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Article

Development and validation of a reversed-phase HPLC method for quantification of 1'-acetoxychavicol acetate content in a nanostructured lipid carrier formulation

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1'-acetoxychavicol acetate (ACA)-loaded nanostructured lipid carriers (NLCs) were formulated for prostate cancer therapy and to determine the optimal therapeutic dose, we developed a rapid, specific, and accurate reversed-phase high-performance liquid chromatography (RP-HPLC) method to quantify the ACA content in NLCs. The method was validated according to International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines. Chromatographic separation of ACA from the lipid components was performed with an Agilent 1220 Infinity LC system and ultraviolet detector using an Agilent Poroshell C18 column (4.6 x 250.0 mm). The mobile phase consisted of acetonitrile and water (80:20 [v/v]) with a flow rate of 0.8 mL/min in isocratic mode. Linearity of the standard curve was assessed at an ACA concentration range of 5-200 µg/mL, and a 1/x weighted linear regression was adopted for the calibration curve. The calculated limits of detection and quantification were 0.59 µg/mL and 1.79 µg/mL, respectively. The mean percent recovery of ACA was 100.02% (relative SD, 2%), and the coefficients of variation for intraday and interday assays were within the values required by the ICH. We also demonstrated robustness of the method by altering the mobile phase ratio and flow rate. Furthermore, we proved specificity of the method for ACA by comparing chromatograms of the blank NLC and ACA-NLC. Hence, we effectively used this validated method to determine the drug-loading capacity and entrapment efficiency of the NLCs.

Keywords: High-performance liquid chromatography. Validation. 1'-acetoxychavicol acetate. Nanostructured lipid carrier. Chemotherapy.

Note: Dr. Nagoor passed away in April 2021, after this manuscript was accepted.

INTRODUCTION

1'-acetoxychavicol acetate (ACA) (Figure 1) is a phytoconstituent isolated from the rhizomes of the Southeast Asian ethnomedicinal plants *Alpinia conchigera* Griff. and *Alpinia galanga* (L.) Willd. (Zingiberaceae) (Janssen, Scheffer, 1985; Barik, Kundu, Dey, 1987; Kondo *et al.*, 1993; Yang, Eilerman, 1999). ACA induces apoptosis-mediated cell death in many cancer cell lines with minimal toxicity in normal cells (In *et al.*, 2012). Studies demonstrated that ACA prevents Ehrlich ascites carcinoma, skin tumor, and adenocarcinoma formation (Tanaka, Kawabata *et al.*, 1997; Tanaka, *et al.*, 1997; Nakamura *et al.*, 1998; Narukawa *et al.*, 2010). Moreover, ACA induces apoptosis of myeloid leukemic cells via mitochondrial and Fas-mediated mechanisms (Ito *et al.*, 2004). *In vitro* studies also showed that ACA induces dose- and time-dependent cytotoxicity in tumor cells, potentially induces cell-cycle arrest at G_0/G_1 phase and suppresses the proliferation and migration rates for oral squamous cell carcinoma (Awang *et al.*, 2010). In short,

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ACA's potential as an effective antitumor agent renders it useful for testing *in vivo*.

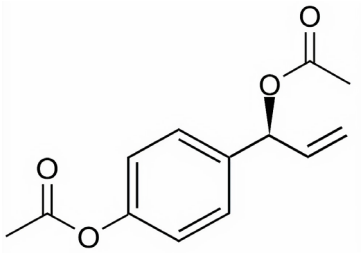

FIGURE 1 - Chemical structure of ACA.

However, in animal studies of ACA in its free form, investigators have encountered several drawbacks, such as poor *in vivo* solubility, resulting in a decline in biological activity. ACA is a hydrophobic compound, which makes delivering it to cancer cells within an aqueous environment challenging. In addition, passive targeting of tumor cells causes inefficient use of free ACA (Arshad *et al.*, 2015). To address these problems, researchers have loaded ACA into nanostructured lipid carriers (NLCs) for delivery to cancer cells. Previously, the cosmetics industry extensively employed NLCs for drug delivery (Müller, Radtke, Wissing, 2002; Beloqui *et al.*, 2016). In the present study, we used a modified NLC to encapsulate ACA for targeted parenteral delivery to cancer cells. Then, we selected the optimized nanoparticle based on its favorable properties of small size, protection against drug degradation, high drug-loading capacity and entrapment efficiency, enhanced dispersion, sustained drug release, and prolonged stability (Pathak, 2009).

To determine the drug-loading capacity and entrapment efficiency of the nanoparticles, a validated methodology for quantitative determination of the drug content is essential. Researchers have previously quantified ACA as a free drug using high-performance liquid chromatography (HPLC) under different chromatographic conditions (Batra *et al.*, 2012; Haque *et al.*, 2017). In addition, some authors have reported on HPLC methods for isolation and purification of ACA from *A. galanga* extracts without specific ACA quantification techniques (Kaur *et al.*, 2010; Baradwaj, Rao, Kumar, 2017). However, quantification of ACA in nanoparticle formulations has neither been validated nor reported. An ACA-loaded NLC is a novel formulation; thus, accurate quantification of ACA in NLCs requires a reliable, validated analytical method. The drug content in nanoparticle-based formulations is commonly determined using ultraviolet (UV) spectrophotometry or HPLC, but HPLC is preferred for its greater sensitivity. Also, interference of contaminants during determination of drug content can be prevented with the component separation technique employed in HPLC. Therefore, we undertook this study to develop and validate a reversed-phase HPLC method to quantify ACA and determine the drug-loading capacity and entrapment efficiency of the NLCs.

MATERIAL AND METHODS

Chemicals and reagents

D, L-1'-acetoxychavicol acetate (ACA) (98.8%) was purchased from LKT Laboratories Inc. (St. Paul, MN, USA). Cocoa butter (White Naturals, Cape Coral, FL, USA), isopropyl myristate (Thermo Fisher Scientific, Waltham, MA, USA), Span 40 (Sigma-Aldrich, St. Louis, MO, USA), and Tween 80 (Merck & Co., Inc., Kenilworth, NJ, USA) were also procured. HPLC-grade water and acetonitrile were purchased from Merck & Co., Inc. All other chemicals and solvents obtained commercially were of analytical or HPLC grade. Before instrumentation, the mobile phase solvents were filtered through a 0.22-µm Millipore membrane filter (Merck & Co., Inc.) and degassed using a vacuum pump.

Instrument

An Agilent 1220 Infinity LC system (Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with column and sample compartment with temperature control, UV-visible diode-array detector, binary pump, autosampler, and variable wavelength detector was used in the study. All analyses were conducted using an Agilent Poroshell C18 column with a 4-µm particle size, 4.6-mm internal diameter, and 250-mm length. The column was equilibrated for 1 h before the analysis. OpenLab CDS

ChemStation Edition (Agilent Technologies, Inc.) was used for data acquisition, analysis, and reporting.

Chromatographic conditions

The chromatographic conditions were standardized by evaluating the peak symmetry of ACA under different mobile phase conditions, flow rates, and UV wavelengths. Methanol or acetonitrile was used as the organic solvent at 10:90, 20:80, and 30:70 (v/v) water:organic solvent proportions. The flow rate was tested at 0.8-1.0 mL/min. In addition, UV wavelengths of 216, 226, and 254 nm were investigated. After testing all parameters and analyzing the ACA peak symmetry, the chromatographic conditions were standardized as follows: the mobile phase consisted of A-water and B-acetonitrile at a proportion of 20:80 (v/v) in isocratic mode, the flow rate was set at 0.8 mL/min, the column temperature was set at 25 °C, the sample injection volume was 10 µL, and the UV wavelength was set at 216 nm. The method run time was 5 min. To quantify ACA concentration, the peak area of UV absorbance was recorded.

Preparation of samples and standard solutions

ACA-NLCs were prepared in triplicate via melt and a high-shear homogenization method (Severino, Santana, Souto, 2012). The lipid phase containing cocoa butter, isopropyl myristate, and ACA was heated to 45 °C before being dispersed together with the aqueous phase containing deionized water, Tween 80, and Span 40 heated to the same temperature. The mixture was then homogenized using a high-shear homogenizer (Heidolph SilentCrusher, Sigma-Aldrich, St. Louis, MO, USA) for 20 min. The resulting sample was immediately kept in a 4 °C refrigerator overnight to allow for the formation of drug-loaded nanoparticles. The samples used to evaluate drug load and entrapment efficiency were obtained as described further in the "Method applicability" section below. As for the standards for calibration curve, ACA stock solution (1 mg/mL) was dissolved in acetonitrile to provide concentrations ranging from 5 to 200 µg/mL. All samples and standard solutions were filtered through Whatman 0.2-µm-pore nylon membrane syringe filters (Whatman, Maidstone, UK) before analysis.

System suitability and method validation

Six replicates of standard ACA solutions (100 µg/mL) were analyzed to determine the system suitability. Factors such as the number of theoretical plates (N) and capacity factor (k') were examined. International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines were followed to validate this analytical method (ICH, 2005) with the following essential parameters: specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy, and robustness.

Specificity

Specificity of the method is the capacity to identify the analyte of interest unequivocally, even in the presence of foreign compounds. This was ensured in the study by comparing chromatograms of blank NLCs and ACA-NLCs, using standard ACA solution as the reference. One milliliter each of the blank NLC and ACA-NLC suspension was subjected to centrifugation (3500 rpm, 1 h, 25 °C; KUBOTA 2800, Tokyo, Japan) in a Vivaspin 6 concentrator (10000 Da MWCO; Sartorius, Göttingen, Germany). The obtained precipitate was resuspended in 1 mL of acetonitrile, diluted 1:10 in acetonitrile, vortexed for 1 min, and filtered through 0.2-µm-pore nylon membrane syringe filters before analysis. The HPLC chromatograms were assessed for interfering peaks at the same retention time as the ACA standard solution. Ideally, the ACA-NLC but not the blank NLC should show a peak at the retention time corresponding to ACA.

Linearity

Linearity of the calibration curve was assessed using linear regression. Eight standard solutions of ACA at varying concentrations (5, 10, 20, 40, 60, 80, 100, and 200 µg/mL; n = 9) were prepared from the stock solution (1 mg/mL) via dilution with acetonitrile. Samples were then injected at a volume of 10 µL into

the HPLC system. The peak area at each concentration was recorded and used for linear regression analysis of the slope from the plot of the mean peak area versus the analyte concentration. Because the correlation coefficient (r^2) from the association between the x and y variables is insufficient to accept the linear regression model, a lack-of-fit test was conducted, and weighted linear regression with 1/x and $1/x^2$ weighting factors was used for the calibration curve (Sonawane *et al.*, 2019). Data analysis was conducted using Excel 2016 software (Microsoft Corporation, Redmond, WA, USA).

The weighting factor was selected by calculating the percent relative error (%RE) using equation 1, where C_{FOUND} is the back-calculated concentration and C_{NOM} is the nominal concentration that represents the standard.

$$\%RE = [(C_{FOUND} - C_{NOM})/C_{NOM}] \times 100 \quad (1)$$

For the F-test, the experimental F-value (F_{EXP}) was determined using equation 2, where MSLF represents the lack-of-fit mean square and MSPE represents the pure error mean square from analysis of variance. The F_{EXP} values were then compared with the tabulated F-value (F_{TAB}) ($F_{6, 64}$) from the F-statistic distribution table at a 95% CI using *c* - 2 numerator and denominator degrees of freedom, where *c* represents 8 levels of concentrations and *n* represents 72 total data points.

$$F_{EXP} = MSLF/MSPE \quad (2)$$

The weighting factor with the lowest total %RE and $F_{EXP} < F_{TAB}$ was selected for the standard calibration curve.

LOD and LOQ

The LOD is the smallest amount of analyte in a sample that can be detected but not required to be quantified, whereas the LOQ is the lowest amount of analyte that can be quantified with predefined accuracy and precision. The LOD and LOQ of the method were calculated based on the slope (S) of the calibration curve and least SD obtained from the response according to equations 3 and 4. This was done in accordance with ICH guidelines.

$$LOD = 3.3 \times SD/S \quad (3)$$

$$LOQ = 10 \times SD/S \quad (4)$$

Precision

Precision is the degree of agreement among multiple measurements of the same homogenous samples under standardized conditions. This can be done by evaluating the repeatability and intermediate precision of the method. Repeatability (intraday precision) was analyzed using standard ACA solutions of low (5 µg/mL), medium (40 µg/mL), and high (100 µg/mL) concentrations on the same day in triplicate. Intermediate precision (interday precision) was similarly investigated on three different days by quantifying the three concentration levels of standard ACA solutions in triplicate. The results were recorded as percent recovery and relative SD (RSD).

Accuracy

The accuracy of an analytical method is the closeness of agreement between the theoretical value and the experimental value. In the present study, the accuracy of the method was tested by spiking blank NLC suspensions with standard ACA solutions at concentrations of 5, 40, and 100 µg/mL. Samples were then mixed thoroughly, diluted 1:10 in acetonitrile, and analyzed. Analyses were performed in triplicate. The results were recorded as percent recovery and RSD.

Robustness

Robustness refers to the ability of an analytical method to remain unchanged by slight alterations in chromatographic parameters. This ensures reliability during normal applications. Robustness of the method was assessed by making minimal changes in experimental conditions, such as the mobile phase proportion (acetonitrile:water ratio, 81:19 and 79:21 [v/v]) and flow rate (0.75 mL/min and 0.85 mL/min). The percent recovery and RSD of standard ACA solutions at 5, 40, and 100 µg/mL were evaluated in triplicate.

Method applicability: drug-loading capacity and entrapment efficiency

ACA-NLCs with various ACA concentrations (0.1-1.0 mg/mL) were formulated in triplicate as described above. After overnight storage at 4 °C, 1 mL of an ACA-NLC suspension was subjected to centrifugation (3500 rpm, 1 h, 25 °C; KUBOTA 2800) in a Vivaspin 6 concentrator. The obtained precipitate was resuspended in 1 mL of acetonitrile, diluted 1:10 in acetonitrile, vortexed for 1 min, filtered through a 0.2-µm-pore filter, and run in the HPLC system to quantify the ACA content. The drug-loading capacity and entrapment efficiency of ACA-NLCs were then measured using equations 5 and 6, respectively, where M_D refers to the mass of the drug in the nanoparticles, M_L refers to the mass of the total lipid, C_P refers to the concentration of the drug in the precipitate, and C_I refers to the initial concentration of the drug added during the formulation of ACA-NLC.

$$DL\ (\%) = M_D/M_L \times 100 \quad (5)$$

$$EE\ (\%) = C_P/C_I \times 100 \quad (6)$$

RESULTS AND DISCUSSION

Method development

Preliminary runs consisted of optimizing the mobile phase composition in isocratic mode at different flow rates. In cases with erratic shaping and tailing of the ACA peak, we rejected the runs. The mixture of acetonitrile and water at the ratio of 80:20 (v/v) with a flow rate of 0.8 mL/min provided the most symmetrical ACA peak. We sought to determine the optimal wavelength for absorbance of ACA among wavelengths of 216, 226, and 254 nm based on previous studies (Niyomkam *et al.*, 2010; Haque *et al.*, 2017) and the wavelengths recommended by the ACA supplier. The highest absorbance was at 216 nm with a peak retention time of 4.06 min (Figure 2). The number of theoretical plates (N= 49,672) and capacity factor (k' = 2.16) were in accordance with the specified limits ($N>2000$ and $2<k'<10$).

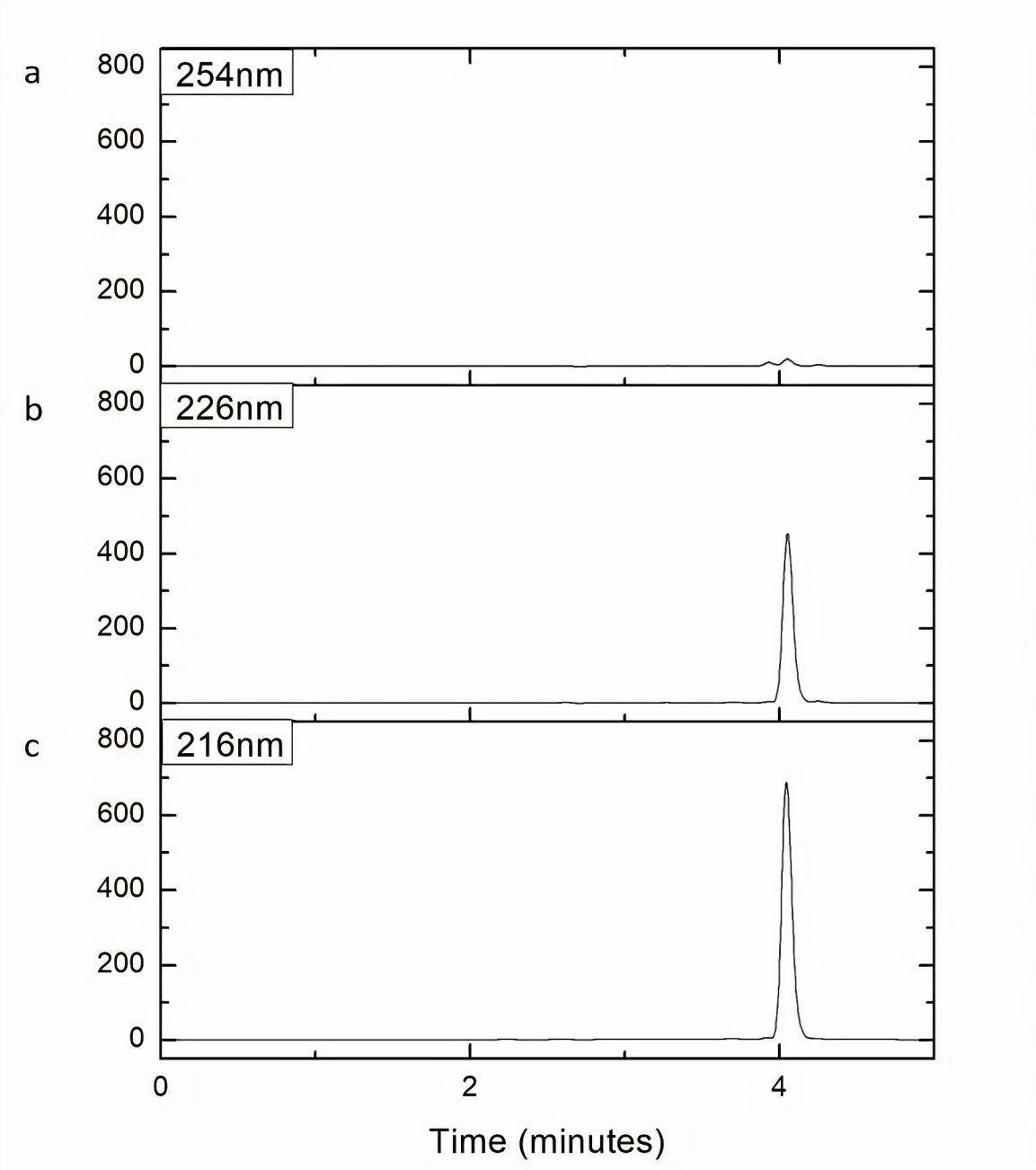

FIGURE 2 - HPLC chromatogram of 100 µg/mL standard ACA solution at UV detection wavelengths of a) 254 nm, b) 226 nm and c) 216 nm. Conditions: mobile phase acetonitrile:water ratio of 80:20 (v/v), flow rate of 0.8 mL/min, column temperature of 25 °C, sample temperature of 25 °C, and injection volume of 10 µL.

Method validation

Specificity

The specificity of the method was assessed by running blank NLCs and ACA-NLCs. ACA-NLCs had the characteristic ACA peak at 4.06 min, whereas the blank NLCs did not have a peak at the same retention time (Figure 3). This shows that the method is specific for ACA without the interference of other constituents from the nanoparticles. This is consistent with previous drug formulation studies that validated the specificity of their

analytical methods. In all those tests, other components from the nanoparticle formulation did not interfere at the retention time for the analyte of interest (Lopes *et al.*, 2017; Savadkouhi *et al.*, 2017).

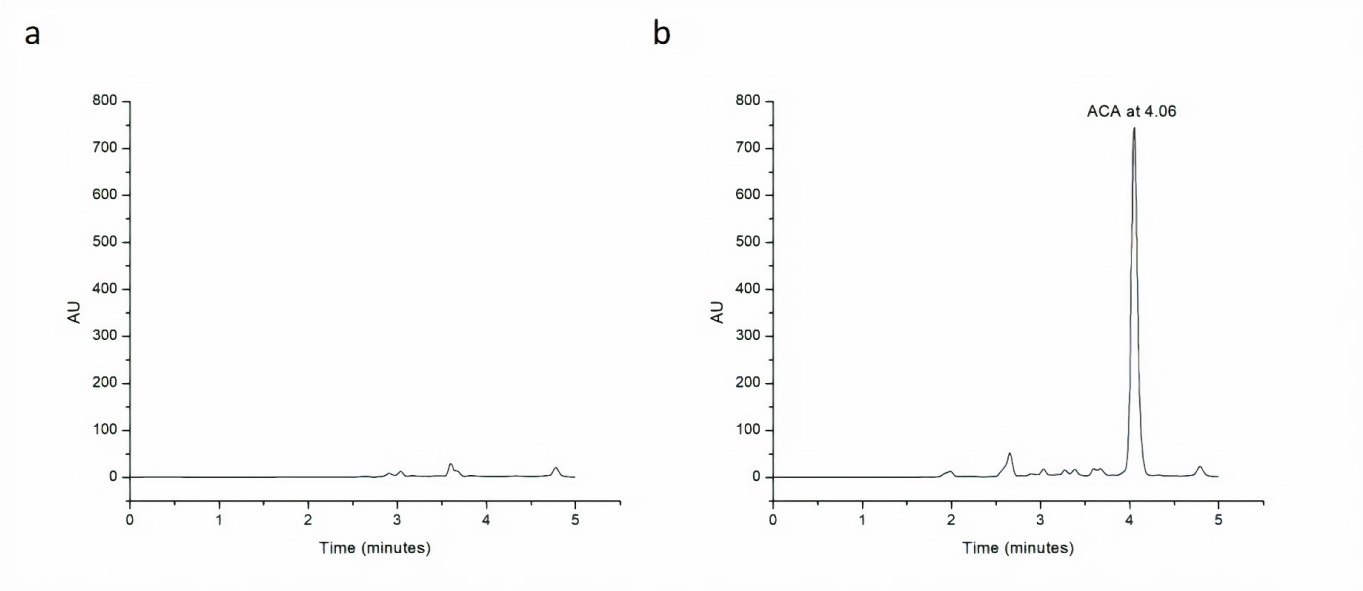

FIGURE 3 - HPLC chromatograms of a) precipitate from blank NLCs and b) precipitate from ACA-NLCs. Conditions: mobile phase acetonitrile:water ratio of 80:20 (v/v), flow rate of 0.8 mL/min, column temperature of 25 °C, sample temperature of 25 °C, and injection volume of 10 µL.

Linearity

We injected standard ACA solutions at eight concentrations ranging from 5 to 200 µg/mL into the HPLC system on 3 random days. The regression equation obtained for the unweighted calibration curve was y = 25.975x + 14.818, and the resulting correlation coefficient (r^2) was 0.9995 (Figure 4). However, the total %RE calculated was 132.4%, and the F_{EXP} for the linearity test was 120.7, which was significantly higher than the F_{TAB} of 2.24 (Table I). This suggests a nonequal variance distribution of the standards range and indicates heteroscedasticity of the data. Hence, inaccurate results of subsequent analyses are possible if unweighted regression is used even though the r^2 value is almost 1 (Boulanger *et al.*, 2003).

Alternatively, we applied the 1/x and $1/x^2$ weighted regressions using the same data set of the ACA standard solutions to select the model with the lowest %RE and F_{EXP}. The results are shown in Table I. The %RE for each weighted regression was lower than that for the unweighted model, but only the 1/x weighting factor exhibited $F_{EXP} < F_{TAB}$. Because the 1/x weighted calibration curve was more suited to homogenizing the variance of the residuals, as demonstrated in a previous study quantifying a different analyte (Sonawane *et al.*, 2019), we selected it for use in subsequent analyses. Therefore, we constructed the calibration curve with ACA standards at the concentration range of 5 to 200 µg/mL using a 1/x weighting factor.

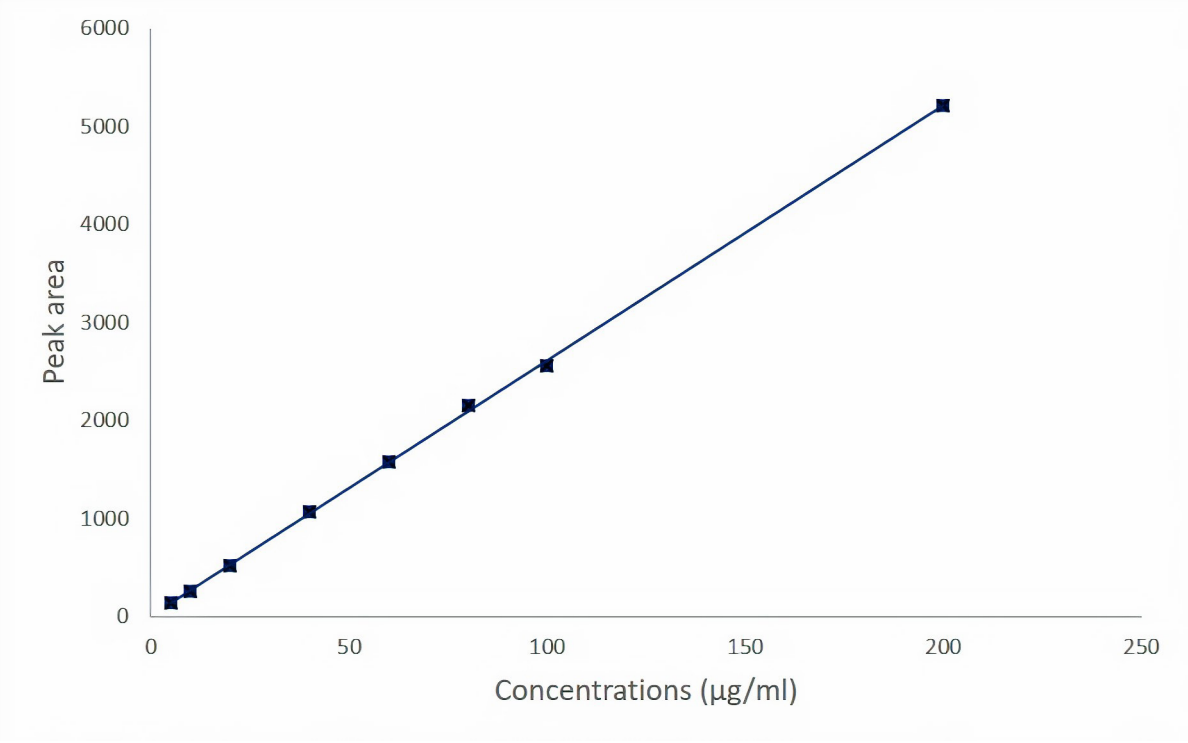

FIGURE 4 - Mean calibration curve of standard ACA solutions at concentrations ranging from 5 to 200 µg/mL (n=3)

TABLE I - Parameters for weighted and unweighted regression models for linearity

Weighting factor	Slope	Intercept	r^2	%RE	F_{EXP}
1	25.975	14.8180	0.9995	132.40	120.70
1/x	26.058	9.4976	0.9995	85.12	0.40
$1/x^2$	26.284	5.7457	0.9981	83.01	2.62

LOD and LOQ

We determined LOD and LOQ for the method based on the slope of the calibration curve and least SD obtained from the response. This was necessary to ensure that the LOD and LOQ can be reliably detected or quantified to prevent erroneous results when applying the method for future quantification (Armbruster, Pry, 2008). Using equations 3 and 4 in "Material and Methods", the LOD was 0.59 µg/mL, and the LOQ was 1.79 µg/mL. To confirm this, we subjected standard solutions of ACA at these concentrations to HPLC analysis. As a result, we detected 0.6 µg/mL ACA and could quantify 1.8 µg/mL ACA with a percent recovery of 100.1% and RSD of 1.85%. Similar to a previous study quantifying halcinonide in lipid nanoparticles, (Lopes *et al.*, 2017), the LOD and LOQ were much smaller than the lowest standard ACA concentration used (5 µg/mL). This showed that the HPLC method is satisfactory for detecting and quantifying ACA within the concentration range of 5 to 200 µg/mL.

Precision

Precision is used to evaluate the degree of agreement between different test results when the

method is used repeatedly with multiple samplings. We assessed intraday (repeatability) and interday (intermediate precision) runs of 5, 40, and 100 µg/mL (n = 3) standard ACA solutions for precision analysis (Tables II and III). The maximum RSD values were 1.91% for the repeatability test and 1.81% for the intermediate precision test. In addition, the percent recovery of ACA for all concentrations ranged from 98% to 104%, indicating that the relative error of the method was low even with repeated analysis. This agrees with previous studies that validated their method precision for quantification of different analyte concentrations according to ICH guidelines (Pecchio *et al.*, 2014; Martins, Mainardes, 2017).

TABLE II – Repeatability for different concentrations of standard ACA solutions

Theoretical concentration (µg/mL)	Mean (± SD*) experimental concentration (µg/mL)	Mean (± SD*) recovery (%)	RSD (%)
5	5.214 ± 0.100	104.28 ± 1.99	1.91
40	40.240 ± 0.120	100.62 ± 0.31	0.31
100	98.430 ± 0.060	98.43 ± 0.06	0.06

*n = 3.

TABLE III - Intermediate precision for different concentrations of standard ACA solutions

Theoretical concentration (µg/mL)	Mean (± SD*) experimental concentration (µg/mL)	Mean (± SD*) recovery (%)	RSD (%)
5	5.042 ± 0.090	100.80 ± 1.83	1.81
40	40.430 ± 0.360	101.10 ± 0.90	0.89
100	98.020 ± 0.270	98.02 ± 0.27	0.28

*n = 3.

Accuracy

Accuracy is defined as the proximity of the results of an experiment to the true value. In this study, we tested the accuracy of the method by spiking blank NLC suspensions with 5, 40, and 100 µg/mL standard ACA solution. The percent recovery for all concentrations ranged from 97% to 101%, and the maximum RSD value was 0.32% (Table IV). The mean percent recovery for all concentrations was 100.02% (RSD, 2%). Hence, the HPLC method was ascertained to have low variability between the theoretical and experimental values of standard ACA solution concentrations at different levels. This is in accordance with the ICH guidelines for accuracy of an analytical method and agrees with the results of previous analytical method validation studies (ICH, 2005; Lopes *et al.*, 2017).

TABLE IV - Accuracy measurement at different concentrations of standard ACA solutions

Theoretical concentration (μg/mL)	Mean (± SD*) experimental concentration (μg/mL)	Mean (± SD*) recovery (%)	RSD (%)
5	5.076 ± 0.010	101.50 ± 0.24	0.24
40	40.340 ± 0.130	100.90 ± 0.32	0.32
100	97.730 ± 0.190	97.73 ± 0.19	0.19

*n = 3.

Robustness

The robustness of an analytical method is its capacity to be insignificantly affected by deliberate variations in chromatographic conditions. In this study, we evaluated the robustness of the method by making small changes to the mobile phase (acetonitrile:water) proportion and flow rate. The maximum RSD was 1.98% when we changed the mobile phase proportion to 79:21 for a 5 μg/mL sample of ACA standard solution. The percent recovery for all concentrations under different conditions ranged from 95% to 107%. None of the changes in the chromatographic conditions affected the RSD significantly (<2%) (Table V), which was consistent with findings of a previous study (Lopes *et al.*, 2017). This demonstrated that the HPLC method is robust and reliable for use in future quantification of ACA content (Fontana, Bastos, Beck, 2010).

TABLE V - Robustness results according to changes in mobile phase and flow rate

Change in original method	Standard ACA solution					
	5 μg/mL		40 μg/mL		100 μg/mL	
	Mean (± SD*) recovery (%)	RSD (%)	Mean (± SD*) recovery (%)	RSD (%)	Mean (± SD*) recovery (%)	RSD (%)
Mobile phase (ACN:H_20, 81:19)	99.58 ± 1.81	1.82	100.90 ± 0.28	0.28	96.93 ± 0.11	0.11
Mobile phase (ACN:H_20, 79:21)	99.57 ± 1.98	1.98	100.10 ± 0.07	0.07	97.28 ± 0.20	0.20
Flow rate (0.85 mL/min)	95.33 ± 1.52	1.59	96.05 ± 0.44	0.46	95.41 ± 0.49	0.51
Flow rate (0.75 mL/min)	102.70 ± 0.64	0.62	107.60 ± 0.54	0.51	106.30 ± 1.01	0.95

*n = 3; ACN, acetonitrile.

Method applicability

Determining the drug load and entrapment efficiency is essential to ensure that the drug-loading capacity of NLCs is maximized without excessively altering the entrapment of drugs during the formulation process. As shown in Table VI, all of the NLC formulations we used had greater than 87% entrapment efficiency. We did not

observe a significant trend in entrapment efficiency with increasing ACA content. As for the drug-loading capacity of the NLC, increasing the drug load was feasible with our NLC formulation, as ACA exhibited a high affinity towards the lipid matrix. Previous studies demonstrated that the entrapment efficiency of their NLC system can be maximized by increasing the drug content (Negi, Jaggi, Talegaonkar, 2013; Ferreira *et al.*, 2015). Although a 5% drug load was the target of the present study, future studies can be conducted to determine the maximum drug-loading capacity of the NLCs without severely affecting their entrapment efficiency.

In addition, we carried out the drug-loading capacity and entrapment efficiency studies by separating the supernatant from the ACA-NLC nanosuspension and measuring the ACA content in the precipitate. This is also referred to as the direct method of quantification, in which the drug content in the lipid phase of the precipitate is measured. The nanoparticle precipitate is solubilized in acetonitrile, vortexed to disrupt the lipid matrix, filtered, and subjected to HPLC for component separation (Gaikwad *et al.*, 2019). Contrary to previous studies that usually used the supernatant to measure drug content in NLCs (Lopes *et al.*, 2017; Martins, Mainardes, 2017), this method prevented technical errors due to the use of highly diluted supernatant samples. It proved to be effective because other components of the lipid phase did not interfere with drug quantification as observed in the specificity chromatograms (Figure 3). Furthermore, no other time-consuming extraction methods were required to isolate ACA from the NLC suspensions because the percent analyte recovery in the accuracy test was high. This means that ACA can be separated from the lipid components of the NLC suspensions during chromatographic runs and does not have to be extracted beforehand.

TABLE VI – Drug-loading capacity and entrapment efficiency of ACA-NLCs

Sample	Initial ACA concentration (mg/mL)	Mean (± SD*) experimental ACA concentration (mg/mL)	Mean (± SD*) drug-loading capacity (%)	Mean (± SD*) entrapment efficiency (%)
NLC1	0.1	0.098 ± 0.002	0.49 ± 0.01	98.23 ± 2.00
NLC2	0.3	0.266 ± 0.014	1.33 ± 0.07	88.69 ± 4.67
NLC3	0.5	0.480 ± 0.008	2.40 ± 0.04	96.01 ± 1.60
NLC4	0.8	0.699 ± 0.023	3.50 ± 0.12	87.39 ± 2.90
NLC5	1.0	0.937 ± 0.020	4.69 ± 0.10	93.73 ± 2.00

*n = 3.

CONCLUSION

This report describes our development of a consistent and viable reversed-phase HPLC method, which is essential for determination of the ACA content in NLCs. Although researchers have isolated and quantified free ACA using HPLC under different chromatographic conditions, this is the first documented study of ACA quantification in nanoparticles. The presence of foreign substances in nanoparticle formulations makes it essential that we perform an accurate separation technique with the NLC sample before quantification of the drug. In the present study, we developed a sensitive and quick method of qualitative and quantitative determination of ACA content in lipid nanoparticles without any drug extraction method. Because a short retention time is preferred in analysis of pharmaceutical compounds, our HPLC method is suitable for entrapment efficiency studies

involving numerous samples. Overall, we successfully validated this method according to all ICH guidelines and showed that it can be used in future studies involving ACA-loaded NLCs.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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