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A Reliable Quantification of Cholesterol and 25-Hydroxycholesterol in Liposomal Adjuvant Formulation by Liquid Chromatography High-Resolution Tandem Mass Spectrometry

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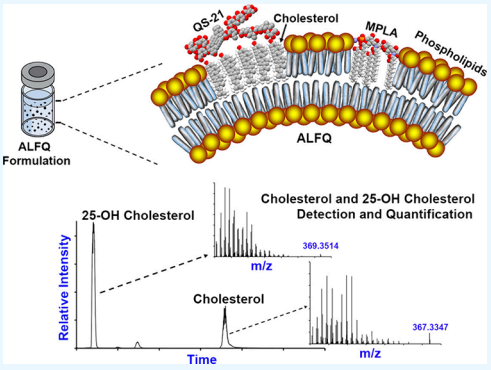
ABSTRACT: Cholesterol, as one of the major components of liposomes, plays a critical role in modulating membrane bilayer permeability, fluidity, and structural stability. Controlling these quality attributes is essential to maintaining the efficacy and fitness of the liposomes in various applications. However, during the manufacture and storage of liposomes, cholesterol has a propensity to undergo oxidative degradation. Hence, an analytical tool that is capable of determining not only the identity and quantity of cholesterol but also its associated degradants is a prerequisite to effective process control and product quality and safety assessments. In this view, a new liquid chromatography electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) method with parallel reaction monitoring (PRM) was developed and qualified to accurately quantify cholesterol and monitor the formation of 25-hydroxycholesterol degradant in liposomal drug formulations without the use of an isotopic internal standard (IS). The method was qualified according to the FDA Quality Guidance for Industry: Q2(R1). Study results showed that the method presents good specificity for cholesterol and 25-hydroxycholesterol detection in the liposomal matrix, good sensitivity characterized by LOD/LOQ in the nanomolar range, and accuracy within the range of 80 to 120%. The described method enables accurate evaluation of in-process and product release samples of Army Liposome Formulation with QS21 (ALFQ).



INTRODUCTION

Liposomes are spherical vesicles made of at least one bilayer of phospholipids and cholesterol in an aqueous environment. These vesicles have been used in a variety of drug delivery applications,[1,2] owing to their biocompatibility and low toxicity and the ability of the vesicular construct to encapsulate both hydrophilic and hydrophobic entities. Successful utilization of liposomes for delivering antimicrobial agents,[3] and macromolecules such as DNA[4] and proteins[5] for diabetes and cardiovascular diseases have been reported. Liposomes have been used also as carriers for chemotherapy targeting agents[6] and MRI contrast agents[7−9] and have been utilized as models for studying biomembranes.[10] Recently, liposomes have been employed as vaccine adjuvants, for example, AS01 has been used in vaccines against malaria,[11] shingles,[12] and respiratory syncytial virus (RSV).[13−15] Army Liposome formulation with QS21 (ALFQ)[16,17] has been used in several phase I vaccine clinical trials targeting malaria antigens,[18] *Campylobacter* diarrhea,[19] SARS-Cov-2,[20] and HIV.[21] The efficacy of liposomes in various applications is influenced by their quality attributes such as size, polydispersity, lamellarity, bilayer fluidity, and encapsulation efficiency, which are controlled by the lipid and cholesterol ratios in the formulation. Hence, quantitative assessments of the liposome components, impurities, and degradants are essential to supporting the process control and product safety and quality.

In liposome formulations, cholesterol, as one of the major components, exerts a profound influence on the properties of the lipid bilayer. It plays a crucial role in the structural stability[22] and modulation of membrane permeability and fluidity.[23,24] For these reasons, high percentages of cholesterol relative to total lipids are often incorporated in liposomal formulation.[16] However, in aqueous solution during the manufacture and storage of liposomes, cholesterol undergoes oxidative degradation to generate oxidative products; for example, 25-hydroxycholesterol formed from autoxidation via hydroperoxide intermediates.[25] The formation of an oxidative product such as 25-hydroxycholesterol has been observed to induce changes in the characteristics of plasma membranes. The presence of the hydroxyl group at C25, in addition to hydroxyl at C3 in 25-hydroxycholesterol alters the amphiphilic

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properties of the molecule causing membrane expansion leading to an increased membrane bilayer.[26] Furthermore, cholesterol functions to abrogate the hemolytic property of QS21 in liposome formulations containing QS21, such as AS01,[11] immune-stimulating complexes (ISCOMs) or Matrix-M,[27−29] and ALFQ,[16,17] which are currently employed as adjuvants in several vaccines. We have shown that detoxification of QS21 in ALFQ is dependent on the amount of cholesterol in the formulation.[17] From this context, it is imperative to monitor the concentration of cholesterol and its degradation product in liposomal adjuvant formulation to support the process and to control the safety and quality of the drug product.

Multiple analytical techniques to determine cholesterol in foods and pharmaceutical samples, including the classical chemical method, gas chromatography, spectrophotometry, capillary electrophoresis, liquid chromatography, and direct mass spectrometry (MS), have been reported.[30−34] Due to the complexity of the liposomal matrix, a more accurate and reliable quantitative analysis of cholesterol and its degradation product requires a separation step combined with an appropriate detection system. To date, cholesterol in liposome formulations and the related lipid nanoparticles were detected and quantified using LC coupled with UV−vis,[35,36] charged aerosol detection (CAD),[37] evaporative light scattering detection (ELSD),[38,39] and MS with different ionization strategies.[31,40,41] The LC-MS/MS-based method has been shown to be reliable for detecting cholesterol and its degradation products in complex matrices. ESI is one of the widely employed MS ionization techniques for quantitative analyses; however, it is not effective in ionizing cholesterol due to its poor proton affinity and low acidity. The use of ESI-MS for accurate cholesterol detection requires an internal standard to compensate signal variabilities or a conversion of cholesterol to cholesteryl ester that tends to form a stable adduct with ammonium ion.[30,42] This paper describes the development and qualification of a sensitive UPLC coupled with the ESI-MS/MS method with parallel reaction monitoring (PRM) mode of detection to accurately determine the concentrations of cholesterol and monitor its degradation in liposomal formulations, without the use of an internal standard or the need of a derivatization step. The utility of this new method has been successfully demonstrated in verifying the concentrations of cholesterol in in-process samples, product release testing, and in monitoring the formation of the degradation product, 25-hydroxycholesterol, as part of stability studies of ALFQ.

RESULTS AND DISCUSSION

Method Development. The described UPLC-MS/MS-based method with PRM detection was developed to accurately quantify the concentration of cholesterol and its degradation product, 25-hydroxycholesterol in liposomal vaccine adjuvants as part of the product formulation stability studies. Since there were no sample pretreatments prior to sample dilution and injection that may potentially hamper accuracy, the method was optimized without employing an isotopic internal standard (IS). The method was optimized based on (i) target analyte chromatographic separation, and (ii) tandem MS detection for accurate and sensitive analyte quantification. Several LC and tandem MS analytical parameters were explored during method optimization. Different formulations of liposomal adjuvants with different lipid ratios and components were tested to investigate and establish the effects of the sample matrix.

Working and Calibration Standards. The final working standard stock solutions with 112 μg/mL cholesterol and 15 μg/mL 25-hydroxycholesterol were prepared in isopropyl alcohol (IPA)/chloroform ($CHCl_3$) mixture (90/10) in the presence of relevant amounts of liposomal matrix containing dimyristoyl phosphatidylcholine (DMPC), dimyristoyl phosphatidylglycerol (DMPG), 3D-PHAD (synthetic monophosphoryl lipid A, MPLA), lysophosphatidylcholine (LMPC), and lysophosphatidylglycerol (LMPG). To minimize the hydrolysis degradation of phospholipids in the matrix and to avoid oxidation of cholesterol, the standard stock solutions were stored at −30 °C. The calibration standards were freshly prepared every experiment by serial dilution of the standard stock solution with IPA/$CHCl_3$ (90/10), in the range of 112.0 to 3.5 μg/mL for cholesterol and 15.0 to 0.5 μg/mL for 25-hydroxycholesterol. We observed that it is necessary to include the liposomal matrix in the working standards to accurately determine the concentrations of target cholesterol and 25-hydroxycholesterol (*vide infra*).

Analyte Separation by LC. Different LC conditions such as mobile phases and gradient conditions, addition of buffer additives, column temperature, and injection volume were explored to attain effective target analyte separation. The final chromatographic conditions employ a Kinetex Phenomenex C18 column (2.1 mm ID × 150 mm, 2.6 mm particle size) with a gradient elution of 95/5 methanol/water and 62/36/2 methanol/dichloromethane/water as mobile phases, with 5 mM ammonium formate, and acidified with 0.1% formic acid.

Under these conditions, cholesterol and its degradation product, 25-hydroxycholesterol, were well separated, as shown in Figure 1A. The extracted ion chromatograms (EIC) in Figure 1B,C showing the different elution times of target cholesterol and 25-hydroxycholesterol, further demonstrated the selectivity of the method. The inclusion of 0.1% formic acid was necessary to facilitate the ionization and hence enhanced the analyte detection. Tailing and fronting of analyte peaks were minimized using 5 mM ammonium formate, without the trade-off of signal suppression. After separation conditions were established, injection volumes in the range of 1 to 5 μL were evaluated based on the associated peak area of each detected ion. The 2 μL injection volume was utilized in the following optimization experiments and in the final method qualification.

Tandem Mass Spectrometry. The target analytes, cholesterol and 25-hydroxycholesterol were detected under ESI positive ionization mode coupled with quadrupole-Orbitrap tandem MS. The precursor ions of cholesterol and 25-hydroxycholesterol were observed to have m/z of 369.3514 and 367.3347, respectively, consistent with the likely formation of $[M+H-H_2O]^+$ ion for cholesterol and $[M+H-2H_2O]^+$ ion for 25-hydroxycholesterol. Relative to the expected m/z of the detected pseudo precursor ions with loss of water, the method detection demonstrated an excellent specificity with mass errors of <3.0 ppm (Figure 2).

Accurate quantification was attained using the PRM mode of detection. The established precursor ions from full-scan experiments were utilized to define a sensitive and specific PRM method for the target analytes. Application of an appropriate normalized collision energy (NCE) resulted in a characteristic fragmentation of each target analyte. For cholesterol, 40 V NCE provided a fragmentation pattern

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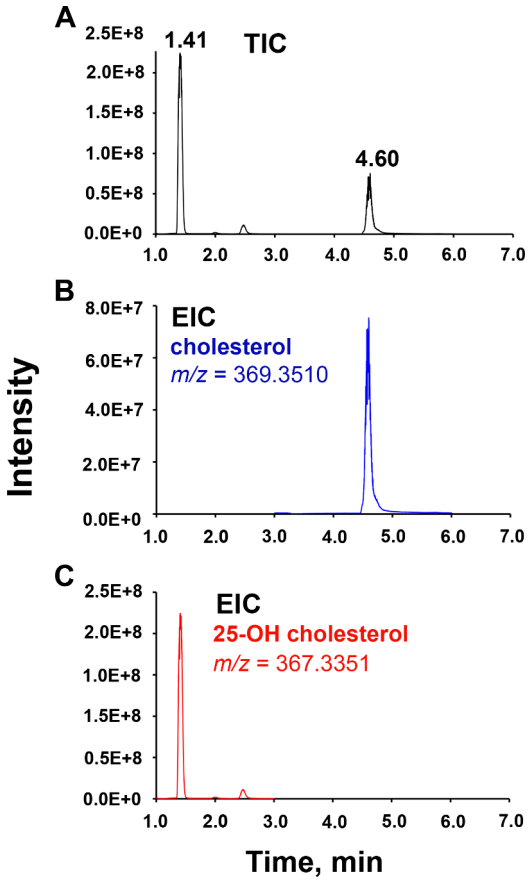

Figure 1. (A) Total ion chromatogram (TIC) trace of cGMP-manufactured ALFQ showing the separation of cholesterol (4.60 min) and 25-hydroxycholesterol (1.41 min). (B, C) Extracted ion chromatograms (EIC) of cholesterol and 25-hydroxycholesterol.

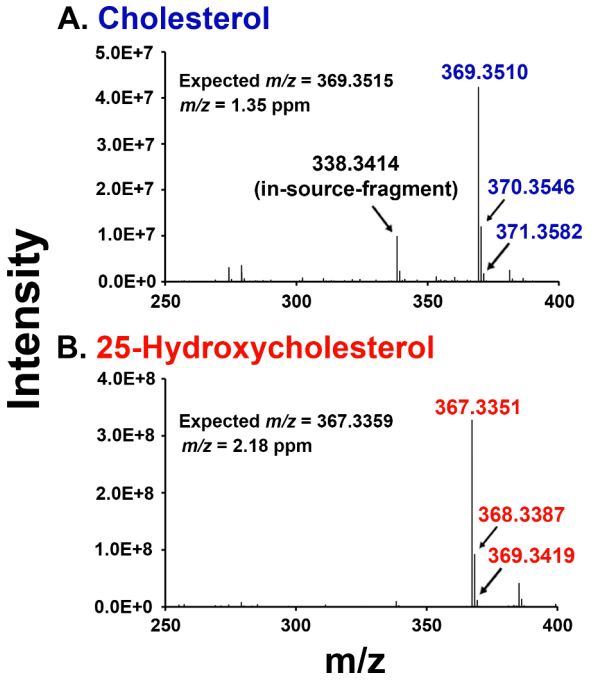

Figure 2. High-resolution mass spectra (HRMS) of cholesterol (A), and the degradation product 25-hydroxycholesterol (B) in ALFQ test samples, highlighting their precursor ions and associated mass detection errors relative to the expected ions.

with three comparable intense peaks with m/z of 95.0859, 147.1168, and 161.1324, along with other minor peaks with m/z of 81.0704, 109.1014, and 135.1169. In the case of 25-hydroxycholesterol, application of 50 V NCE resulted in a fragmentation pattern with a base peak of m/z 95.0859, together with other peaks similar to those observed in cholesterol (Figure S1). The observed fragmentations of cholesterol and 25-hydroxycholesterol in MS were similar to the previous reports.[43,44]

As shown in Table 1, the final PRM method employs a mass transition of m/z 369.35 > 147.02 with NCE of 40 V at 3.6 to 5.6 min for cholesterol and m/z 367.34 > 95.09 with 50 V NCE at 0.4 to 2.4 min for 25-hydroxycholesterol. The method with these PRM conditions exhibits selective and stable mass spectrometry detection.

Table 1. PRM Parameters

analyte	transition (m/z)	NCE (V)
cholesterol	369.3510 → 147.1165	40
25-hydroxycholesterol	367.3351 → 95.0857	50

Matrix Effects. The numerical matrix effect (ME) associated in measuring cholesterol and 25-hydroxycholesterol in liposomes was investigated by comparing the slopes of the calibration curves generated from a standard mix prepared in IPA/$CHCl_3$ containing a liposomal matrix (DMPC, DPMG, 3D-PHAD, LMPC, and LPMG), with that of the neat standard mix that contains only cholesterol and 25-hydroxycholesterol in IPA/$CHCl_3$. The remarkable differences in the calibration curve slopes, which translate to a % ME of ~62% (Table S1), suggest a significant signal suppression within the linear/dynamic range of the method. Hence, to obtain an accurate quantification of target analytes, the working standards are prepared in the presence of the liposomal matrix consisting of relevant ratios of DMPC, DPMG, 3D-PHAD, LMPC, and LPMG. This liposomal matrix was incorporated in the working and calibration standards for the following optimization and method qualification experiments.

The effect of the sample matrix in the quantification of target analytes under the described analytical conditions was further studied by determining the percent (%) recoveries for cholesterol and 25-hydroxycholesterol measurements in different types of formulation, such as Army Liposome Formulation with 55% mol cholesterol (ALF55) and ALFQ, in comparison with a neat mixture of cholesterol and 25-hydroxycholesterol in IPA/$CHCl_3$. As shown in Figure 3A, the % recoveries for cholesterol measurements in ALFQ were relatively lower compared to that in a neat solution, with a significant signal suppression of ~14% (Table S2). Likewise, for the ALF55 sample, the cholesterol % recoveries were significantly lower compared with that of a neat solution, suggesting a remarkable signal suppression of ~19%. Although there are apparent significant differences in the % recoveries for cholesterol measurements in ALFQ and ALF55 relative to that of the neat solution, their values are within 80 to 120%. In the case of 25-hydoxycholesterol measurements, the % recoveries were investigated in 20% spiked ALF55 and ALFQ samples, in comparison with a neat solution of cholesterol and 25-hydroxycholesterol in IPA/$CHCl_3$. Consistent with cholesterol measurements, there was a slight signal suppression in 25-hydroxycholesterol detection of ~4 and ~9% in ALFQ and

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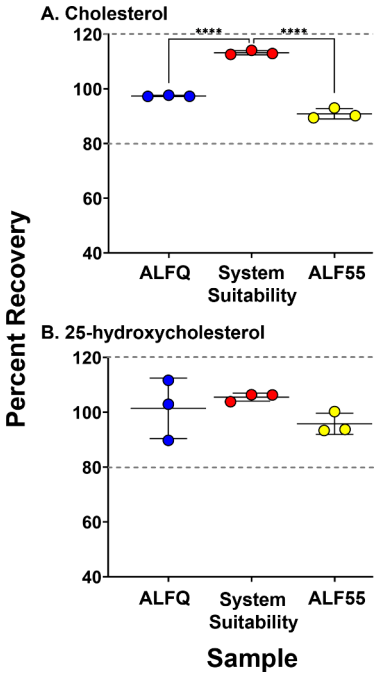

Figure 3. Comparison of percent (%) recoveries of cholesterol (A) and 25-hydroxycholesterol (B) measurements by the described method for different sample matrices.

ALF55, respectively, relative to that of a neat solution (Figure 3B).

Overall, the observed effects of the sample matrix suggest the need to incorporate the liposomal matrix in working and calibration standards or the use of an internal standard. We observed that the use of a liposomal matrix in the calibration standards resulted in excellent accuracy for cholesterol and 25-hydroxycholesterol measurements in liposomal vaccine adjuvants.

System Suitability. The suitability of the final method without the use of an IS was initially established based on consistency of signals and chromatographic retention time (RT). This method showed a signal variance as % relative standard deviation (RSD) of <10% (n = 10), and a RT variance of <1% (n = 10). These initial results show stability of mass spectrometry detection under PRM and consistent LC separation of the analyte. These results further show that the use of a relatively volatile diluent such as a combination of IPA and $CHCl_3$ did not affect the consistency of the signal and did not pose significant errors in the quantification of cholesterol and 25-hydroxy cholesterol, as described below in the method accuracy and precision studies.

Method Qualification. The described method was qualified following *FDA Guidance for Industry: Q2(R1) Validation of Analytical Procedures*, based on detection sensitivity (LOD/LOQ), linearity/dynamic range, selectivity/specificity, and measurement accuracy and precision.[45]

Selectivity/Specificity. The selectivity of the method using tandem MS with PRM detection was established using ALFQ, a system suitability solution (a neat solution of cholesterol and 25-hydroxycholesterol), and ALFQ spiked with 5% 25-hydroxycholesterol. There are no other peaks detected and identified other than the target analytes, cholesterol and 25-hydroxycholesterol (Figure 1A–C and Table 2). A quantitative recovery of 25-hydroxycholesterol in the spiked samples further verified the selectivity of PRM detection. The presence of a liposomal matrix in the standard calibration solutions did not interfere with analyte detection.

Linearity/Dynamic Range and LOD/LOQ. The linear/dynamic ranges of the method observed at 3.5 to 112.0 μg/mL for cholesterol and 0.5 to 15.0 μg/mL for 25-hydroxycholesterol can be described by a linear and a quadratic model, respectively, with both equal weighing schemes (Table 3).

Table 3. Linearity/Dynamic Range

analyte	conc. range (μg/mL)	curve fit (weighing scheme)	R^2	S/N, LL*	% recovery
cholesterol	3.51 to 112.44	linear (equal)	0.9990 to 0.9998	377	80 to 120%
25-OH cholesterol	0.47 to 15.01	quad (equal)	0.9991 to 0.9994	388	80 to 120%

These linear ranges were characterized by regression coefficients, R^2 > 0.9991, and accuracies of all calibration points within 80 to 120%. Below the lowest limit of quantification (LLOQ), the % recoveries were not within the acceptable limit of 100 ± 20%.

The method limit of detection (LOD) and limit of quantification (LOQ) for detecting cholesterol and 25-hydroxycholesterol are summarized in Table 4. These values were determined from five (5) sets of calibration standards including the LLOQ, following eqs 1 and 2 (experimental section). For 25-hydroxycholesterol, the LOD and LOQ were not warranted; instead, LLOQ was considered. Sensitivity studies showed that cholesterol and 25-hydroxycholesterol can be detected in the liposomal matrix in the nanomolar range.

Table 4. Method LOD and LOQ

	analyte	
parameter	cholesterol	25-OH cholesterol
mean ± SD LOD (μg/mL)	1.80 ± 0.89	0.47[a]
mean ± SD LOQ (μg/mL)	5.44 ± 2.68	0.47[a]

[a]LOD and LOQ were not warranted; instead, LLOQ was reported.

Table 2. Specificity of the Detection

sample	analyte	mass info (m/z)	conc. (μg/mL)	area	RT	S/N
system suitability solution	cholesterol	369.3510 > 147.1165	14.06	4,682,428	4.26	759.20
	25-OH cholesterol	367.3351 > 95.0857	1.88	6,768,661	1.36	1,308.35
lab-grade ALFQ	cholesterol	369.3510 > 147.1165	10,822.33	13,311,094	4.26	1,672.78
	25-OH cholesterol	367.3351 > 95.0857	BDL	NA		NA
lab-grade ALFQ (spiked with 5% 25-OH cholesterol)	cholesterol	369.3510 > 147.1165	10,822.33	4,317,011	4.27	5,042.65
	25-OH cholesterol	367.3351 > 95.0857	541.5	13,108,091	1.36	3,403.52

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Accuracy and Precision. The mean accuracy and precision of the method were established using freshly prepared lab-grade ALFQ at different dilutions (1:150, 1:300, 1:600) and ALFQ spiked with 5, 10, and 20% 25-hydroxycholesterol. Table 5 shows that at all dilution conditions, cholesterol measurements exhibited % recoveries in the range of 94 to 113%, while that of 25-hydroxycholesterol measurements showed recoveries in the range of 80 to 105%, all of which are within the acceptable limit. Overall, the described method exhibits good accuracy for measuring cholesterol and a good impurity assay for the presence of the degradation product 25-hydroxycholesterol in a liposomal adjuvant formulation.

Table 5. Percent Recoveries Associated with Cholesterol and 25-Hydroxycholesterol Measurements in Freshly Prepared Lab-Grade ALFQ

accuracy studies for cholesterol measurements			
	cholesterol conc. (μg/mL)		
dilution of test samples	calculated[a]	theoretical	% recovery
1:150	10,252.55 ± 391.03	10,822.33	94.74 ± 3.61
1:300	12,185.10 ± 246.42	10,822.33	112.59 ± 2.28
1:600	11,419.40 ± 891.51	10,822.33	105.52 ± 8.24
accuracy studies for 25-hydroxycholesterol measurements			
	25-hydroxycholesterol conc. (μg/mL)		
spiked level	calculated	theoretical	% recovery
20%	2,197.35 ± 238.92	2,166.00	101.45 ± 11.03
10%	1,137.35 ± 114.23	1,083.00	105.02 ± 10.55
5%	435.45 ± 48.09	541.50	80.42 ± 8.88
0%	N.D.[b]		

[a]The calculated concentrations are the actual measured concentrations after accounting sample dilutions. [b]N.D. = nondetected.

The method precision was described in terms of within-run and between-run repeatability expressed as % RSD of the measured concentrations and chromatographic RT from a series of analyses.

The within-run sample repeatability (n = 12) for measuring cholesterol and 25-hydroxycholesterol was characterized by % RSD <4% and ∼10%, respectively, with RT repeatability of % RSD <1% for both analytes (Table 6). The between-run repeatability of the method shown in Table 7 exhibits a % RSD of ∼11%.

The results from the accuracy studies characterized by % recoveries within the acceptable limit of 100 ± 20% showed that the use of an IS in this analytical system is not required to attain a reliable and accurate quantification. Repeatability studies have shown the stability of mass spectrometry detection and consistency of quantification, thus further supporting the suitability of IPA/$CHCl_3$ as a sample diluent.

Application in Real Samples. The qualified method was used to accurately evaluate in-process samples and release of manufactured ALFQ. This method is currently used to monitor the formation of 25-hydroxycholesterol degradation product, as part of the stability studies of cGMP-manufactured ALFQ and ALF55.

Table 7. Between-Run Measurement Variability

day	cholesterol conc. (μg/mL)
day 1	12,185.00
day 2	9,762.90
day 3	10,279.00
mean ± SD	10,742.33 ± 1275.84
% RSD	11

EXPERIMENTAL SECTION

Chemical Reagents and Instrumentation. DMPC, DMPG, 3D-PHAD, cholesterol (plant derived), LMPC, LMPG, and 25-hydroxycholesterol were purchased from Avanti Polar Lipids, LLC, Alabaster, Alabama. Lab-grade ALF55 and ALFQ were prepared following the established procedures.[46−49] cGMP-grade ALF55 was manufactured under contract by Avanti Polar Lipids, and ALFQ was manufactured from ALF55 at the Pilot Bioproduction Facility (PBF) at WRAIR. Methanol, water, formic acid, and ammonium formate (all Optima) used for mobile phases were purchased from Fisher Chemicals, Asheville, North Carolina. Quantitative analyses were done using a Thermo Scientific Vanquish Flex UHPLC system coupled with Q-Exactive Quadrupole-Orbitrap Mass Spectrometer, controlled by Xcalibur software version 4.4. The data were processed using a Thermo Scientific TraceFinder 5.1.

Working and Calibration Standards. A tertiary working standard for the quantitation of cholesterol and 25-hydroxycholesterol in liposomal formulation were prepared in the liposomal matrix consisting of DMPC, DMPG, 3D-PHAD, and cholesterol and their degradation products, LMPC, LMPG, and 25-hydroxycholesterol in 90:10 IPA/$CHCl_3$. This tertiary stock solution was stored at −30 °C to prevent the hydrolysis decomposition of phospholipids and oxidative degradation of cholesterol. From the above tertiary solution, a calibration standard mixture in liposomal matrix with cholesterol concentration in the range of 112.00 to 3.50 μg/mL, and 25-hydroxycholesterol in the range of 15.00 to 0.47 μg/mL was prepared by a serial dilution with IPA/$CHCl_3$. A fresh set of calibration standards was made for every analysis.

Liposomal Test Samples. The described analytical method for quantifying cholesterol and 25-hydroxycholesterol in liposomal formulations was employed in both lab-grade and cGMP-manufactured ALF55 and ALFQ samples. Different levels of sample dilutions in LC-MS grade IPA/$CHCl_3$, 1:150 (low), 1:300 (mid) and 1:600 (high) were investigated to establish the appropriate assay dilution condition. The 1:300 assay dilution was employed in the final and optimized method.

Optimized UPLC-MS/MS Conditions. Quantitative analysis of cholesterol and 25-hydroxycholesterol was accom-

Table 6. Within-Run Sample Preparation and Injection Variability (n = 12)

	cholesterol		25-hydroxycholesterol	
sample	conc. (μg/mL)	RT (min)	conc. (μg/mL)	RT (min)
mean ± SD	9,816.28 ± 215.84	4.30 ± 0.01	1,137.35 ± 114.23	1.36 ± 0.01
% RSD	2.20	0.23	10.04	0.74

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plished in Thermo Scientific Vanquish UHPLC coupled with a Q-Exactive Quadrupole-Orbitrap detector. The analytical separation was carried out in a Kinetex Phenomenex C18 column (2.1 mm ID × 150 mm, 2.6 μm particle size), using methanol/water (95/5) with 0.1% formic acid and 5 mM ammonium formate (A), and methanol/dichloromethane/water (62/36/2) with 0.1% formic acid and 5 mM ammonium formate (B) as mobile phases with a constant flow of 0.4 mL/min at a controlled column temperature of 40 °C. The UPLC gradient used is described in Table S3 (Supporting Information). The injection volume was set at 2 μL.

All data were acquired using a positive electrospray ionization (ESI) in PRM mode, with detection parameters of m/z 369.3510 > 147.1165 at 4.26 min (chromatographic RT) and m/z 367.3351 > 95.0857 at 1.40 min for cholesterol and 25-hydroxycholesterol, respectively. The electrospray and source settings were as follows: 2.5 kV (capillary voltage), 320 °C (capillary temperature), 25 AU (sheath gas flow rate), 10 AU (Aux gas flow rate), and 300 °C (Aux gas temperature).

Analytical Method Qualification. A qualification study following the ICH quality guidelines for validation[45] was conducted to establish the selectivity/specificity, sensitivity, linearity, precision, and accuracy of the method to quantify cholesterol and 25-hydroxycholesterol in liposomal drug products.

System Suitability. Exploratory studies were conducted to determine the appropriate system suitability solution matrix and concentrations that will be employed to ensure the suitability of the equipment during the assay. A mixture of 14.00 μg/mL cholesterol and 1.90 μg/mL 25-hydroxycholesterol in IPA/$CHCl_3$ with the liposomal matrix provided good signals for both analytes and was utilized as a standard system suitability solution. The overall assay suitability was evaluated based on the relative standard deviation (%RSD) of the chromatographic RT and peak area of the six (6) injections at the beginning and at the end of the sequence.

Specificity. The ability of the method to detect the target analytes selectively and specifically, in the presence of other components, matrix, and solvents, was established using system suitability solutions, freshly prepared lab-grade ALFQ samples, and 25-hydroxycholesterol-spiked ALFQ formulations.

Linearity and Dynamic Range. The linear or dynamic ranges of the described quantitative method were explored in wide concentration ranges of cholesterol (112.00 to 1.75 μg/mL) and 25-hydroxycholesterol (15.0 to 0.2 μg/mL) in liposomal matrix. The linearity and linear/dynamic ranges were evaluated based on the regression coefficient (R^2 > 0.995) and % difference of the calculated concentrations relative to the theoretical calibration concentrations (% difference <20%) from three (5) sets of calibration standards. Based on the above criteria, the final linear/dynamic ranges of the method for quantitation of cholesterol and 25-hydroxycholesterol were observed at 112.00 to 3.5 μg/mL and at 15.0 to 0.5 μg/mL, respectively.

LOD/LOQ. The parameters used to calculate LOD and LOQ were extracted from the linearity studies. LOD was established from the standard error of the y-intercept (σ) and the slope (S) of the linear calibration curve using eq 1. The signal-to-noise (S/N) ratio of the LOD should be ≥3.

$$\mathrm{LOD} = \frac{3.3\sigma}{S} \tag{1}$$

The LOQ on the other hand was determined using eq 2. The LOQ should exhibit an S/N of ≥10. Both LOD and LOQ was established from five (5) independent sets of calibration standards.

$$\mathrm{LOQ} = \frac{10\sigma}{S} \tag{2}$$

Accuracy. The ability of the method to provide the correct calculated and determined concentrations of the target analytes relative to the expected and true values was established. Three (3) levels of assay dilution conditions in the presence of the sample matrix were explored, 1:150, 1:300, and 1:600, and the accuracy was established based on the percent (%) recoveries at each dilution relative to the nominal concentration (10,822 μg/mL) of cholesterol in ALFQ test samples. The accuracy of the method to quantitate the degradation product, 25-hydroxycholesterol was established based on the % recoveries relative to the theoretical values at 5, 10, and 20% spike levels.

Precision. Sample preparation repeatability using 12 sample preparations of cGMP-manufactured ALFQ at appropriate assay dilution within a single run and injection repeatability from one sample preparation injected 12× were used to establish the method variability. The between-run method precision was established from three (3) independent measurements of cholesterol concentrations in the same ALFQ test sample performed in three (3) different days. The precision was defined based on % RSD of the calculated concentrations and chromatographic RT, associated in sample preparation and sample injection within a single run, and the % RSD of the calculated cholesterol concentrations analyzed in multiple runs at different days.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c01524.

Tandem MS spectra of cholesterol and 25-hydroxycholesterol; raw data for analyzing matrix effects; slopes of the calibration curves from standards prepared in the liposomal matrix and in neat solvent IPA/$CHCl_3$; comparison of percent recoveries associated with cholesterol and 25-hydroxycholesterol measurements in ALFQ and ALF55 versus in neat sample solution; and UPLC gradient for the quantification (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. The preparation of lab-grade liposomes (ALF55 and ALFQ), working standards, and analytical method development and qualification were performed at the Laboratory of Antigen and Adjuvant Research, U.S. Military HIV Research Program, Walter Reed Army Institute of Research. cGMP grade ALF55 was manufactured at Avanti. All authors have given approval of the final version of the manuscript.

Notes

The authors declare no competing financial interest.

Material has been reviewed by the Walter Reed Army Institute of Research. There is no objection to its presentation and/or publication. The opinions or assertions contained herein are the private views of the author and are not to be construed as official or as reflecting true views of the Department of the Army or the Department of Defense.

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