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Research Article

SIMPLE STABILITY-INDICATING VALIDATED HPLC METHOD FOR DIOSGENIN IN COSMECEUTICAL FORMULATIONS WITH LONG TERM STABILITY APPLICATION FOR LIGHT AND HEAT

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ABSTRACT

A rapid, simple, precise and accurate stability-indicating analytical HPLC method was developed and validated for determination of diosgenin in cosmeceutical formulations. Modified extraction with suggested analytical method resulted in assurance of full recovery in all types of formulations. In this study, a Luna-C_{18} (4.6 mm x 150 mm, 5 µm particle size) column was employed using a mixture of distilled water and acetonitrile (15:85, v/v) as a mobile phase with a wavelength of 210 nm. Method validation was conducted based on ICH and USP guidelines. For a selectivity test, this method was shown to separate well with numerous interferences that were commonly employed as formulation excipients. An adequate linearity relationship (r^2=0.999) was obtained for diosgenin concentrations ranging from 0.05 to 1.0 mg mL^{-1}. For all formulations, the accuracy percentage ranged from 99.21 to 101.52% and the precision was lower than 2%. These acceptance criteria indicated satisfactory validation results. Moreover, the proposed method was further applied for long-term and accelerated stability experiments in all formulations in forced stress conditions of light and heat.

Keywords: Diosgenin, Excipients, Tetrahydrofuran, Long-term stability, Accelerated stability

INTRODUCTION

Diosgenin (25R-Spriost-5-en-3β-ol) is an aglycone of dioscin, which is a steroidal saponin that occurs abundantly in natural plants like wild yams, including *Dioscorea wild* species[1-2]. As a steroidal metabolite, diosgenin has been used early as a starting material for various synthetic steroidal drugs in the pharmaceutical industry. Moreover, its estrogenic effect on mammalian glands has been demonstrated[3], and it has been employed as a component of combined oral contraceptive pills (COCP) [4]. It has also been shown to lead to cholesterol reduction[5] and to have antioxidant ability[6]. Published studies have reported that diosgenin is a potent chemotherapeutic agent against several cancers including osteosarcoma and colon carcinoma[7-8]. Recently, as an active substance, diosgenin in bulk extract of wild yam was widely used in skin care formulas meant to reduce the sign of aging in relation with keratinocyte proliferation[9]. Also, Lee et al. demonstrated that diosgenin had an inhibitory effect against melanogenesis that occurred through activation of phosphatidylinositol-3-kinase pathway (PI3K) signaling[10], suggesting its possible application for a skin lightening effect.

Several methods have been reported to be useful for determination of diosgenin from various approaches, including HPTLC, HPLC, LC ESI-MS and ELISA[11-16]. LC ESI-MS method was the most sensitive but it cannot be commonly employed to analytical laboratories and institutes due to requirement of unusual instrument and experienced expert. One of them, HPLC is the most universal quantification method to any laboratories for routine analysis and easily adaptable even in complex matrixes without disturbance of excipients[17-18].

However, reported HPLC methods with extraction procedure were practically not adaptable in complex preparations like commercial cosmetic formulations due to overlapping of diosgenin peak with formulation excipients and showed limited recovery during extraction process. Also, to date, there have been no reports regarding HPLC determination of diosgenin in formulations. Thus, the present study was to develop a simple, rapid, precise and accurate reversed phase-HPLC for indication of the stability of diosgenin so that it can be easily adapted in analytical laboratories and/or to various types of formulations.

In this study, development and validation of the method used for analysis of diosgenin were conducted according to the requirements of the ICH and USP guidelines including specificity, linearity, accuracy, precision, limit of detection and limit of quantification without any interference from complex excipients[19-20]. In the extraction process of diosgenin, incomplete solubilization occurred for all formulations. However, diosgenin was completely extracted by employing THF, which is a miscible polar aprotic strong solvent, as a diluent of methanol. Finally, the proposed method was successfully applied to stability studies of each formulation over six months of exposure to thermal and light stress conditions, suggesting that diosgenin is a stable substance and the method is applicable to other stability studies and quality control applications in the cosmeceutical/pharmaceutical field.

MATERIALS AND METHODS

Materials and reagents

HPLC grade acetonitrile and THF were purchased from Fisher Science (USA). HPLC grade methanol was purchased from J.T. Baker (NJ, USA). A diosgenin standard (≥99%) was purchased from Sigma Aldrich. All reagents were used without any further purification. All cosmetic ingredients, which included disodium EDTA, glycerin, butylene glycol, sodium hyaluronate, carbomer, acrylates/C10-30 alkyl crosspolymer, allantoin, sorbitol, methyl paraben, ethanol, PEG-60 hydrogenated castor oil, phenyltrimethicone, lecithin, glyceryl stearate/PEG-100 stearate, cyclomethicone, glyceryl staearate, propyl paraben, polysorbate60, glyceryl stearate, sorbitan sesquioleate, stearic acid, cetearyl alcohol, cetyl alcohol, cetylethylhexanoate, mineral oil, dimethicone, cyclopenasiloxane, and propyl paraben, were purchased from Daebong LS (Rep. of Korea).

HPLC Instrument and Condition

A Waters HPLC system with a 600 controller, 996 photodiode array detector, 616 pump, and a 717 autosampler was used in this experiment. Data acquisition was achieved using the Waters Empower software. All chromatographic separations were conducted on a Phenomenex Luna C_{18} column (150 mm X 4.6 mm, 5 µm) at ambient temperature with detection at 210 nm. The mobile phase consisted of distilled water and acetonitrile (15:85, v/v). All HPLC experiments were conducted using an injection volume of 20 µL, an autosampler and a total run time of 30 min for each sample.

Preparation of diosgenin supplemented commercial formulations

Cosmetic formulations for the development, validation and application studies were prepared as shown in Table 1.

Previously, all pre-mixture solutions were made at 75°C with adequate homogenization. Skin was formulated by mixing the A and B phases at 25°C with sufficient agitation followed by the addition of triethanolamine, citric acid and sodium citrate to adjust the pH. Lotion and cream were prepared by suspension of the A and B phases at 75°C to achieve homogeneity in a highly thickened solution, after which triethannolamine and phenoxyethanol were added under the same conditions. All formulations contained 400 µg mL^{-1} of diosgenin in the dispersed state.

Table 1: Formulation components for skin, lotion and cream preparations

Phase	Components	Percentage in each formulation (w/w)		
		Skin	Lotion	Cream
A	Distilled water	87.31	89.12	82.53
	Pre-mixture[a]	6.53	-	-
	Pre-mixture[b]	-	3.54	-
	Pre-mixture[c]	-	-	1.03
B	Pre-mixture[d]	5.90	-	-
	Pre-mixture[e]	-	6.60	-
	Pre-mixture[f]	-	-	15.70
	Diosgenin	0.04	0.04	0.04
C	Triethanolamine	0.14	0.20	0.20
D	Citric acid	0.03	-	-
	Sodium citrate	0.05	-	-
	Phenoxyethanol	-	0.50	0.50

[a] Disodium EDTA 0.03%, Glycerin 3.00%, Butylene glycol 3.00%, Sodium hyaluornate 0.30%, Carbomer 0.10% Acrylates/C10-30 Alkyl Crosspolymer 0.10%)

[b] (Allantoin 0.10%, Sorbitol 1.00%, Butylene glycol 2.00%, Carbomer 0.10%, Acrylates/C10-30 Alkyl Crosspolymer 0.10%, Methylparaben 0.20%)

[c] (Disodium EDTA 0.03%, Allantoin 0.10%, Sodium hyaluornate 0.50%, Carbomer 0.20%, Methylparaben 0.20%)

[d] (Ethanol 5.00%, PEG-60 hydrogenated castor oil 0.40%, Phenyltrimethicone 0.30%, Methylparaben 0.20%)

[e] (Lecithin 0.50%, Ethanol 1.00%,Glyceryl stearate/PEG-100 stearate 1.50%, Cyclomethicone 3.00%, Glyceryl stearate 0.50%, Propylparaben 0.10%)

[f] (Polysorbate60 1.20%, Glyceryl stearate 0.80%, Sorbitan sesquioleate 0.30%, Glyceryl stearate/PEG-100 stearate 2.00%, Stearic acid 0.50%, Cetearyl alcohol 2.00%, Cetyl alcochol 1.50%, Cetylethylhexanoate 1.00%, Mineral oil 3.00% Dimethicone, 0.30%, Cyclopentasiloxane 3.00%, Propylparaben 0.10%)

Preparation of standard and test solutions

Diosgenin standard (Sigma≥99%) was purchased and prepared. Briefly, 10 mg of diosgenin was precisely weighed and then dissolved in 10 mL of methanol mixed with tetrahydrofuran in a ratio of 1 to 1 as a stock solution. This 1 mL of stock solution was subsequently diluted in 10 mL of the same solvent above as a standard solution. For the test solution, 2.5 g of each formulation were also precisely weighed and dissolved in 10 mL of methanol mixed with 50% THF for all formulations. Both the standard and test solution were filtered using a 0.22 µm pore-sized syringe filter for further HPLC injection. All possible weighing errors were compensated by multiplying dilution factor inversely.

Validation of the method

All validation processes were conducted based on the ICH and USP guidelines[21-22]. To assess the specificity and selectivity of diosgenin in each formulation, a chromatographic run of test solutions prepared from skin, lotion and cream formulations was conducted using the developed method while simultaneously identifying the diosgenin peak using a photo-diode array spectrum.

A linearity study was also conducted over a concentration range of 50.0 - 1000.0 µg mL^{-1}, covering the analytical working concentration during chromatographic separation. Five concentrations, 50, 100, 200, 500, and 1000 µg mL^{-1}, were selected and prepared by dilution of the stock solution. Triplicate injections were then made for each concentration and the obtained peak area was used to plot the standard curve against the concentration of diosgenin.

The accuracy and recovery were evaluated by preparing a placebo of cosmetic skin, lotion and cream formulations that differed only in that they had diosgenin concentrations of 320, 400 and 480 µg mL^{-1} covering a range of 80-120% of the claimed concentration. Triplicate injections were made for each concentration and recovery (%) in skin, lotion and cream formulations and the results were assessed by comparison with standard solutions of diosgenin prepared daily.

Repeatability and intermediate precision were evaluated by analyzing prepared skin, lotion and cream formulations containing 400 µg mL^{-1} of diosgenin. Repeatability was assessed based on the R.S.D. (%) of the peak area of diosgenin obtained from six replicate injections at 100% of the claimed concentration. Intermediate precision was conducted using the same procedure as for the repeatability experiment; however, analyses were conducted on two different days and evaluated by R.S.D. (%).

LOD and LOQ were determined by direct calculation based on the signal to noise ratio measured during the chromatographic run. LOD and LOQ were determined as the concentration at which the signal to noise ratio was 3:1 and 10:1, respectively. Specifically, 50 µg mL^{-1} of the diosgenin stock solution was diluted in serial units of 10 µg mL^{-1}, after which triplicate injections of the test solution and methanol:THF (1:1) as a blank for noise assessment were analyzed.

To ensure adequate sustainability of performance between the method developed here and the HPLC instrument for daily analysis, a system suitability test (SST) was conducted during development and validation of the method. The following evaluation parameters were considered, capacity factor (*K*), asymmetry (*A*), tailing (*T*), theoretical plates (*N*) and R.S.D. (%) of retention time/peak area/peak height.[19-20] Data from six replicates of injections were obtained and calculated to determine the R.S.D. (%) value.

Long term and accelerated stability evaluation

The proposed method was employed to observe the long-term and accelerated stability of diosgenin in skin, lotion and cream formulations to evaluate the persistence of diosgenin by elapsed time based on the stability testing guideline of ICH[21]. For a long-term study defined to monitor at 25°C, each formulation containing 400 µg mL^{-1} of diosgenin was kept at room temperature with/without artificial shade provided by aluminum foil for a six month period. An accelerated stability study under a forced

thermal condition was also conducted using the same procedure, but the samples were maintained at 50°C in an oven. At appropriate time intervals (0.5, 1, 2, 4 and 6 months), each sample was withdrawn and used to prepare a test solution for determination of the diosgenin to verify the degree of thermal and light induced influences.

RESULTS AND DISCUSSION

Method development

The preceding optimization was necessary before method validation and separation of diosgenin under various conditions, while SST test was simultaneously conducted for all formulations.

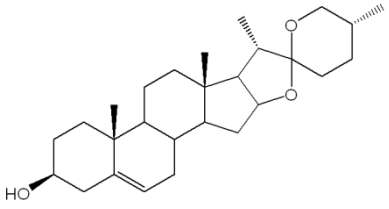

Fig. 1: The structure of diosgenin

As shown in Fig. 1, the analyte diosgenin only has one conjugated double bond, indicating a lack of specific chromospheres. Because of this property, diosgenin could not be detected, even at higher wavelengths. Accordingly, the detection range of diosgenin is limited to its low wavelength region (< 220 nm), which has poor sensitivity due to absorption competition with the mobile phase. However, when considering the signal to baseline ratio of the mobile phase, wavelength selection at 210 nm was found to be appropriate for quantification.

Evaluation of the mobile phase revealed that acetonitrile produced a better baseline at 210 nm than methanol and was more advantageous in that it contributed low pressure to the chromatographic system. To improve the peak shape, the addition of an ion paring agent such as acetic acid, trifluoroacetic acid and buffer at different concentrations was conducted; however, considerable effects were not observed (data not shown).

Through variation testing of the composition in water and acetonitrile based on previous report, [2, 12-14] isocratic elution using a water:acetonitrile (15:85) mobile phase showed affordable resolution of excipients and adequate retention time during a chromatographic run time of 30 min.

In previous study,[2,13] methanol was used as diluent to extract diosgenin from each source without any recovery problem. But, in our study, a test solution extracted by methanol did not achieve sufficient recovery for all formulations due to disturbance of complex excipients, indicating that methanol is not an ideal diluent for non-soluble diosgenin. Other extracting agents and additives including ethanol, trifluoroacetic acid, phosphate buffer, etc. were evaluated to improve the solubility during the extraction of diosgenin. One of these, THF, was employed at different ratios. Methanol mixed with THF at a ratio of 1:1 showed the greatest extraction capacity of all formulations, indicating that it is a proper extraction solvent (Table 2). Following this optimization, validation of the method was evaluated.

Table 2: Recovery efficiency obtained by suspending various ratios of tetrahydrofuran to methanol when preparing samples from skin, lotion and cream formulations

Ratio of tetrahydrofuran to methanol (%)	Skin Recovery (%) ±SD	Lotion Recovery (%) ±SD	Cream Recovery (%) ±SD
0	88.09 ± 1.1366	84.63 ± 1.0335	46.98 ± 0.7398
10	91.08 ± 0.8352	88.91 ± 1.0825	51.43 ± 0.7840
30	95.35 ± 1.1060	95.46 ± 1.4451	80.59 ± 1.0501
50	100.38 ± 0.7775	101.18 ± 1.2620	99.70 ± 1.2820

Method Validation

System suitability

To ensure adequate performance of the method, system suitability parameters of the analyte peak were evaluated using the ICH and USP guidelines.[22-23] As shown in Table 3, all obtained parameters fell in a range of conventionally accepted criteria. The capacity factor was within 2.2 < K' < 6.8. In addition, the value of asymmetry and tailing factor was lower than 1.5 and the theoretical plate value for column efficiency was 5421. All the R.S.D. values of the retention time/area/peak height were lower than 2%, indicating that the obtained value provided satisfactory results and the system was working properly.

Table 3: System suitability parameters of diosgenin standard

Parameters	Value
Capacity factor (*K'*)	6.085
Asymmetry (*A*)	1.13
Tailing (*T*)	1.05
Theoretical plates (*N*)	5421
R. S. D. of retention time (%)[a]	0.846
R. S. D. of area (%)[a]	0.928
R. S. D. of peak height (%)[a]	1.163

[a] (n=6)

Specificity

The proposed method was evaluated to verify the ability to separate the diosgenin peak with possible interference of commonly used excipients including antioxidants, thickening agents, preservatives, pH regulators and emollients. Chromatographic analysis revealed good separation of the diosgenin peak and no interference with the excipients in any formulations, even in densely mixed lotion and cream formulations (Fig. 2).

Linearity

Evaluation of the linearity was conducted at concentrations ranging from 0.05 to 1.00 μg mL^{-1}, which covered the working concentrations. The correlation efficient value (r^2=0.999) was higher than the acceptance criteria of 0.995 according to the USP guideline. Other related parameters for the regression equations obtained by least squares treatment of the results are shown in Table 4.

Accuracy

To measure the accuracy of the proposed method, three known concentrations of diosgenin were added to each formulation and their calculated recovery was compared to the actual concentration (Table 5). This calculated recovery value ranged from 99.21 to 101.52%, indicating that this method worked properly with excellent accuracy in skin, lotion and cream formulations as model cosmetic preparations without any disturbance of excipients.

Precision

The precision of this method was assessed based on the R.S.D. (%) value obtained from intra- and inter-day analysis of six replicate injections (Table 6). All values for the intra- and inter-day precision study were lower than 2%, confirming that the precision of the method was sufficient. In addition, no significant differences in repeatability among formulation types were observed.

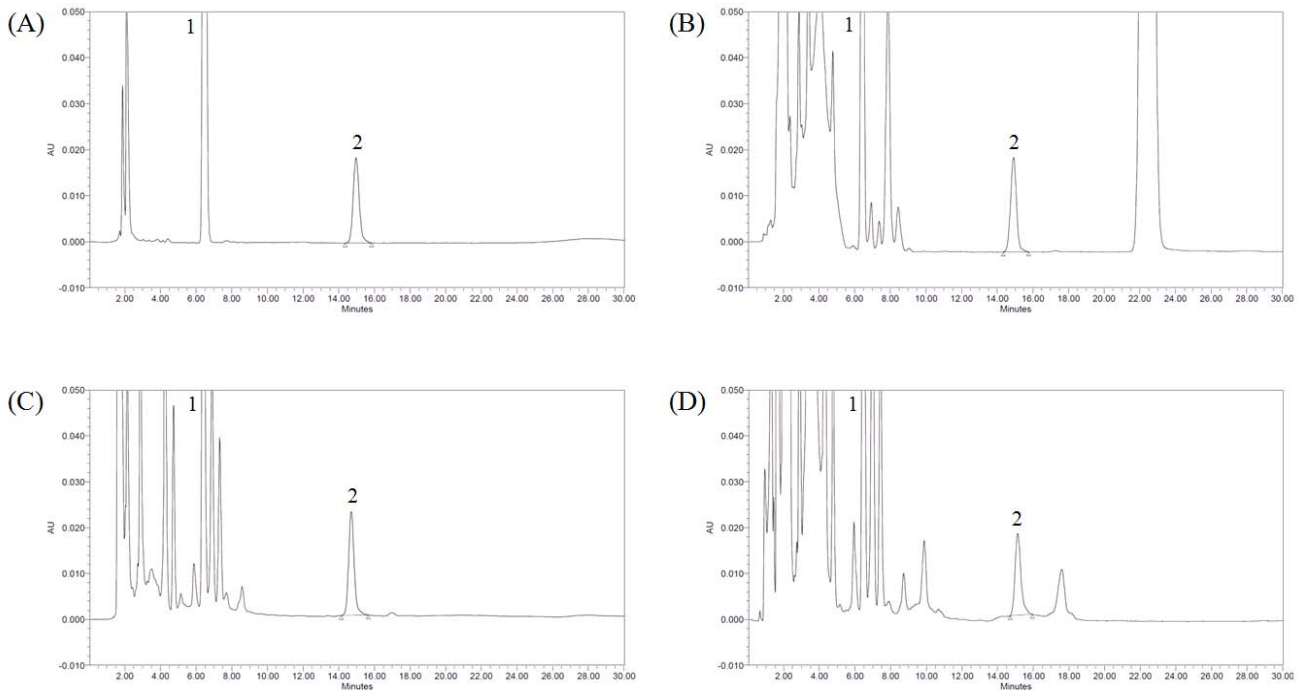

Fig. 2: Chromatographic separation of diosgenin in (A) standard, (B) skin, (C) lotion and (D) cream solution using proposed method. (1) Tetrahydrofuran (2) Diosgenin

Table 4: Statistical analysis of regression data and limit of detection and quantification

Parameters	Value
Regression equation (*Y*)[a]	
Calibration range	50-1000 µg mL^{-1}
Slope (b)	2059.7
Standard deviation of the slope (S_b)	106.46
Relative standard deviation of the slope %	5.4745
Confidence limit of the slope[b]	1956.72 – 2162.19
Intercept (a)	-15330
Correlation coefficient (r)	0.9999
Standard error of the estimation	7189.665
Limit of detection[c]	~10 µg mL^{-1}
Limit of quantification[c]	~30 µg mL^{-1}

[a] Y=a + bC, where C is the concentration of diosgenin in µg mL^{-1} and Y is the area of peak

[b] 95% confidence limit

[c] direct calculation of signal to noise ratio

Table 5: Accuracy of data obtained from skin, lotion and cream formulations in a concentration range of 320 to 480 µg mL^{-1} of diosgenin.

Type of formulation	% of aimed concentration (µg mL^{-1})	Prepared concentration (µg mL^{-1})	Measured concentration ± SD (µg mL^{-1})	Recovery (%) ± SD
Skin	80	320	318.42 ± 3.1847	99.51 ± 0.9952
	100	400	400.90 ± 3.2501	100.23 ± 0.8125
	120	480	487.28 ± 3.9164	101.52 ± 0.8159
Average ± SD				100.42 ± 0.8745
Lotion	80	320	321.78 ± 1.5551	100.56 ± 0.4860
	100	400	401.53 ± 2.0919	100.38 ± 0.5230
	120	480	484.27 ± 2.4088	100.89 ± 0.5018
Average ± SD				100.61 ± 0.5036
Cream	80	320	321.67 ± 3.4672	100.52 ± 1.0835
	100	400	401.73 ± 3.1472	100.43 ± 0.7857
	120	480	476.22 ± 2.7315	99.21 ± 0.5691
Average ± SD				100.06 ± 0.8127

Table 6: Precision data in skin, lotion and cream formulations

Type of formulation	% of aimed concentration (µg mL^{-1})	Prepared concentration (µg mL^{-1})	Intra-day Measured R.S.D. (%)[a]	Intra-day Measured R.S.D. (%)[b]
Skin	100	400	0.933	0.809
Lotion	100	400	1.019	0.513
Cream	100	400	1.294	0.957

[a] (n=6); [b] (n=12)

Detection and quantification limit

LOD and LOQ values were estimated by direct calculation using the designated signal to noise ratio. As shown in Table 6, the LOD was found to approximately 10 µg mL^{-1} and the LOQ was about 30 µg mL^{-1}. This inferior sensitivity resulted from the intrinsic low absorbance of diosgenin, which made its spectrometric quantification difficult at levels below the LOD, necessitating another approach.

Application of method for skin, lotion and cream formulations

Based on the ICH stability testing guideline[21], a long term experiment was conducted at room temperature and an accelerated experiment was conducted at 50°C using the proposed method.

Thermal and light influences on diosgenin stability were evaluated in each formulation over a six month period. Chromatographic analysis during the designated interval revealed that there was no decrease in diosgenin concentration when compared to the initial period for all formulations (Fig 3). These results show that diosgenin is a stable substance against heat and light with no production of degradation compounds. In addition, no effects of formulational differences on diosgenin stability were observed.

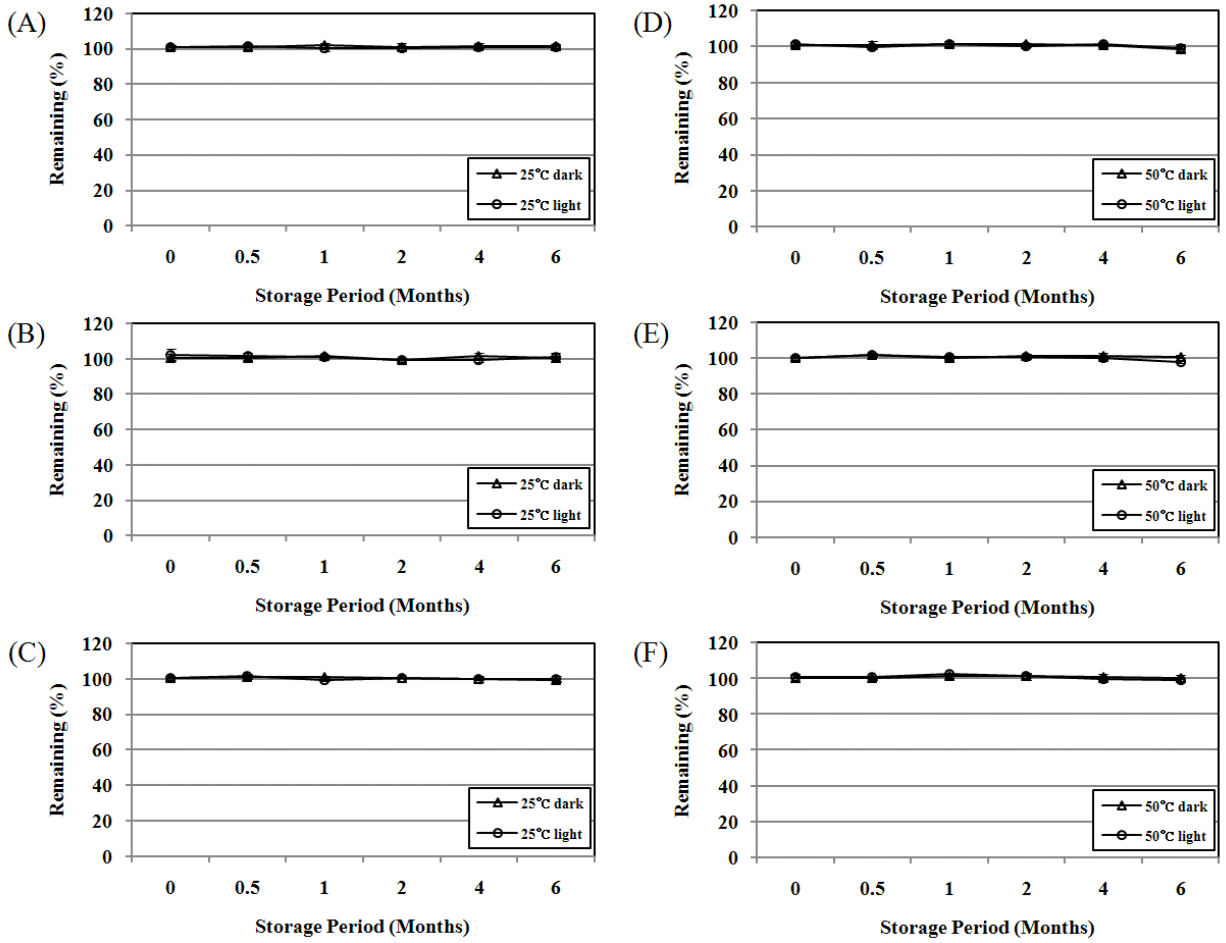

Fig. 3: Application of the proposed method for evaluation of long term stability (25°C) in skin (A), lotion (B) and cream (C) and accelerated stability (50°C) in (D) skin, (E) lotion and (F) cream with/without light (Mean ± S.D., n=3)

CONCLUSION

Confirmation of the stability of the main active substance in finished products is essential in the area of cosmeceuticals and pharmaceuticals to ensure and suggest the period of effectiveness, expiration date and safety to consumers.

In this study, a simple, rapid, accurate and precise stability-indicating HPLC method was developed and validated for the analysis of diosgenin in skin, lotion and cream types of cosmetic formulations as model preparations. During the sample preparation step, diosgenin was extracted from all formulations without loss and with full recoveries, indicating suitable analysis. This method can be practically applied to other types of cosmeceutical formulations, as well as pharmaceutical preparations for other stability studies and routine quality control applications as the need for diosgenin analysis increases.

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