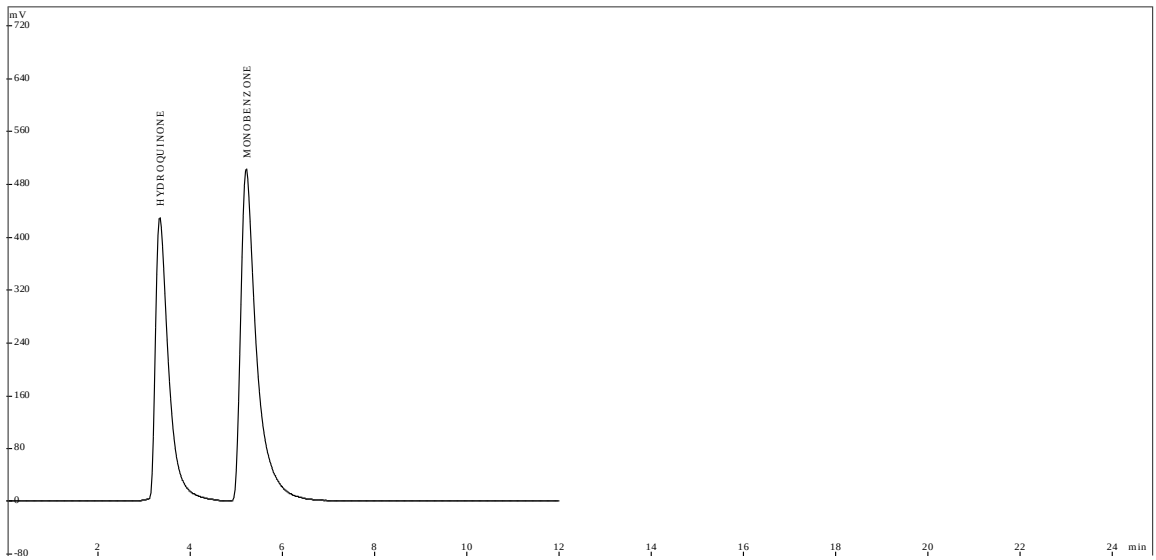
Weigh accurately cream about 1.0g and transfer in to a 50 mL volumetric flask, and add 30 mL of mobile phase, The solutions were sonicated for 20 minutes on ultra-sonicator, dilute to volume with mobile phase and filter.

Take 1 mL from above solution in 20 mL volumetric flask and dilute to mark with mobile phase.

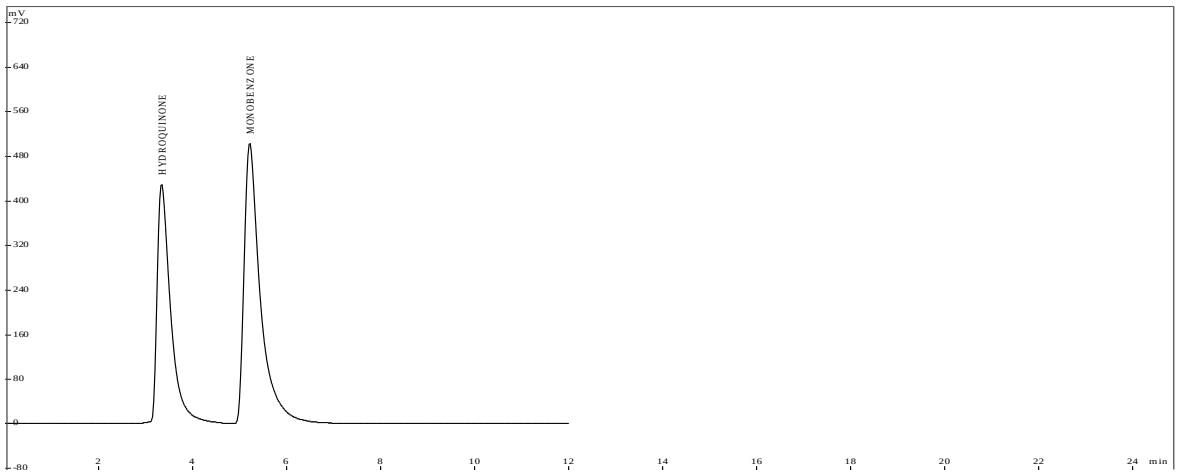
Procedure: Filter both Sample and Standard Solution with 0.2 µ filter paper and inject 20 µL.



CHROMATOGRAM OF MOBILE PHASE



CHROMATOGRAM OF STANDARD



CHROMATOGRAM OF SAMPLE

METHOD VALIDATION

As per ICH guideline the method was validated and following parameters were evaluated, along with Ruggedness.[22-25]

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

Table 1: Analysis of sample.

Contents	Label claim	Found %w/w	Assay % of label amount
Hydroquinone	4.0	4.002	100.05
Monobenzone	5.0	4.977	99.55

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	Monobenzone
Precision of the method (n = 6)	1.09%	0.96%
Theoretical Plates	4125	4652
Resolution Factors	8.092	8.214
Tailing factor	1.000	1.000
Retention time	3.38	5.14

LINEARITY

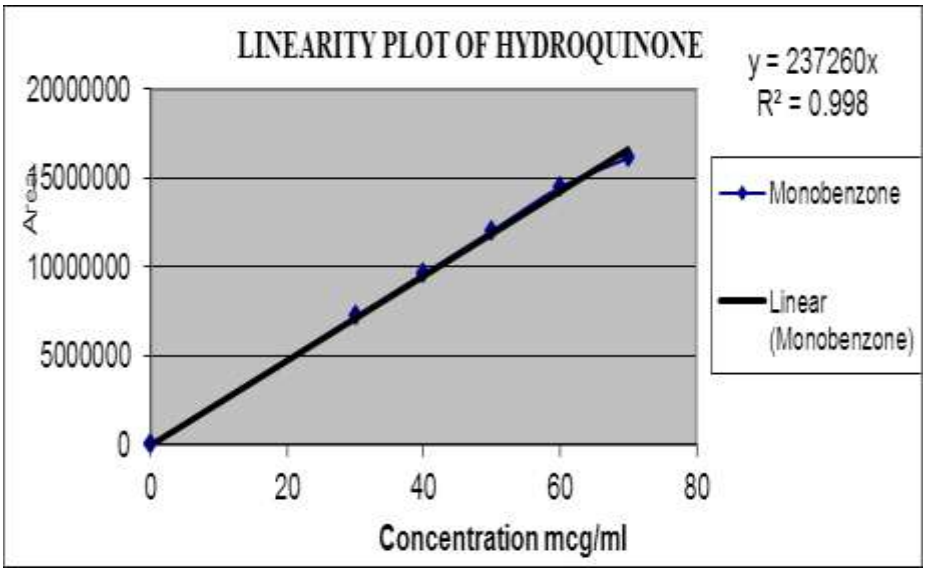
Linearity of the method was established by analysis of combined standard solution. The range of an analytical procedure is the interval between the upper and lower concentrations (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

Linearity of the proposed method was established by using series of standard solutions of Hydroquinone and Monobenzone 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 Monobenzone and its correlation coefficient is 0.998 and 0.998 and linearity graph is shown in Graph No 1.

Table No. 3: Linearity and Statistical analysis data for Hydroquinone.

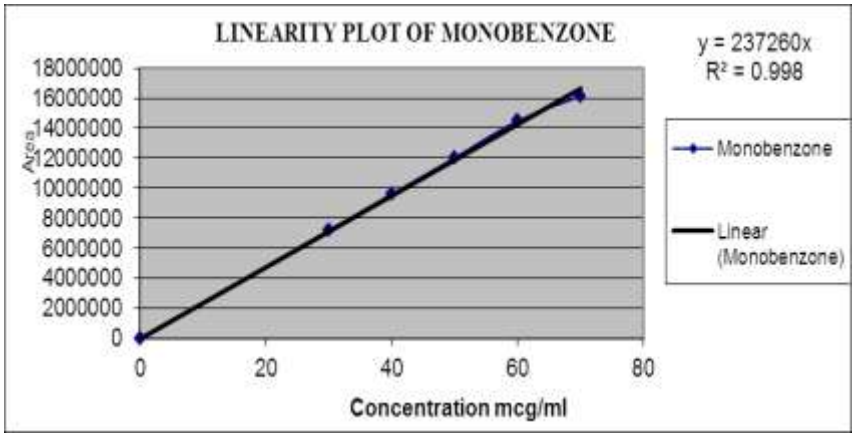
S. No	Concentration (µg/mL)	Area
1.	24µg/mL	5143935
2.	32µg/mL	6858643
3.	40µg/mL	8573275
4.	48µg/mL	10207962
5.	64µg/mL	12012585
Correlative Coefficient(r^2)		**0.998**



Graph No 1: Linearity Graph of Hydroquinone.

Table No. 4: Linearity and Statistical analysis data for Monobenzone.

S. No.	Concentration (µg/mL)	Area
1.	30µg/mL	7220028
2.	40µg/mL	9626692
3.	50µg/mL	12033371
4.	60µg/mL	14490059
5.	70µg/mL	16146752
Correlative Coefficient(r^2)		**0.998**



Graph No 2: Linearity Graph of Monobenzone.

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 Monobenzone was found to be 1.0µg/mL and 2.0µg/mL respectively. The LOQ is the smaller concentration of the analyte response that can be quantified accurately the LOQ was 5 µg/mL and 10 µg/mL respectively.

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 standard drug was added at 80%, 100% and 120% levels. Each level was repeated three times. The contents of Hydroquinone and Monobenzone found by proposed method is shown in the Table 5. The mean recoveries of Hydroquinone and Monobenzone 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 %		Amount Added (mg)	Amount Recovery (mg)	Area	% Recovery	Mean%
80%	1	32.0mg	31.72mg	6858549	99.15%	99.2%
	2	32.0mg	31.89mg	6858654	99.67%	
	3	32.0mg	31.60mg	6857944	98.78%	
100%	1	40.0mg	39.71mg	8573275	99.28%	99.12%
	2	40.0mg	39.72mg	8573958	99.31%	
	3	40.0mg	39.92mg	8573249	99.82%	
120%	1	48.0mg	47.64mg	10287934	99.26%	98.94%
	2	48.0mg	47.32mg	10286451	98.59%	
	3	48.0mg	47.50mg	10286549	98.97%	

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

Accuracy Level %		Amount Added (mg)	Amount Recovery (mg)	Area	% Recovery	Mean%
80%	1	40.0mg	39.22mg	9626659	98.05%	98.86%
	2	40.0mg	39.92mg	9625986	99.80%	
	3	40.0mg	39.50mg	9624862	98.75%	
100%	1	50.0mg	49.18mg	12033371	98.36%	98.93%
	2	50.0mg	49.76mg	12036595	99.52%	
	3	50.0mg	49.46mg	12035285	98.92%	
120%	1	60.0mg	59.61mg	14440025	99.36%	99.04%
	2	60.0mg	59.19mg	14416852	98.66%	
	3	60.0mg	59.46mg	14445526	99.10%	

PRECISION STUDIES

Precision of method was studies by analysis of multiple sampling of homogeneous sample. The precision of analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogenous Sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility. Precision should be investigated using homogenous 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

Sample	Hyroquinone	Monobenzone
Sample 1	99.25%	99.24%
Sample 2	98.97%	99.28%
Sample 3	99.12%	98.31%
Sample 4	99.52%	99.69%
Sample 5	99.61%	98.87%
%RSD	0.27%	0.52%

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 Monobenzone was noted. The factor selected were flow rate, pH 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 describe in Table 8.

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 the 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	
	Flow rate ml/min	Hydroquinone	Monobenzone
0.8	-0.1	3.8	5.6
1.0	1	3.3	5.1
1.2	+0.1	2.8	4.5
pH of the mobile phase			
3.4	-0.1	3.1	4.8
3.5	1	3.3	5.1
3.6	+0.1	3.5	5.4
% Acetonitrile in the mobile phase			
55	-0.1	3.5	54
60	1	3.3	5.1
65	+0.1	3.1	4.8

Table 9: Ruggedness of the method.

	Hydroquinone	Monobenzone
Between instrument **I** and **II**		
Instrument	% Content	% Content
I	99.51%	99.29%
II	99.87%	98.96%
% Error	0.36%	0.33%
Between instrument **I** and **II**		
Analist	% Content	% Content
I	100.07%	99.90%
II	99.73%	99.19%
% Error	0.34%	0.71%

CONCLUSION

Based on the results, it is concluded isocritic RP-HPLC method was successfully developed for the assay of Hydroquinone and Monobenzone in 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 for the individual and simultaneous determination of Hydroquinone and Monobenzone 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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