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Development and Validation of a Simple and Selective Chromatographic Method for Quantification of Ceramide-NP in Skin-Simulating Liposome Formulations

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Synopsis

Numerous topical products containing ceramide to restore the skin barrier function have been available on the market in recent years. A simple, rapid, and selective analytical method could contribute to the development of innovative formulations such as skin-simulating liposomes, which contain ceramides as stratum corneum components. The similarity of the physicochemical structure of ceramide and lipid excipients in liposome formulation complicates the separative and quantitative analysis. Therefore, this study aims to develop and validate a selective high-pressure liquid chromatography (HPLC) method for the simultaneous quantification of lipids in ceramide-containing skin simulating liposome formulations. The separation was performed with a reversed-phase C18 column (150 mm x 4.6 mm, 5 μm), the mobile phase system consisted of methanol: acetonitrile (60:40, v/v), the flow rate of 0.5 ml/min, the oven temperature at 45°C, injection volume of 10 μL, and UV detection at 210 nm. The method was linear ($r^2 > 0.99$) in the range of concentration from 80 μg/mL to 480 μg/mL with acceptable precision, accuracy, and selectivity for the liposome formulations. The limit of detection (LOD) values of ceramide NP (Cer-NP) was 7.90 μg/mL. The limit of quantification (LOQ) values of Cer-NP was 24.06 μg/mL. The study indicates that the method is efficient and has accuracy and selectivity for the simultaneous quantification of Cer-NP in skin-simulating liposome formulations.

INTRODUCTION

The formulations consisting of skin components such as ceramides, cholesterol, and other natural lipids have recently attracted attention as lipid replacement therapy in atopic dermatitis.[1] Numerous products that contain skin lipids such as ceramide, fatty acids, and cholesterol have been available on the market to restore the skin barrier.[2] The pharmaceutical and cosmeceutical industries have mostly preferred the production of liposome formulations for skin barrier recovery.[3] Hence, a reliable analytical method is still a requirement for the quantification of ceramides and lipid components in formulation development studies.

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Even though several analytical methods including ceramides have been described in the literature, each of them is only for lipid compounds obtained from complex materials.[4-11] The low solubility of ceramide subtypes in many solvents is an issue to quantify ceramides in the topical formulation. Therefore, the available methods have certain limitations to apply to topical formulation development studies. The quantification methods and evaluations in terms of formulation development studies are given in Table I.

The similarities in the structural identity of ceramides and phosphatidylcholines used in the compositions of liposomes lead to difficulty to determine these compounds selectively in their qualitative and quantitative analysis.[12] The chemical structure of phosphatidylcholine, Cer-NP, and cholesterol are given in Figure 1.

The fatty acid chain and polar head group are the characteristics of both ceramides and phosphatidylcholines. Additionally, separated analysis of skin lipid domain components, including ceramide, phospholipids, and cholesterol, is relatively time-consuming and high cost. To our knowledge, there are no reports of a selective analytical method that quantify the ceramide in a liposome formulation with lipid excipients.

Table I
HPLC-Based Qualitative and Quantitative Analytical Methods Including Ceramide Derivatives in Literature

Evaluation	Mobile phase	Stationary phase	Ceramide source	Reference
• Obtained from cell line • Poor solubility in methanol • Not suitable for topical formulation	MeOH/$NH_4CH_3CO_2$	C8	HepG2 cell line	[4]
• Obtained from cell line • Involving fluorescence ceramide derivatives • Not for the topical formulation	Methanol NH_4HCO_2/aq. NH_4HCO_2	C8	Ovary cancer cell line	[5]
• Necessity of derivatization process • Insolubility in water • Formulation is not liposome based	Water/MeOH	ODS AQ	Oat	[6]
• Specificity for protein-bound ceramide derivatives • Not for the topical formulation	MeOH/$NH_4CH_3CO_2$	BEH C18	Human s. corneum	[7]
• Poor solubility in methanol • Not for the topical formulation	MeOH	C18	Commercial	[8]
• *p-phenyl benzoyl* derivatization • Obtained from rat • Not for the topical formulation	Hexane/ethyl acetate	Si-10	Spargue-Dawley rat s. corneum	[9]
• Not for the topical formulation	Hexane/ethanol	Si-60	Human s. corneum	[10]
• Obtained from yeast • Not for the topical formulation	Hexane/ethanol	CN	*S. cerevisiae*	[11]

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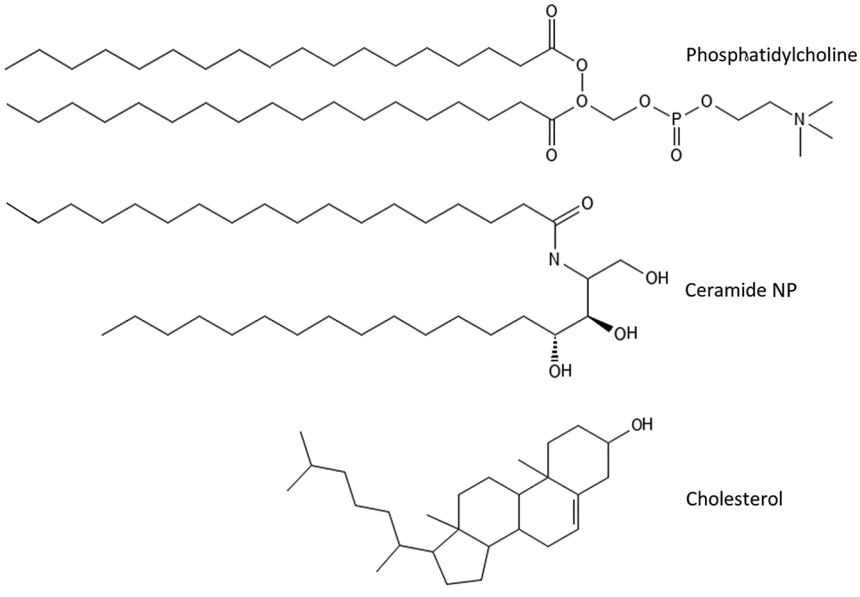


Figure 1. The chemical structure of phosphatidylcholine, Cer-NP, and cholesterol.

The skin-stimulating liposome formulation previously prepared by our group was used to prepare analysis samples in the current study. The development and validation of a simple, rapid, precise, cost-effective, and selective isocratic HPLC method was developed for the quantification of Cer-NP in the skin-simulating liposome formulation for the following formulation development studies.

MATERIALS AND METHODS

MATERIALS

Cer-NP and Phospholipon 90G (phosphatidylcholine) were kindly gifted by Evonik GmBH (Essen, Germany) and Lipoid GmBH (Ludwigshafen, Germany), respectively. Cholesterol was provided by Sigma-Aldrich (Missouri, USA). HPLC-Grade methanol and acetonitrile were purchased from Merck KGaA (Darmstadt, Germany). The total assays were performed using ultrapure Milli-Q water (Massachusetts, USA).

METHOD DEVELOPMENT

Preparation of the samples. The skin-simulating liposome formulation containing phosphatidylcholine Cer-NP, and cholesterol obtained in our previous work by the thin-film hydration method was used in the preparation of the lipid solution samples.[13] Then, the liposomes were verified via their particle size and polydispersity index (PDI) by Zetasizer Nano ZS (Malvern, U.K.). The particle size was with great size (about 720 nm) to increase accumulation in the upper skin layers.[14] The PDI was in narrow size distribution (0.187) to improve colloidal stability. The particle size and PDI results of the skin-simulating

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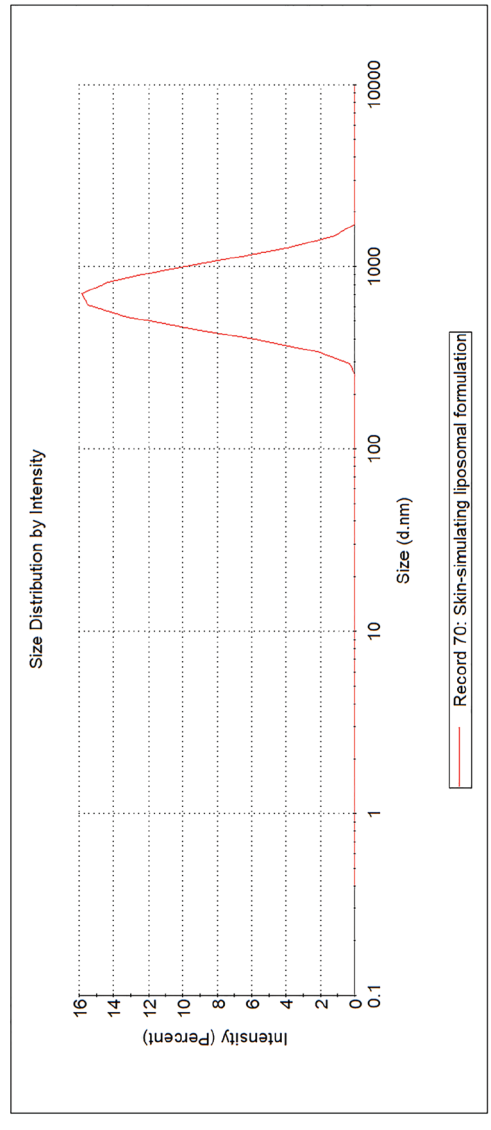


Figure 2. The particle size and PDI of the skin-simulating liposome formulation.

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liposome formulation were given in Figure 2. The liposomes were centrifuged for 30 minutes at 3,000 rpm and parted from the dispersion medium. Then, the liposome was dissolved in a mixture of methanol and tetrahydrofuran (1:1, v/v). Cer-NP was quantified with a suitable dilution of sample solutions by HPLC.

Preparation of standard solutions. The Cer-NP is poorly soluble in hydrophilic or aqueous solutions because of its high lipophilicity.[15] Therefore, the stock solutions of Cer-NP were prepared by dissolving the lipids in methanol/tetrahydrofuran (1:1) at 480 μg/mL. The standard solutions were performed by suitable dilution of stock solutions in methanol/tetrahydrofuran at various concentrations (80, 200, 320, 400, and 480 μg/mL).

ANALYTICAL EQUIPMENT AND CHROMATOGRAPHIC

The lipid solution sample was analyzed at an HPLC-equipped DAD-detector (LC-20AD Model, Shimadzu, Kyoto, Japan) in a range of 190 nm and 800 nm wavelength, to choose an appropriate wavelength for the next analysis. The reversed-phase C-18 (150 mm × 4.6 mm, 5 μm) column (*Macherey-Nagel*, Dueren, Germany) was used as a stationary phase. The mobile phase ingredients were chosen as methanol ($P' = 3.0$) and acetonitrile ($P' = 3.1$) based on their reversed phase polarity.[12] The different chromatographic conditions include various compositions of mobile phase (from 100% to 60% of methanol in acetonitrile), flow rate (0.5 mL/min, 0.8 mL/min, and 1.0 mL/min), and injection volume (10 μL, 50 μL, and 80 μL) were tested, proposing to obtain chromatographic peaks with the acceptable analytical performance of the lipids (Table II).

The system's suitability was tested by the tailing factor (T_f) and the number of theoretical plates (N). The oven temperature was maintained at 45°C, and detection was performed at 210 nm wavelength. The sample volumes of 10 μL were injected in each analysis. The analysis was carried out for 20 minutes.

VALIDATION OF METHOD

The developed method was validated considering parameters of specificity, linearity, accuracy, precision, the LOD, and the LOQ according to ICH Q2(R1).[16,17]

Specificity. The selectivity was investigated by injecting mobile phase (blank) solution, phosphatidylcholine, Cer-NP, cholesterol samples, and liposome formulation, separately. Then, the analysis was evaluated using a blank solution to identify the peak of Cer-NP and other lipid components. Additionally, the lipid components in the liposomes were analyzed as described in *Section Analytical Equipment and Chromatographic Conditions*. All samples were performed in three replicates. The results of peak area and retention time were statistically analyzed using a one-way analysis of variance (ANOVA) with a significance level of 0.05.

Linearity. The solutions consisting of Cer-NP were prepared at the range of liposome formulation concentration from 80 μg/mL to 480 μg/mL, performing at least three replicates. Then, the assays were analyzed to evaluate the linearity of Cer-NP. The data were fitted using linear least squares regression. The correlation coefficient significance and

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Table II
The Chromatographic Conditions of Trials; Mobile Phase System, Flow Rate and Injection Volume, and Results; the Retention Times, Tailing Factors, and the Number of Theoretical Plates of Cer-NP Chromatograms

	HPLC conditions				Retention time (min)			Tailing factor	Number of theoretical plates	Suitability
	Mobile phase system (MeOH:ACN)	Flow rate	Stationary phase	Injection volume	Phosphatidylcholine	Cer-NP	Cholesterol	Cer-NP	Cer-NP	Peak quality
Trial 1	10:0	1	C18	100	5.7	6.3	8.3	1.162	320.7	Peak depletion
Trial 2	9:1	1	C18	100	4.9	6.7	8.6	1.336	2569.6	Peak depletion
Trial 3	8:2	1	C18	100	6.7	7.3	9.1	1.169	5969.9	Overlapping
Trial 4	7:3	1	C18	100	7.1	8.2	9.9	2.693	1257.1	Overlapping
Trial 5	6:4	1	C18	100	7.3	8.9	10.4	1.383	4759.4	Unqualified
Trial 6	9:1	0.8	C18	80	6.6	ND	8.4	ND	ND	Peak depletion
Trial 7	9:1	0.8	C18	50	6.4	ND	8.2	ND	ND	Peak depletion
Trial 8	9:1	0.8	C18	10	6.4	ND	8.1	ND	ND	Peak depletion
Trial 9	9:1	0.5	C18	80	10.3	ND	13.2	ND	ND	Overlapping
Trial 10	9:1	0.5	C18	50	10.3	ND	13.1	ND	ND	Overlapping
Trial 11	9:1	0.5	C18	10	10.4	ND	13.2	ND	ND	Overlapping
Trial 12	6:4	0.8	C18	80	6.9	8.4	10.3	1.303	3594.0	Suitable
Trial 13	6:4	0.8	C18	50	6.6	8.1	9.9	ND	ND	Unqualified
Trial 14	6:4	0.8	C18	10	6.9	8.9	10.3	1.258	5983.8	Suitable
Trial 15	6:4	0.5	C18	80	10.5	13.1	15.9	ND	ND	Unqualified
Trial 16	6:4	0.5	C18	50	10.5	13.0	15.8	0.851	3792.8	Suitable
Trial 17	6:4	0.5	C18	10	10.7	13.5	15.8	1.194	5913.4	Suitable
Trial 17'	6:4	0.5	C8	10	11.6	ND	ND	1.067	ND	Peak depletion

Note: ND = Not determined.

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proportionality tests were evaluated based on the residual variance using the student t-test ($p = 0.05$). The residues were calculated based on the difference between theoretical and experimental values, which were estimated from the calibration curve.[18]

LOD and LOQ. The LOD and LOQ values of the method were calculated from the calibration curves according to the following equations based on the standart deviation of the response and slope:[17]

$$LOD = \frac{SD \times 3.3}{S}$$

$$LOQ = \frac{SD \times 10}{S}$$

SD is the mean standard deviation of y-axis interception values of calibration curves

S is the mean angular coefficient of calibration curves.

Precision. The precision of the method was evaluated for two levels: repeatability (intra-assay) and intermediate (inter-assay) precision. The intra-assay precision was performed with liposome analytes at 80%, 100%, and 120% (320, 400, 480 μg/mL) using three replicates within a day which qualified the ICH Q2(R1) specifications.[16] The inter-assay precision was performed on three different days at analyte concentrations of (320, 400, and 480 μg/mL) in three replicates using refresh samples prepared by the same analysis. The results of precision were calculated as the coefficient of variation (CV) in each level for each analyte concentration, using the following equation:

$$CV = (\textit{standard deviation of the peak areas for each analyte} / \textit{average of the peak areas for each analyte}) \times 100$$

Accuracy. The accuracy of the method was carried out at 80%, 100%, and 120% of liposome analytes (320, 400, 480 μg/mL) of Cer-NP based on ICH Q2(R1).[16] The liposome formulation including a 600 μg/mL concentration of analytes was prepared with the appropriate dilutions. The experiments were performed in three replicates. The results of accuracy were calculated based on the peak areas of Cer-NP in terms of recovery (R), as described in the following equation:

$$R = (\textit{measured concentration in the liposomes} / \textit{measured concentration in the solutions}) \times 100$$

RESULTS AND DISCUSSION

METHOD DEVELOPMENT AND OPTIMIZATION OF CHROMATOGRAPHIC CONDITIONS

Three compounds (phosphatidylcholine, Cer-NP, and cholesterol) exhibited the maximum absorption at 210 nm; hence the wavelength was standardized in all studies. The main

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variations in the chromatographic conditions and lipids' representative chromatogram of the developed HPLC method are presented in Table II and Figure 3, respectively.

During the study, the major problem was peak overlapping, which is derived from the physicochemical similarity of the phosphatidylcholine and Cer-NP.[19] Namely, cholesterol has saturated and unsaturated cyclic hydrocarbons. However, phosphatidylcholine and Cer-NP similarly have a long fatty acid chain and polar head group, which state the movement through the column.[20] Furthermore, the steric hindrance derived from branching and cycling indicates partitioning to the pore of column material; hence, cholesterol might have distinctness because of its cyclic hydrocarbons.[12] Additionally, the functional groups on the backbone affect the affinity of the analyte with the stationary phase.[12] Herein, the polarity of the head group on phosphatidylcholine could arise from its phosphate ions while Cer-NP's polarity was based on its hydroxyl groups.

On the other hand, the solubility of phosphatidylcholine, Cer-NP, and cholesterol have differences based on their lipophilicities. Cer-NP has solubility problems in various solvents and aqueous media.[21] Even though cholesterol has a significantly lower lipophilic character (log P of 8.7) rather than the other compounds (phosphatidylcholine: 12.9, Cer-NP: 12.4), the chemical structure specified the retention time. Considering the chemical structure similarity of phosphatidylcholine and Cer-NP, their retention times are expected to be very similar. Hence, various compositions of mobile phase, flow rates, and injection volumes were tested to prevent the overlapping of phosphatidylcholine, Cer-NP, and cholesterol peaks, which could improve the retention times of the peaks of these compounds. The retention times of lipid materials were studied in different conditions. In addition, tailing factors (T_f) and the number of theoretical plates (N) of the Cer-NP peak were presented in Table II. To improve peak resolution, the serial mobile phase composition (trials 1–5) was tested with the mobile phase consisting of the mixture of methanol: acetonitrile from 60:40 (v/v) to 100:00 (v/v). These subsequent modifications in the mobile phase composition with a reduction of its polarity caused an increase in lipid retention times. Trial 5 resulted in low resolution (2.32) with the phosphatidylcholine peak still in conflict with the Cer-NP peak. Thereby, the flow rate was fixed to be 0.8 mL/min in trials 12–14, and, then 0.5 mL/min in trials 15–17. Also, the injection volume was decreased from 100 μL to 10 μL, to get sharper peaks (trials: 12–17). The suitable method was repeated with a C8 column with

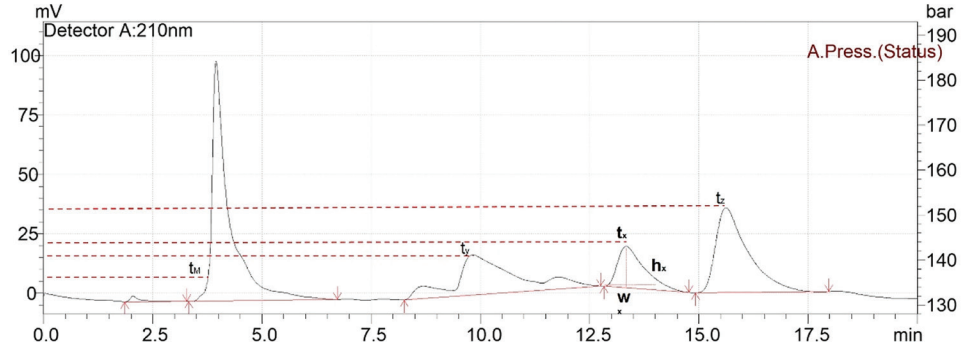


Figure 3. Representative HPLC chromatogram of the lipid components in skin-simulating liposome formulation.

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the same column dimension and pore diameter to show the stationary phase effect. The phosphatidylcholine showed an approximate retention time of 11.648, when Cer-NP and cholesterol were detected at 4.58 and 5.18, individually.

Based on the tailing factor and the number of theoretical plate parameters, the method in which the mixture of methanol: acetonitrile (60:40, v/v) as mobile phase with 0.5 mL/min flow rate and 10 μL of injection volume showed adequate peak separation for the lipid components. In this final method shown in Figure 3, the retention times of phosphatidylcholine, Cer-NP, and cholesterol were 10.7 minutes, 13.5 minutes, and 16.0 minutes, respectively.

The system suitability parameters are determined based on USP guidelines. The resolution of phosphatidylcholine and Cer-NP was 4.14, which had been a problem in the other trials. The tailing factor of Cer-NP was less than 1.5 as defined by USP.[22,23] The number of theoretical plates was 5913.479 in agreement with the limit of the FDA's established parameter of $N > 2.000$.[24] The acceptance criteria of system suitability on USP and Trial 17 values are listed in Table III.

VALIDATION OF METHOD

Specificity. The specificity is a defining parameter that has been the ability of the method to quantify the analyte of interest in the presence of interferences.[16] Herein, it was proposed to determine whether a contamination peak derived from the manufacturing process formed in the Cer-NP retention times during the preparation of liposome formulation. In the set condition analysis, no contamination peak was detected in the Cer-NP retention times. Moreover, the peak area and retention time of Cer-NP from the liposome formulation showed no significant difference in comparison with the standard solution of Cer-NP, confirming the method selectivity ($p \leq 0.05$). The specificity chromatogram was shown in Figure 4.

Linearity. The stock solution was prepared at a lipid concentration of skin-simulating liposome formulation. The linearity of the method was evaluated at five concentration points by diluting the standard stock solution to get solutions over the range of 80 μg/mL and 480 μg/mL for each of all lipids according to ICH Q2 (R1).[16] The results were plotted graph concentration versus an area to evaluate the correlation coefficient, which has shown a high

Table III
The Acceptance Criteria of System Suitability and Method Values

Acceptance criteria		Method values	Equation	Suitability
Number of theoretical plate	>2000	5913.479	$N = 16\,(t_x/w_x)$	Suitable
Selectivity factor	>1	1.42 (Phosphatidylcholine and Cer-NP) 1.21 (Cer-NP and Cholesterol)	$\alpha = (t_y - t_m)/(t_x - t_m)$	Suitable
Resolution	>1.5	4.14 (Phosphatidylcholine and Cer-NP)	$R = (t_y - t_x)/0.5(w_y + w_x)$	Suitable
Tailing factor	<2	1.19	$T_f = w_{0.05}/2f$	Suitable
Capacity factor	0.5 – 20	2,64	$K = (t_x - t_m)/t_m$	Suitable
RSD	<2	1,13	= SD × 100/mean	Suitable

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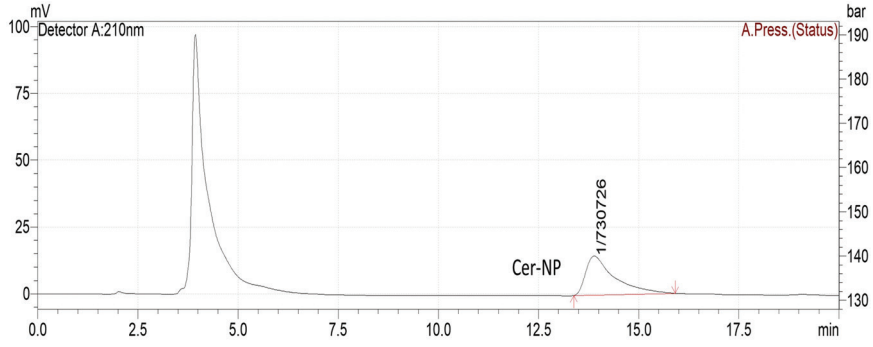


Figure 4. Specificity chromatogram of Cer-NP.

probability of correlation between peak area and concentration. The correlation coefficient of the calibration curve was more than 0.99, which met the validation requirements for this parameter.[16] The linear regression of calibration curves produced the equations presented in Figure 5, where y was the peak area and x was the analyte concentration in μg/mL.

The slope of the calibration curve was different from zero according to student's t-test as recommended, and its high value (1691.8) indicated an appropriate response of the method against changes in the concentrations. The calculation of residues resulted in random data distribution with no tendency. The proportional test calculated following student's t-test showed that the independent terms (−16933) could be attributed to the acceptable systematic error of the method. Overall, based on these results HPLC method is indicated to be linear and to provide all requirements of international protocols for pharmaceutical analytical methods.

LOD and LOQ. The LOD value of Cer-NP was 7.90 μg/mL. The LOQ value was 24.06 μg/mL. These values were sufficient to quantify Cer-NP in the skin-simulating liposome formulation assays such as encapsulation efficiency, in vitro performance tests, etc.

Precision. The precision of the method was facilitated to assess variability because of random errors which cannot be controlled as those related to reagents glassware and sample preparation. The precision results (intra- and inter-day) are presented in Table IV. Cer-NP exhibited acceptable results (from 0.99% to 4.41%) in accordance with the literature[25,26] for intra- and inter-day analysis.

Accuracy. The accuracy of an analytical procedure expresses the closeness of agreement between the value that is accepted either as a conventional true value or an accepted reference value and the value found.[16] The mean recovery could be within 90% to 110% of the theoretical value for non-regulated products.[27] The accuracy results of the three analytes ranged from 97.03% to 110.21% (Table IV). Herein, it indicated that there was a proximity between experimental and theoretical concentration values of the analytes.

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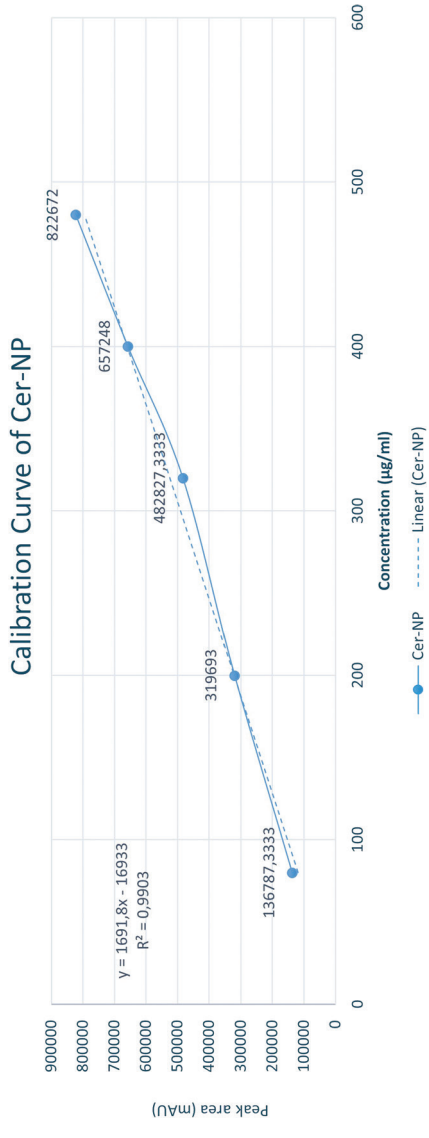


Figure 5. The calibration curves of Cer-NP in the range of 80 µg/mL and 480 µg/mL. ($n = 3$).

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Table IV
The Results of Accuracy, Intra-, and Inter-Day Analysis

	Intraday (CV, %)			Interday (CV, %)			Accuracy (%)		
Analyte	Level 1	Level 2	Level 3	Level 1	Level 2	Level 3	Level 1	Level 2	Level 3
Cer-NP	1.03	0.99	1.66	3.01	1.94	4.41	110.21±1.13	107.83±1.07	97.03±1.61

CONCLUSION

Ceramides are used in the composition of cosmetic or dermatological products to restore the skin barrier. However, the quantification methods of ceramides in liposome formulations including phosphatidylcholines and cholesterol have been relatively time-consuming because the hydrophobicity of ceramides, depending on fatty acyl chain length, can cause turbidity in various alcoholic solvents. In addition, the similarity of the physicochemical structure of ceramides and lipid excipients used in the composition of liposome formulations complicates their separation and quantification in the analysis. Therefore, a simple analytical method to selectively determine Cer-NP in liposome formulations is still required. In this work, a simple, cost-effective, and selective chromatographic method was developed to quantify simultaneously Cer-NP in the skin-stimulating liposomes, which has been previously formulated as a promising carrier for recovering the skin barrier previously. Then, the method was validated in terms of its specificity, linearity, accuracy, precision, LOD, and LOQ. The results showed that the HPLC method developed could be useful to analyze simultaneously Cer-NP in the liposome formulations.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this paper.

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