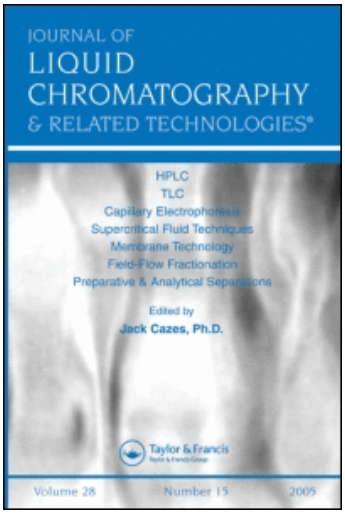

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Development and Validation of HPLC Assay of a New Molecule, 6-methyl-3-phenethyl-3, 4-dihydro-1H-quinazoline-2-thione from Solid Lipid Nanoparticles and its Topical Formulations

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Abstract: A rapid and sensitive high performance liquid chromatography (HPLC) method has been developed and validated for the quantitative determination of a new molecule, 6-methyl-3-phenethyl-3,4-dihydro-1H-quinazoline-2-thione from solid lipid nanoparticles and its topical formulations according to the loading amount. This new molecule is going to be developed as skin whitening agents with topical formulations such as creams, ointments, and gels. The analyses were carried out on a Kromasil 60 CN column (10 μm, 250 mm × 4.6 mm) using a mobile phase composed of water-acetonitrile-diethylamine (600:400:4,v/v) adjusted to pH3.5 with phosphoric acid, following at a flow rate of 2 mL/min. Methyl benzoate was used as the internal standard (IS). The method offers excellent linearity with regression coefficient $r^2 > 0.998$, good repeatability, reproducibility, and relatively short analysis time (20 min). The sample preparation was carried out by a simple extraction of a new molecule with mobile phase, subsequently sonicated for 10 min. The above HPLC conditions resolved the diluted new molecule, pharmaceutical excipients, and potential degradants within 20 min, with a new molecule eluting at about 12.7 min and IS at 4.2 min. System

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suitability parameters and validation parameters including method precision, accuracy, and linearity were setup. The relative standard deviations (RSD) of precisions in the solid lipid nanoparticles consisting of 0.25 g of a new molecule were satisfactory to 0.48%. The detects of quantification (LOD) and the limits of quantification (LOQ) for the new molecule in topical formulations, including 0.25 g of solid lipid nanoparticles, were 2.52 μg/mL and 5.37 μg/mL and the mean recovery value was 100.67% with a RSD less than 0.78%. The proposed procedures were successfully applied for the determination of the new molecule in solid lipid nanoparticles and its topical formulations and control during stability studies.

Keywords: 6-methyl-3-phenethyl-3,4-dihydro-1H-quinazoline-2-thione, HPLC analysis, New molecule, Topical formulations, UV detection, Validation

INTRODUCTION

Melanin is related to the color of the skin and hair, its major role is protection against UV. However, an excessive accumulation of melanin causes hyperpigmentation such as melasma, post-inflammatory melanoderma, and solar lentigo.[1] Melanin is formed through a series of oxidative reactions involving the amino acid tyrosine, in the presence of the enzyme tyrosinase. Tyrosinase, also known as polyphenol oxidase (PPO), is a multifunctional oxidase containing copper that is widely distributed in microorganisms, animal tissues, and plant materials, and is responsible for melanin biosynthesis.[2] Therefore, it has been known that many tyrosinase inhibitors are useful in cosmetics as skin-whitening agents, and also as remedies for pigmentation disturbances[3,4] and, for example, arbutin,[5] ellagic acid,[6] and kojic acid[7] inhibit melanogenesis by inhibiting tyrosinase activity. However, a recent study showed that kojic acid has serious side effects such as cytotoxicity, skin cancer, and dermatitis, and has been banned for cosmetic use in many countries.[8] Considering this toxic effect, some new molecules having a potential as skin whitening agents should be developed.

Recently, we synthesized a new molecule for skin depigmentation, such as 6-methyl-3-phenethyl-3,4-dihydro-1H-quinazoline-2-thione.[9] This molecule (Figure 1) showed an inhibition effect of melanin formation via cAMP/protein kinase A, cGMP/protein kinase G, 1-oleoyl-2-acetlglycerol/protein kinase C, and decrease of tyrosine content, generally known for melanin synthesis. Subsequently, this molecule might have a potential for topical formulations as a skin whitening agent.

This molecule is to develop as a skin whitening agent in the form of topical formulations including solid lipid nanoparticles to show its optimal efficiency for skin whitening. However, there is no report on a validated HPLC method which separates this new molecule. Therefore,

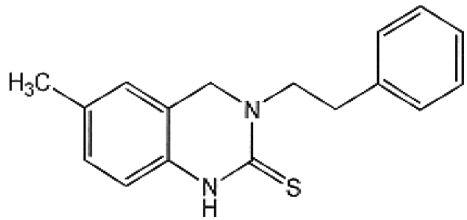

Figure 1. The structure of a new molecule, 6-methyl-3-phenethyl-3,4-dihydro-1H-quinazoline-2-thione.

the new and validated analysis method should be established for synthesis of this molecule and quality control of solid lipid nanoparticles and its topical formulations. This work aims to assay the new molecule from the solid lipid nanoparticles and its topical formulations, according to the loading amount via the validated HPLC method, and to determine the degradation profile under the accelerated stress conditions, according to the ICH guideline.

EXPERIMENTAL

Apparatus

The liquid chromatographic system used in the present study, consisted of an Shiseido Nanospace instrument equipped with a quaternary solvent delivery system and a model Shiseido 3002 UV-VIS detector, model 3002 Pump (Shiseido, Japan). A Rheodyne autosampler syringe loading sample injector with a 20 mL sample loop was used for the analysis injection. Chromatographic data were collected and processed using Shiseido SMC 21 software (Shiseido, Japan). The separation was performed at ambient temperature, on a reversed phase Kromasil CN column (250 mm $\times$ 4.6 mm; 10 μm particle size). All experiments were employed in the isocratic mode.

Reagents and Solutions

All solutions were prepared with analytical reagent grade compounds. Tween80 (Yakuri Pure Chemical, Japan), water, and acetonitrile were purchased from Burdick & Jackson, HPLC grade (SK Chemical, Korea). The mobile phase was prepared by the addition of water and acetonitrile by B&J, HPLC grade and diethylamine (Junsei Chemical, Japan),

phosphoric acid (Yakuri Pure Chemical, Japan). The internal standard (IS) was methyl benzoate (Sigma, USA).

Sample Preparations

For preparation of the test solution according to the loading amount, an amount equivalent to 15 mg of a new molecule from the solid lipid nanoparticles and its topical formulations was transferred into a 50 mL volumetric flask and adjusted with a dilution solution, which was composed of tween 80, water, and acetonitrile (5:5:40). After filtration of the solution, a 10 mL of sample taken from the filtrate was transferred to a 50 mL volumetric flask, added with 100 μL of methyl benzoate, and adjusted with acetonitrile to give the fixed concentration of 0.06 μg/mL. For preparation of the standard solution, 15 mg of a new molecule was transferred into a 50 mL volumetric flask, followed by the preparation procedure of test solution as above.

Chromatographic Conditions

The samples were separated isocratically on the Kromasil CN 10 μm (4.6 mm × 250 mm). The column temperature is maintained at 40°C. The mobile phase consisted of water, acetonitrile, and diethylamine solution in a ratio of 600:400:4, adjusted to pH 3.5 with phosphoric acid. This phase was filtered through 0.45 μm membrane and degassed by ultrasonification, prior to use. Solvent delivery was employed at a flow rate of 2.0 mL/min. The detection of the analysis was carried out at 226 nm. The volume of injection was 20 μL.

Validation Procedure

A system suitability test was performed by six replicate injections of the standard solutions at a concentration of 0.06 μg/mL. The standard plots were constructed with seven concentrations in the range of 36–84 μg/mL prepared in triplicates to test linearity. The ratio of the peak area signal of a new molecule to that of IS was plotted against the corresponding concentration to obtain the calibration graph. The linearity was evaluated by linear regression analysis that was calculated by the least square regression method. The precision of the assay was studied with respect to repeatability. Repeatability was calculated from seven replicate injections of a freshly prepared new molecule in the same equipment at a concentration of 100% (36 μg/mL) of the intended test concentration

value on the same day. Peak area ratios of a new molecule to that of IS were determined and precision was reported as %RSD. Recovery studies were performed to demonstrate the accuracy of the proposed method and to see whether there is interference from excipients used in the preparation of solid lipid nanoparticles or its topical formulations. The standard solution of a new molecule and the test solution from solid lipid nanoparticles or its topical formulations were prepared as mentioned above in sample preparations, diluted in six different concentrations of 60% (0.036 mg/mL), 80% (0.048 mg/mL), 90% (0.054 mg/mL), 110% (0.066 mg/mL), 120% (0.072 mg/mL), and 140% (0.084 mg/mL), and analyzed three times for each concentration under the chromatographic system conditions. Recovery results were expressed with the area of the test solution/the area of the standard solution $\times$ 100.

Accelerated Stress Tests

The new molecule and solid lipid nanoparticles, as well as its topical formulations, were exposed to the stress condition of 60°C, 75% RH for 7 days, to figure out the degradants or related substances from them.

RESULTS AND DISCUSSION

Validation of Methods

The aim of method validation was to confirm that the present method was suitable for its intended purpose as described in ICH guidelines Q2A and Q2B.[10] The described method has been extensively validated in terms of specificity, linearity, accuracy, precision, and system suitability. For validation of this method, methyl benzoate has been chosen as the most appropriate IS in the present analysis, owing to stability among several substances, and it did not interfere with the matrix of pharmaceutical samples and was well separated from a new molecule. Moreover, a significant advantage of this IS was its elution time, which was shorter than that of a new molecule resulting in short run time of less than 5 min. A typical chromatogram for a new molecule and IS using the proposed method is shown in Figure 2. A sharp and symmetrical peak was obtained with a good baseline for each molecule, thus facilitating the accurate measurement of the peak area. The average retention time for a new molecule and IS were found to be 12.82 ± 0.06 and 4.24 ± 0.05 min, respectively, each molecule was clearly separated and their corresponding peaks were sharply developed at reasonable retention times.

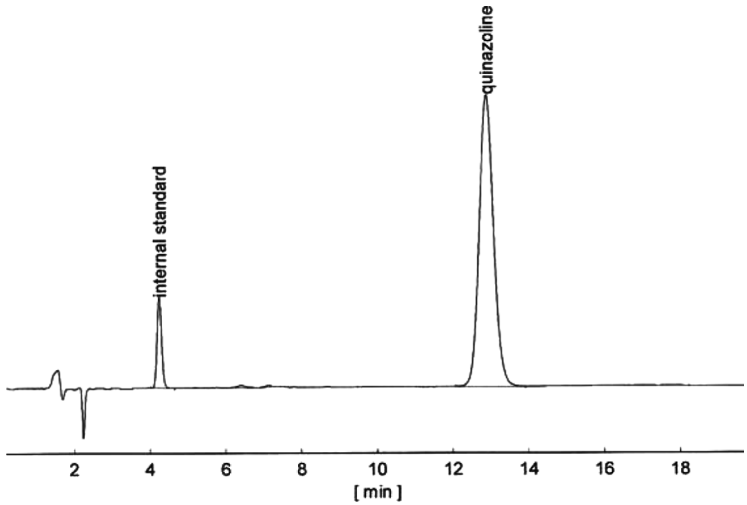

Figure 2. HPLC chromatogram of a new molecule and internal standard.

System Suitability Test

System suitability tests are an integral part of a liquid chromatography method and they were used to verify that the proposed method was able to produce good resolution between the peaks of interest with high reproducibility. The system suitability was determined by making six replicate injections from freshly prepared standard solutions whenever the solid lipid nanoparticles and its topical formulations were analyzed and examining each solute for their peak area, resolution, and tailing factors. System suitability requirements of a new molecule were %RSD of peak areas less than 2% and retention time less than 1%, peak resolution factor greater than 8.0 between two adjacent peaks between a new molecule and IS, tailing factor less than 2.0. The results of the system suitability test in comparison with the required specifications[11] were shown in Tables 1A–4A. According to the results presented, it showed the proposed method fulfilled the requirements within the accepted limits.

Precision

The precision of the proposed method was assessed by repeatability performing seven replicate injections of 0.06 mg/mL of standard solution of a new molecule. The %RSD value of measurements for solid lipid nanoparticles including 0.25 g or 1g of a new molecule was 0.48% or 0.66%. The %RSD value of measurements for topical formulations including

Table 1. Validation results from solid lipid nanoparticles including 0.25g of a new molecule

A. System suitability test

Composition & specification	Retention time (min)	Precision (% RSD)		Tailing factor	Resolution factor
		RT	Area		
A new molecule	12.89	0.24%	0.10%	1.56	13.03
IS	4.25	0.13%	0.12%	1.11	—
Specification	—	Not more than 1.0%	Not more than 2.0%	Not more than 2.0	Not less than 8.0

B. Precision

Assay (%)	AVE	SD	RSD
99.60			
99.54			
100.68			
100.64	100.12%	0.48	0.48%
99.86			
100.44			
100.05			

C. Accuracy

Assay (%)	Theory assay (%)	Detection assay	Recovery
60	58.86	59.10	100.41
80	78.43	78.82	100.50
90	89.69	91.76	102.31
100	98.12	98.40	100.29
110	109.80	110.56	100.69
120	121.57	121.32	99.79
140	139.22	140.19	100.70

D. Linearity and range

Theory concentration (μg/mL)	Area unit (calculated by Internal Standard)
35.32	6.0568
47.06	8.0811
53.81	9.0943
58.87	10.1032
65.88	11.1087
72.94	12.0987
83.53	14.1398

(*Continued*)

Table 1. Continued

E. Stability

	% Recovery				
Day	1	2	3	Average	RSD
1	99.03	98.77	98.44	98.75	0.30
3	100.02	100.07	99.90	100.00	0.09
7	99.61	99.78	99.53	99.64	0.13

Table 2. Validation results from solid lipid nanoparticles including 1g of a new molecule

A. System test

Composition & specification	Retention time (min)	Precision (% RSD)		Tailing factor	Resolution factor
		RT	Area		
New Molecule	12.81	0.29%	0.14%	1.23	12.01
IS	4.27	0.15%	0.12%	1.06	—
Specification	—	Not more than 1.0%	Not more than 2.0%	Not more than 2.0	Not less than 8.0

B. Precision

Assay (%)	AVE	SD	RSD
100.23 99.34 100.11 101.01 99.16 100.14 100.43	99.72%	0.66	0.66%

C. Accuracy

Assay (%)	Theory assay (%)	Detection assay	Recovery
60	59.45	59.88	100.72
80	79.54	79.56	100.03
90	89.11	91.23	102.38
100	99.23	99.67	100.44

(*Continued*)

Table 2. Continued

C. Accuracy

Assay (%)	Theory assay (%)	Detection assay	Recovery
110	109.75	110.11	100.33
120	121.44	121.57	100.11
140	139.07	140.01	100.68

D. Linearity and range

Theory concentration (μg/mL)	Area unit (calculated by Internal Standard)
35.32	6.0462
47.06	8.2345
53.81	9.2235
58.87	10.1123
65.88	11.1232
72.94	12.2284
83.53	14.2826

E. Stability

	% Recovery				
Day	1	2	3	Average	RSD
1	100.34	100.39	100.21	100.32	0.10
3	100.11	100.07	99.93	100.04	0.09
7	99.33	99.46	99.30	99.36	0.09

0.25 g or 1g of solid lipid nanoparticles was 0.68% or 0.63% Tables 1B–4B. The % RSD of assay results obtained in the precision study was satisfied; the % RSD should be not more than 2.0%.

Accuracy

The data for accuracy were expressed in terms of percentage recoveries of a new molecule from the solid lipid nanoparticles or its topical formulations according to the loading amount. These results are summarized in Tables 1C–4C. The mean recovery data of a new molecule from solid lipid nanoparticles containing 0.25 g or 1g of it were within the range of 99.79 to 102.31% (Table 2C), 100.03 to 102.38% (Table 2C), those of a new molecule from its topical formulations including 0.25 g or 1g of solid lipid nanoparticles were 99.80 to 102.22% (Table 3C), 100.01 to 101.72% (Table 4C).

Table 3. Validation results from topical formulation composed of solid lipid nanoparticles including 0.25g of a new molecule

A. System suitability test

Composition & specification	Retention time (min)	Precision (% RSD)		Tailing factor	Resolution factor
		RT	Area		
New Molecule	12.80	0.20%	0.11%	1.11	12.23
IS	4.27	0.11%	0.09%	1.00	—
Specification	—	Not more than 1.0%	Not more than 2.0%	Not more than 2.0	Not less than 8.0

B. Precision

Assay (%)	Average assay	SD	RSD (N.M.T. 2.0%)
100.23 99.50 101.02 100.29 98.99 99.44 100.09	99.94%	0.68	0.68%

C. Accuracy

Assay (%)	Theory assay (%)	Detection assay	Recovery
60	58.34	59.44	101.89
80	78.78	79.33	100.70
90	89.34	91.32	102.22
100	99.45	99.47	100.02
110	109.34	110.09	100.69
120	120.73	120.49	99.80
140	139.88	140.63	100.54

D. Linearity and range

Theory concentration (μg/ml)	Area unit (calculated by Internal Standard)
35.32	6.0472
47.06	8.3753
53.81	9.1129
58.87	10.1293
65.88	11.1324
72.94	12.1129
83.53	14.1401

(*Continued*)

Table 3. Continued

E. Stability

	% Recovery				
Day	1	2	3	Average	RSD
1	99.44	99.09	98.96	99.16	0.25
3	99.02	99.28	99.57	99.29	0.28
7	98.21	98.72	98.01	98.31	0.37

Table 4. Validation results from topical formulation composed of solid lipid nanoparticles including 1g of a new molecule

A. System suitability test

Composition & specification	Retention time (min)	Precision (% RSD)		Tailing factor	Resolution factor
		RT	Area		
Quinazoline	12.77	0.30%	0.19%	1.44	13.28
Istd (m.b)	4.22	0.13%	0.11%	1.29	—
Specification	—	Not more than 1.0%	Not more than 2.0%	Not more than 2.0	Not less than 8.0

B. Precision

Assay (%)	Average assay	SD	RSD (N.M.T. 2.0%)
99.11 100.34 101.19 100.09 99.75 100.23 100.21	100.13%	0.63	0.63%

C. Accuracy

Assay (%)	Theory assay (%)	Detection assay	Recovery
60	60.32	60.45	100.22
80	79.88	79.89	100.01
90	88.92	90.45	101.72
100	99.63	99.81	100.18
110	109.32	109.41	100.08

(*Continued*)

Table 4. Continued

C. Accuracy

Assay (%)	Theory assay (%)	Detection assay	Recovery
120	121.01	121.61	100.50
140	139.92	140.50	100.41

D. Linearity and range

Theory concentration (μg/ml)	Area unit (calculated by Internal Standard)
35.32	6.1432
47.06	8.3845
53.81	9.3762
58.87	10.4723
65.88	11.3895
72.94	12.7376
83.53	14.4739

E. Stability

	% Recovery				
Day	1	2	3	Average	RSD
1	101.22	100.86	101.03	101.04	0.18
3	99.63	99.91	99.90	99.81	0.16
7	98.72	98.70	99.04	98.82	0.19

Linearity

The seven point calibration graphs were constructed covering a concentration range 0.036–0.084 mg/mL of a new molecule in solid lipid nanoparticles and its topical formulations according to the loading amount. The linear relationships between the ratio of the peak area signal of a new molecule to that of IS versus the corresponding drug concentration were observed, as shown by the results presented in Tables 1D–4D. The correlation coefficiency of a new molecule from solid lipid nanoparticles containing 0.25 g or 1 g was to be greater than 0.9983 or 0.9984, while as that of a new molecule from its topical formulations including 0.25 g or 1g of solid lipid nanoparticles was to be greater than 0.9980 or 0.9985.

Stability Studies Against Accelerated Stress Tests

Solid lipid nanoparticles and its topical formulations were stable under the condition of 60°C, 70% RH, during 7 days based on the proposed

method in this study. Tables 1E–4E showed all the solid lipid nanoparticles and its topical formulations maintained their content with 98.75 to 101.04% under this validated HPLC method.

CONCLUSION

The statistical evaluation of the proposed method revealed its good linearity, reproducibility, and its validation for different parameters, and led us to the conclusion that it could be used for the rapid and reliable determination of a new molecule, 6-methyl-3-phenethyl-3,4-dihydro-1H-quinazoline-2-thione and solid lipid nanoparticles, as well as its topical formulations. Its chromatographic run time of 20 min could allow the analysis of a large number of samples in a short period of time. The validated HPLC method has been proven to be simple and rapid, accurate, precise, and specific, and could be applied for the determination of a newly synthesized molecule in pharmaceutical formulations. Also, this proposed method could be suitable for the screening of formulated samples in routine quality control applications.

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