Designation: E3323 – 22

Standard Test Method for Lipid Quantitation in Liposomal Formulations Using High Performance Liquid Chromatography (HPLC) with an Evaporative Light-Scattering Detector (ELSD)[1]

This standard is issued under the fixed designation E3323; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This test method describes an analytical technique to quantify lipid components that are often present in liposomal formulations as major components.

1.2 This test method uses high performance liquid chromatography (HPLC) to separate lipids in liposomal formulations and evaporative light-scattering detection (ELSD) to quantify the individual components.

1.3 This test method quantifies three major organic components in liposomal formulations: cholesterol, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG 2000), and hydrogenated soy L-α-phosphatidylcholine (HSPC).

1.4 This test method can estimate the absolute concentration of cholesterol, DSPE-PEG 2000, and HSPC and their ratio (DSPE-PEG 2000: HSPC: cholesterol) in liposomal formulations.

1.5 This test method describes preparation of calibration standards and samples, HPLC and ELSD instrumentation, method development and method validation, sample analysis, and data reporting.

1.6 The detection limits and quantitation limits for the analytes (lipid components) in this test method are in the range of 2 μg⁄g to 4 μg/g and 7 μg⁄g to 10 μg/g, respectively. The analytical measurement ranges for cholesterol, DSPE-PEG 2000, and HSPC are 10 μg⁄g to 165 μg/g, 10 μg⁄g to 300 μg/g, and 10 μg⁄g to 200 μg/g, respectively.

1.7 Significant digits and rounding of all reported values have been performed according to the guidelines as established in Practice D6026.

1.8 *Units*—The values stated in SI units are to be regarded as the standard. Where appropriate, c.g.s units in addition to SI units are included in this standard.

1.9 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety, health, and environmental practices and determine the applicability of regulatory limitations prior to use.*

1.10 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

D1193 Specification for Reagent Water

D1356 Terminology Relating to Sampling and Analysis of Atmospheres

D6026 Practice for Using Significant Digits and Data Records in Geotechnical Data

D7439 Test Method for Determination of Elements in Airborne Particulate Matter by Inductively Coupled Plasma–Mass Spectrometry

E131 Terminology Relating to Molecular Spectroscopy

E177 Practice for Use of the Terms Precision and Bias in ASTM Test Methods

E456 Terminology Relating to Quality and Statistics

E682 Practice for Liquid Chromatography Terms and Relationships

E2490 Guide for Measurement of Particle Size Distribution of Nanomaterials in Suspension by Photon Correlation Spectroscopy (PCS)

E3025 Guide for Tiered Approach to Detection and Characterization of Silver Nanomaterials in Textiles

E3080 Practice for Regression Analysis with a Single Predictor Variable

[1] This test method is under the jurisdiction of ASTM Committee E56 on Nanotechnology and is the direct responsibility of Subcommittee E56.08 on Nano-Enabled Medical Products.

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[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

3. Terminology

3.1 *Definitions:*

3.1.1 *accuracy, n*—closeness of agreement between a test result and an accepted reference value.

3.1.1.1 *Discussion*—The term accuracy, when applied to a set of results, involves a combination of random components and a common systematic error or bias component. **E177**

3.1.2 *aerosol, n*—suspension of solid particles or liquid droplets or both in a gaseous medium. **D1356**

3.1.3 *analyte, n*—chemical constituent of interest in an analytical procedure. **E3025**

3.1.4 *analytical instrument qualification, n*—collection of documented evidence that an instrument performs suitably for its intended purpose (**1**).[3]

3.1.5 *baseline noise, n*—combination of high-frequency signal fluctuations and low-frequency signal drift that affect baseline stability.

3.1.5.1 *Discussion*—These signal fluctuations can originate from line-voltage fluctuations, shot noise (Poisson noise) from electronic circuits, improper solvent degassing, temperature instability, and other nonequilibrium effects. Noise is representative of detector response that is not related to responses from analytes or matrix interferences.

3.1.6 *calibration curve, n*—relationship between measured response values and analytical concentrations of a standard or reference material. **D7439**

3.1.6.1 *Discussion*—A set of calibration standards are used to construct a calibration curve, and the concentration of analyte present in an unknown sample can be determined by comparing the detector response with the calibration curve.

3.1.7 *calibration standards, n*—set of solutions with known analyte concentration used to construct calibration curves.

3.1.8 *carryover effect, n*—systematic error that is derived from the preceding sample injection being introduced into the next sample affecting accurate quantitation.

3.1.9 *cholesterol, n*—steroidal organic compound that stabilizes the lipid bilayer in liposomal formulations.

3.1.10 *chromatogram, n*—graphical presentation of detector response plotted as a function of elution time or effluent volume as the sample components elute from the column and reach the detector.

3.1.10.1 *Discussion*—In the case of evaporative light-scattering detection (ELSD), the detector response is often expressed as voltage (mV) over a range of elution time (t), where voltage is a function of the intensity of light scattered by nonvolatile particles inside the optical chamber. For analysis, the characteristic response of ELSD for an eluting analyte is typically evaluated from the peak area under the curve that is recorded in the chromatogram. This peak area (A) can be expressed mathematically as an integral of detector response for analytes over an elution time interval from t_1 to t_2:

$$A(t) = \int_{t_1}^{t_2} (S)dt \tag{1}$$

Where $A(t)$ and $S(t)$ = Peak area and the instantaneous detector's response at time, t, respectively (**2**).

3.1.11 *coefficient of determination, n*—statistical measure of the linear relationship between X and Y calculated by:

$$r^2 = \frac{\left(\sum_{i=1}^{n} X_i Y_i\right)^2}{\left(\sum_{i=1}^{n} X_i\right)\left(\sum_{i=1}^{n} Y_i\right)} \tag{2}$$

Where n = number of observations. **E131**

3.1.12 *evaporation, n*—process by which an element or compound transitions from its liquid state to its gaseous state.

3.1.12.1 *Discussion*—In the ELSD technique, only the solvent and volatile buffer components of the HPLC-effluent are removed during the evaporation process and less-volatile or nonvolatile analytes are left behind as dried particles. This process is also called "desolvation". The evaporation process depends on various factors including gas pressure, flow rate of the carrier gas, nature of the solvents, and temperature of the evaporation tube. It is important to choose the appropriate mobile phase components that are volatile; nonvolatile buffers are not compatible with this test method.

3.1.13 *intermediate precision, n*—closeness of agreement between test results obtained under specified intermediate precision conditions. **E177**

3.1.14 *intermediate precision conditions, n*—conditions under which test results are obtained with the same test method using test units taken at random from a single quantity of material that is as nearly homogeneous as possible and with changing conditions such as operator, measuring equipment, location within the laboratory, and time. **E177**

3.1.15 *limit of detection, n*—least amount of analyte in a sample that can be detected but not necessarily quantitated under the stated experimental conditions.

3.1.15.1 *Discussion*—The limit of detection is usually expressed as the concentration of the analyte in the test sample.

3.1.16 *limit of quantitation, n*—least amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy.

3.1.16.1 *Discussion*—The limit of quantitation is usually expressed as the concentration of the analyte in the test sample.

3.1.17 *linearity, n*—ability of the analytical method (within a certain range) to obtain test results that are directly proportional to the concentration (amount) of the analyte in the sample (**3**).

3.1.17.1 *Discussion*—To establish response linearity, a minimum of six analyte concentrations are recommended. Regression analysis by the method of least squares (r^2) provides a mathematical estimate of the degree of linearity.

3.1.18 *lipids, n*—diverse group of organic compounds that are soluble in organic solvents but are insoluble in water.

3.1.18.1 *Discussion*—In this test method, lipids refer to cholesterol, DSPE-PEG 2000, and HSPC. The chemical structures of these three lipids are presented in Appendix X1.

[3] The boldface numbers in parentheses refer to a list of references at the end of this standard.

3.1.19 *liposomal formulation, n*—product designed to assist in the delivery of an active pharmaceutical ingredient, either encapsulated or intercalated in a liposome.

3.1.19.1 *Discussion*—Formulated products can contain vesicles having a single lipid bilayer (unilamellar), multiple concentric lipid bilayers (multilamellar), or a mixture of unilamellar and multilamellar vesicles.

3.1.20 *liposome, n*—synthetic vesicle composed of a one or more bilayers formed by amphipathic molecules such as phospholipids that enclose a central aqueous compartment. Adapted from **(4)**.

3.1.21 *matrix blank, n*—substance that closely matches the samples being analyzed with regard to matrix components.

3.1.21.1 *Discussion*—Ideally, the matrix blank contains all the sample components except the analyte(s) of interest and is subjected to all sample-processing operations including all reagents used to analyze the test samples. The matrix blank is used to determine the presence or absence of any significant interference as a result of the matrix, reagents, and equipment.

3.1.22 *matrix effect, n*—influence of one or more components from the sample matrix on the measurement of the analyte concentration or mass.

3.1.22.1 *Discussion*—Matrix effects may be observed as increased or decreased detector responses compared with those produced by simple solvent solutions of the analyte **(5)**.

3.1.23 *method validation, n*—process used to confirm that an analytical procedure used for a specific test is suitable for its intended purpose.

3.1.24 *mobile phase, n*—liquid used to elute sample components through the column that may consist of a single component or a mixture of components.

3.1.24.1 *Discussion*—The term eluent is often used for the preferred mobile phase. **E682**

3.1.25 *nebulization, n*—process to convert the solution of target analyte components to a fine-spray via a nebulizer.

3.1.26 *peak area, n*—area under a peak obtained from integration of a detector signal above the baseline for a given component.

3.1.27 *peak resolution, n*—measure of chromatographic separation of two components in a mixture calculated by:

$$R_s = 2 \times \frac{(t_{R2} - t_{R1})}{(w_1 + w_2)} \quad (3)$$

where:

R_s = peak resolution,
t_{R2} and t_{R1} = retention time of the two components 1 and 2 ($t_{R2} > t_{R1}$), and
w_1 and w_2 = corresponding widths at the bases of the peaks obtained by extrapolating the relatively straight sides of the peaks to the baseline.

3.1.28 *precision, n*—closeness of agreement between independent test results obtained under stipulated conditions. **E177**

3.1.29 *range, n*—interval between the upper and lower concentrations of the analyte in a sample for which it has been demonstrated that the analytical procedure has an acceptable level of accuracy, precision, and linearity.

3.1.30 *regression analysis, n*—statistical procedure used to characterize the association between two or more numerical variables for prediction of the response variable from the predictor variable. **E3080**

3.1.30.1 *Discussion*—The objective is to obtain a regression model for use in predicting the value of the response variable for given values of the predictor variable. In this test method, the response variable is the ELSD signal [light-scattering unit (LSU)] and the predictor variable is mass concentration.

3.1.31 *repeatability, n*—precision of test results from tests conducted within the shortest practical time period on identical material by the same test method in a single laboratory with all known sources of variable conditions controlled at the same levels. Adapted from **E177**

3.1.32 *reproducibility, n*—precision of test results from tests conducted on identical material by the same test method in different laboratories. Adapted from **E456**

3.1.33 *robustness, n*—measure of change in the outcome of an analytical procedure with deliberate and systematic variations in any or all of the key method parameters that influence it. Adapted from **E2490**

3.1.34 *solvent blank, n*—solution containing all reagents used in sample dissolution in the same quantities used for preparation of blank and sample solutions.

3.1.34.1 *Discussion*—The solvent blank is used to assess contamination from the laboratory environment and characterize spectral background from the reagents used in sample preparation. **D7439**

3.1.35 *specificity, n*—ability to assess unequivocally the analyte in the presence of components that may be expected to be present in the test sample.

3.1.35.1 *Discussion*—Typically, these might include impurities, degradants, matrix, and so forth **(3)**.

3.1.36 *system suitability, n*—determination of instrument performance in a particular procedure (for example, sensitivity and chromatographic retention) by analyzing a set of appropriate reference standards before the analytical run.

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *test sample, n*—final form of the sample that is used for testing.

3.2.1.1 *Discussion*—In this test method, the sample solubilized in methanol followed by appropriate dilution by solvent is defined as the test sample.

3.2.2 *test unit, n*—unit or portion of a material that is obtained from a primary material following a sampling procedure to acquire test result(s) for the property(-ies) to be measured.

3.2.2.1 *Discussion*—In this test method, the original liposomal formulation to be tested for lipid quantitation is defined as the test unit.

3.3 *Acronyms:*

3.3.1 *Cal*—Calibration

3.3.2 *CRM*—Certified reference material

3.3.3 *DSPE-PEG*—1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-methoxy(polyethylene glycol)

3.3.4 *ELSD*—Evaporative light-scattering detector

3.3.5 *HPLC*—High performance liquid chromatography

3.3.6 *HSPC*—Hydrogenated soy L-α-phosphatidylcholine

3.3.7 *ID*—Inside diameter

3.3.8 *LOD*—Limit of detection

3.3.9 *LOQ*—Limit of quantitation

3.3.10 *LSU*—Light-scattering unit

3.3.11 *OSHA*—Occupational Safety and Health Administration

3.3.12 *QA*—Quality assurance

3.3.13 *QC*—Quality control

3.3.14 *RCF*—Relative centrifugal force

3.3.15 *RSD*—Relative standard deviation

3.3.16 *SD*—Standard deviation

3.3.17 *SLM*—Standard liter per minute

3.3.18 *ULOQ*—Upper limit of quantitation

3.3.19 *UV*—Ultraviolet

4. Summary of Test Method

4.1 Over the past few decades, several liposomal drug formulations have been approved for clinical use (**6**, **7**). An ongoing effort from pharmaceutical industries, academic institutions, foundations, and industry partners has been made to develop new nanoscale liposomal formulations with improved drug efficacy, and many products are currently being assessed in clinical trials (**7-11**). The critical quality attributes to consider for these nanomaterials include size and shape heterogeneity, chemical composition, and physicochemical stability of ingredients present in the liposome formulation (**12**).

4.2 This test method describes an analytical method for the separation and quantitation of cholesterol, DSPE-PEG 2000, and HSPC in liposomal formulations.

4.3 This test method is based on the combination of two well-established analytical techniques: (*1*) chromatographic separation of analytes (a mixture of cholesterol, DSPE-PEG 2000, and HSPC) via HPLC using a designated column, and (*2*) quantitation of analytes present in the effluent via ELSD in which the scattering intensity of the incident light is correlated with the total mass of the analytes present in the injected sample. The ELSD detector can serve as a universal detector for a range of nonvolatile or semi-volatile analytes including those that do not have ultraviolet (UV)-absorbing chromophores (**13**, **14**).

4.4 A calibration curve from six calibration standards of cholesterol, DSPE-PEG 2000, and HSPC is developed by following the procedure described in this test method. As ELSD shows a nonlinear response with analyte concentration, logarithmic transformation is performed to obtain a linear regression model. Hence, log (peak area) versus log (concentration) is plotted for each analyte to obtain the corresponding calibration curve. The slope and intercept obtained from linear regression analysis are used to quantify the individual mass (concentration) of lipid components in an unknown liposome sample.

4.5 This test method describes the specific test conditions, sample preparation, method validation, and data analysis requirements.

5. Significance and Use

5.1 Lipid composition in a liposomal formulation is an important aspect during synthesis of liposomes, which determines stability, surface characteristics, drug encapsulation, and drug release capabilities. The cholesterol component plays a key role in controlled drug release by adding stability to the liposome. A small variation in the lipid composition can significantly alter the parameters mentioned above (**15**).

5.2 Variation in the lipid composition in the liposomal formulation may influence the safety and efficacy of the product. Therefore, chemical composition of the liposomes shall be determined.

5.3 The pharmaceutical industry and regulatory agencies require QC, QA, specifications, thorough characterization, and quantification of lipid components (**16**, **17**).

5.4 This test method can be used to ascertain variations in the lipid component profiling of various liposomal formulations. However, this test method does not intend to identify chemical degradation products (**18**).

5.5 Analyzing the stability of analytes and their chemical degradation profiles as a result of oxidation or hydrolysis is beyond the scope of this test method (**18**, **19**).

6. Interferences

6.1 Method interferences may be introduced by impurities present in reagents, glassware, and other apparatus used during sample preparation and instrumental analysis. These impurities may result in high baseline noise or interfering peaks. The presence and magnitude of method interferences are determined by routine analysis of solvent and laboratory blanks.

6.2 To avoid heterogeneity in pH of the solution, eluents and buffer salts shall be properly mixed (sonication is recommended). It is also recommended that the solvent reservoir bottle be cleaned routinely, and the appropriate bottles be filled with freshly prepared mobile phase solutions.

6.3 Aerosol formation in ELSD requires a constant supply of dry and filtered gas that is free from particulate matter and nonvolatile hydrocarbons. The most commonly used gas is nitrogen. The inlet gas shall be filtered through a 0.01 µm filter to remove particulate matter, and an appropriate gas adsorbent trap should be used to remove nonvolatile hydrocarbons and moisture, and thereby minimizing baseline noise. The use of gases that allows either combustion of solvents or oxidation of target analytes should be avoided.

6.4 Keep the autosampler injection port and column clean to avoid carryover or ghost peaks. Contamination of glass containers or vials used for this test method should be avoided.

6.5 Fluctuations in detector response can adversely affect data quality. To avoid this issue, stabilize the detector at the experimental conditions (for example, nebulization temperature, evaporation temperature, and nitrogen flow rate) for 30 min. This will also minimize baseline noise.

6.6 Set the temperature of the column compartment as recommended in the test method and equilibrate the column while stabilizing the detector response (see 6.5).

6.7 The presence of bubbles in the HPLC tubing causes pressure fluctuations. Solvent purging (2.00 mL/min) for 15 min in which the column is bypassed is recommended to remove interferences caused by the presence of air bubbles.

6.8 Excipients present in the test samples may interfere with the detection of cholesterol, DSPE-PEG 2000, or HSPC. Therefore, the matrix effect can be assessed by comparing the detector response for a known amount of standard analyte spiked in the matrix blank and for the same amount of analyte spiked in a solvent blank. If any matrix effect is observed by this comparison, optimization of the sample preparation procedure to remove the interfering compounds from the test sample or modification of the chromatographic parameters (for example, solvent gradient or eluent flow rate) to avoid the coelution of target analytes and interfering excipients are recommended. The Bligh-Dyer method, as described in Appendix X3, could be adopted to remove water-soluble excipients in test liposomal formulations, if necessary.

6.9 Chemicals with high purity shall be used for the preparation of lipid calibration standards. When feasible, it is recommended that higher-order reference standards (for example, CRMs) are used in calibration. If reference materials are not available, high-quality crystalline or lyophilized chemicals of known purity can be used for this purpose.

6.10 All the stock solutions, calibration standards (calibration levels), and test samples should be stored either at 0 °C to 4 °C or –20 °C as recommended in this test method to avoid degradation of target analytes.

7. Apparatus

7.1 *HPLC,* with in-line degasser module.

7.2 *ELSD.*

7.3 *Deactivated BEH (bridged ethylene hybrid) C18 column,* with 13 nm pore size, 3.5 µm particle size, and 3 mm ID × 150 mm column length or other equivalent stable C18 column that can resolve the peaks for analytes and potential interference with a peak resolution ≥1.5.

7.4 *Analytical balance* that can accurately weigh with ≤0.0001 g readability.

7.5 *Vortex mixer.*

7.6 *Mechanical pipettors,* covering ranges from 2 µL to 20 µL, 100 µL to 200 µL, and 100 µL to 1000 µL and 10 mL.

7.7 *Glass amber vials,* 10 mL and 20 mL.

7.8 *Autosampler amber vials,* 2 mL.

7.9 *Solvent reservoir bottle,* 1 L.

7.10 *Bottle top vacuum filter system,* pore size 0.2 µm.

7.11 *Ultrasonic water bath.*

8. Reagents and Materials

8.1 *Purity of Reagents*—Reagent-grade chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society where such specifications are available.[4] Other grades may be used, provided it is first ascertained that the reagents are of sufficiently high purity to permit their use without lessening the accuracy of the determinations.

8.2 *Reagents*—All reagents should be of LC-MS grade and solvents should be prefiltered with a ≤0.2 µm filter. Other grades (for example, HPLC grade) may be used, provided it is first ascertained that the reagents are of sufficiently high purity to permit their use without lessening the accuracy of the determinations.

8.2.1 *Ammonium acetate,* LC-MS grade.

8.2.2 *Acetonitrile,* LC-MS grade.

8.2.3 *Methanol,* LC-MS grade.

8.2.4 *Purity of Water*—Unless otherwise indicated, references to water shall be understood to mean reagent water as defined by Type 1 of Specification D1193. Use deionized water (>18 MΩ cm) Type 1 high-purity water.

8.3 *Materials:*

8.3.1 *Lipid Standards:*

8.3.1.1 *Cholesterol,* ≥99 % pure, powder form.

8.3.1.2 *HSPC,* ≥99 % pure, consisting of C16:0 (HSPC 1) and C18:0 (HSPC 2) fatty acids, powder form.

8.3.1.3 *DSPE-PEG 2000,* ≥99 % pure, powder form.

9. Hazards

9.1 Because the ELSD produces an aerosol, the outlet of the ELSD unit shall be vented to a suitable fume hood or external exhaust.

9.2 Follow laboratory safety protocol and proper protective measures while handling liposomal formulations.

9.3 This test method uses methanol, chloroform, and acetonitrile that exhibit various levels of toxicity through inhalation and skin contact. All organic solvents used in this test method should be handled in a chemical fume hood. Avoid inhalation of the solvents. All the organic wastes should be disposed of appropriately.

9.4 The waste of the sample extract should be handled with proper care and precaution. Follow laboratory safety protocol to appropriately dispose of used test sample. The user is advised to follow relevant local regulatory requirements (for example, OSHA), suppliers' safety data sheets, institutional requirements, and recommended procedures pertaining to safe handling and disposal of all chemicals used in this test method.

[4] *ACS Reagent Chemicals, Specifications and Procedures for Reagents and Standard-Grade Reference Materials*, American Chemical Society, Washington, DC. For suggestions on the testing of reagents not listed by the American Chemical Society, see *Analar Standards for Laboratory Chemicals*, BDH Ltd., Poole, Dorset, U.K., and the *United States Pharmacopeia and National Formulary*, U.S. Pharmacopeial Convention, Inc. (USPC), Rockville, MD.

10. Preparation of Mobile Phases

10.1 *Mobile Phase A: Acetonitrile/Water (90/10 v/v) + 5 mmol/L Ammonium Acetate:*

10.1.1 Filter deionized water (>18 MΩ cm) through a 0.2 µm membrane using a bottle top vacuum filter system. This step is not needed in the case of a water purification system with an attached 0.2 µm filter.

10.1.2 Rinse an empty solvent bottle (1 L) with deionized water thoroughly and dry the bottle.

10.1.3 In a 1 L volumetric flask, transfer 3.85 g ± 0.02 g of ammonium acetate quantitatively, dissolve the salt thoroughly with ≈800 mL of deionized water, equilibrate the solution at room temperature, and then fill the flask with deionized water to the 1 L graduation mark. This will provide 50 mmol/L solution of ammonium acetate.

10.1.4 Transfer 100 mL of 50 mmol/L ammonium acetate and 900 mL of LC-MS-grade acetonitrile in the clean and dry 1 L bottle. The volumes are measured using a graduated cylinder (100 mL and 1 L, respectively).

10.1.5 Degas the solution for 10 min to 15 min using an ultrasonic bath before using it as mobile phase A. The use of an in-line degasser module further helps to achieve a stable baseline during an analytical run.

10.1.6 Transfer the degassed solution (mobile Phase A) to an empty, clean, and dry 1 L reservoir attached to the HPLC.

10.2 *Mobile Phase B: Methanol + 5 mmol/L Ammonium Acetate:*

10.2.1 To prepare 1 L of mobile Phase B, weigh 385 mg ± 10 mg of ammonium acetate and transfer it quantitatively to an empty 1 L volumetric flask, fill the flask with ≈800 mL of LC-MS-grade methanol to dissolve ammonium acetate, equilibrate the solution at room temperature, and then fill the flask with methanol to the 1 L graduation mark to make a homogeneous 5 mmol/L ammonium acetate solution in methanol.

10.2.2 Transfer the methanolic 5 mmol/L ammonium acetate solution to a clean and dry 1 L bottle.

10.2.3 Sonicate the solution to homogenize the analytes as needed and degas the solution for 10 min to 15 min using an ultrasonic bath. Use of an in-line degasser module further helps to achieve a stable baseline during an analytical run.

10.2.4 Transfer the degassed methanol with 5 mmol/L ammonium acetate to an empty, clean, and dry 1 L reservoir on HPLC.

NOTE 1—Sparging with helium can be used as an alternative to the ultrasonic degassing and in-line vacuum degassing combination as recommended in this test method.

11. Preparation of Calibration Standards

NOTE 2—*Gravimetric Measurements*—All working solutions in this test method are prepared gravimetrically using an analytical balance (0.0001 g accuracy). The concentration of an analyte is expressed in units of µg analyte per g of solution. Although the volumetric preparation shows close agreement with the gravimetric preparation, it is known that a 1 % to 5 % error can be introduced during small volume transfers and, hence, bias the quantitation results. The analytical balance provides better measurement resolution (that is, more significant figures) than mechanical pipettes and offers better accuracy.

NOTE 3—Conditioning the pipette tip with appropriate solvents before transfer of calibration standards for weighing, working promptly with the stock solutions, and weighing the volatile liquids in securely capped containers using a secondary container are highly recommended practices.

NOTE 4—The user is required to provide a certificate of analysis or equivalent alternative information on the source, purity, storage conditions, retest/expiration date, and lot/batch number of reference standards or high-purity chemicals to ensure quality and stability (**20**).

11.1 Powder stocks of the analytes stored in the freezer at –20 ºC should be allowed to equilibrate at ambient temperature before weighing.

11.2 Deionized water (>18 MΩ cm) should be filtered through a 0.2 µm membrane using a bottle top vacuum filter system before use. This step is not needed in the case of water purification system with an attached 0.2 µm filter.

11.3 *Preparation of Individual Stock Solutions*—Prepare individual stock solutions (≈1000 µg/g) of cholesterol, DSPE-PEG 2000, and HSPC in separate amber glass vials (20 mL).

11.3.1 Rinse three empty 20 mL amber glass vials with deionized water and dry thoroughly.

11.3.2 Label each vial with the corresponding analyte *j*, where *j* = cholesterol, DSPE-PEG 2000, and HSPC. Put a cap on each bottle.

11.3.3 Weigh each capped vial on an analytical balance and record the mass to ±0.1 mg as W_{0j}.

11.3.4 Individually weigh 10 mg ± 2 mg of cholesterol, DSPE-PEG 2000, and HSPC on an analytical balance, transfer each analyte to the appropriately labeled vial, put a cap on each vial, and record the mass of the capped vial + analyte as W_{1j}.

11.3.5 Transfer 12.5 mL (≈10.0 g) of LC-MS-grade methanol using a mechanical pipettor to the vial from 11.3.2 labeled as cholesterol. Put a cap on each vial.

11.3.6 Repeat 11.3.5 for DSPE-PEG 2000 and HSPC.

11.3.7 Weigh each capped vial containing, analyte *j*, and methanol on an analytical balance and record the mass to ±2 mg as W_{2j}.

11.3.8 Dissolve the solids in each vial thoroughly by vortex mixing for 1 min.

NOTE 5—Complete solubilization and formation of a homogeneous solution may require an additional 5 min bath sonication.

11.3.9 Store the vials of the individual stock solutions at –20 °C until needed. Stock solutions are stable up to four months under this condition.

11.3.10 The individual stock concentrations of the three components, C_j, are calculated using Eq 4. The purity (*j*) % for each component should be the value from the manufacturer's certificate of analysis:

$$C_j = \frac{\text{Mass of the analyte}}{\text{Total mass of solution}} = \text{(purity (}j\text{)\%)} \times [(W_{1j} - W_{0j}) / (W_{2j} - W_{0j})] \times 10^6 \text{ µg/g} \quad (4)$$

Where *j* = cholesterol, DSPE-PEG 2000, or HSPC.

11.4 *Preparation of Individual Stock Solutions*—Prepare individual stock solutions (≈500 µg/g) of cholesterol, DSPE-PEG 2000, and HSPC in separate amber glass vials (20 mL).

11.4.1 Rinse three empty 20 mL glass vials with deionized water and dry thoroughly.

11.4.2 Label each vial with the corresponding component (analyte) *j*, where *j* = cholesterol, DSPE-PEG 2000, or HSPC. Put a cap on each vial.

11.4.3 Follow 11.3.3 – 11.3.8 (with a lesser amount of analyte *j*, for example, 5 mg ± 1 mg) to obtain *Cj* values of 500 µg/g.

11.4.4 The concentration of each analyte *j* in the individual stock solutions is calculated using Eq 4.

11.4.5 Store the vials of the individual stock solutions at –20 °C until needed. Stock solutions stored under this condition are stable up to four months.

11.5 *Preparation of Calibration Standards for Individual Analyte:*

11.5.1 Six calibration standards (that is, calibration levels) of individual analytes: (*1*) cholesterol with target concentrations ranging from 5 µg/g to 175 µg/g (for example, 5 µg/g, 10 µg/g, 25 µg/g, 50 µg/g, 100 µg/g, and 175 µg/g); (*2*) DSPE-PEG 2000 with target concentrations ranging from 5 µg/g to 300 µg/g (for example, 5 µg/g, 10 µg/g, 50 µg/g, 100 µg/g, 150 µg/g, and 300 µg/g); and (*3*) HSPC with target concentrations ranging from 5 µg/g to 200 µg/g (for example, 5 µg/g, 10 µg/g, 50 µg/g, 100 µg/g, 150 µg/g, and 200 µg/g) are prepared from the 1000 µg/g and 500 µg/g stock solutions (see 11.3 and 11.4 respectively). ELSD has stronger analytical sensitivity for cholesterol compared to DSPE-PEG 2000 and HSPC, therefore the highest calibration level for cholesterol is targeted at ≈175 µg/g. In this test method, all measurements are gravimetric, and therefore, the user does not need to transfer the exact volume of methanol or analyte stock solution to reach the exact target analyte concentration but does need to record the accurate mass and calculate the concentration of each calibration standard by following the example described in 11.5.7 – 11.5.9.

11.5.2 Rinse one empty 10 mL glass vial with deionized water and dry thoroughly.

11.5.3 Rinse one measuring cylinder with deionized water and dry thoroughly.

11.5.4 For calibration Level 1, C_j^{cal} = 5 µg/g, where *j* = cholesterol, DSPE-PEG 2000, or HSPC. Label the 10 mL vial as Level 1 (cholesterol, DSPE-PEG 2000 or HSPC). Put a cap on the vial.

11.5.5 For that level, weigh the capped vial on an analytical balance and record the mass to ±0.1 mg as W_{0j}.

11.5.6 Add the volume of the stock solution with analyte *j* = cholesterol to the vial as given in Table 1 for Level 1. Place a cap on the vial. Record the mass as A_{1j} in units of g.

11.5.7 Add the volume of methanol to the vial as given in Table 1 for Level 1. Place a cap on the vial. Record the mass as A_{2j} in units of g. The three masses, W_{0j}, A_{1j}, and A_{2j}, will be required to calculate the actual mass of analyte *j* present in the solution and the total mass of analyte *j* plus solvent (that is, methanolic solution of analyte *j*).

11.5.8 The concentrations of six stock solutions for three analytes, C_j (µg/g) (≈1000 µg/g and ≈500 µg/g) are precalculated in 11.3.10 and 11.4.5, respectively. These values are used in 11.5.9 to calculate the concentration of a calibration standard.

11.5.9 The final concentration of the calibration standard for analyte *j* is determined by Eq 5:

$$C_j^{cal}(\mu g/g) = C_j \times [(A_{2j} - A_{1j})/(A_{1j} - W_{0j})] \quad (5)$$

11.5.10 Repeat 11.5.2 – 11.5.9 for calibration levels 2 to 6, using volumes of each analyte stock solution and methanol shown in Table 1.

11.5.11 Repeat 11.5.2 – 11.5.10 for the other two analytes, *j* = DSPE-PEG 2000 and HSPC.

11.5.12 Homogenize each individual analyte + methanol solution for calibration levels 1 to 6 by vortex mixing. Store the calibration solutions at 0 °C to 4 °C (for one day) or –20 °C for longer storage. The solutions are stable up to one month at –20 °C.

11.5.13 A representative table for the six calibration standards with the target analyte concentrations is provided in Table 1.

NOTE 6—The concentrations provided in Table 1 are the examples of target concentrations for various calibration levels. Eq 5 shall be used to obtain mass-based concentrations for any target analyte concentration.

11.5.14 The individual calibration plots are used only to verify the calibration range in which the regression model and the fit remains linear, and to choose the concentration range for preparing calibration standards containing mixture of the three analytes.

11.6 *Preparation of Calibration Standards of Analyte Mixtures:*

11.6.1 Mixtures of cholesterol, DSPE-PEG 2000, and HSPC are utilized to construct calibration curves containing a minimum of six calibration levels, and the constructed calibration curve within the bracketed range is used for method validation and the quantitation of all three analytes present in the test samples.

11.6.2 Each calibration standard (level) is obtained by combining the three stock solutions of individual analytes (either ≈1000 µg/g or ≈500 µg/g stock) in a 1:1:1 mass ratio with methanol in a 10 mL vial. Example of solution preparation parameters for various concentration levels (mass ratio of cholesterol: DSPE-PEG 2000: HSPC ≈ 1:1:1) are summarized in Table 2.

TABLE 1 Example of Six Calibration Standards for Individual Analyte Prepared from Two Stock Solutions[A] (1000 and 500 µg/g)

Cal. level	Target conc. of analyte in methanol (µg/g)	Conc. of stock used (ppm, µg/g) = C_j	Total mass of the target cal. standard (g)	Amount of analyte needed (C_j)		Amount of methanol added (mL)
				Mass (µg)	Stock Volume (mL)	
1	5	500	4	20	0.05	5.00
2	10	500	4	40	0.10	4.95
3	50	500	4	200	0.25	4.80
4	100	500	4	400	0.50	4.55
5	150	1000	4	600	0.76	4.29
6	300	1000	4	1200	1.52	3.35

[A] To estimate the volume of analyte stock solution, the density of methanol is used as the density of stock. Other calibration standards with intermediate analyte concentration, for example, concentrations of 25 µg/g, 75 µg/g, 175 µg/g, or 200 µg/g can be prepared similarly.

TABLE 2 Example of Calibration Standards of Analyte Mixtures Prepared from Two Stock Solutions[A] (1000 and 500 µg/g)

Cal. level	Target conc. of each analyte in the mix (µg/g)	Conc. of stock used (ppm, µg/g) = C_j	Total mass of the target cal. standard (g)	Amount of analyte needed (C_j)		Amount of methanol added (mL)
				Mass (µg)	Stock Volume (µL)	
1	5	500	2	10	25	2.43
2	10	500	2	20	50	2.38
3	15	500	2	30	75	2.30
4	25	500	2	50	125	2.15
5	50	1000	2	100	125	2.15
6	75	1000	2	150	188	1.95
7	200	1000	2	400	500	1.00
8	300	1000	2	600	750	0.25

[A] To estimate the volume of analyte stock solution, the density of methanol is used as the density of stock. The analyst is not limited to example concentrations, and any six concentrations (evenly spaced) can be used to establish a linear fit with $r^2 \geq 0.995$. Other calibration standards with intermediate analyte concentration, can be prepared similarly.

11.6.3 To prepare the eight levels of the calibration standard mixtures shown in Table 2, follow the steps similar to those described in 11.5.2 – 11.5.7.

11.6.4 Rinse the eight empty 10 mL glass vials with deionized water and dry thoroughly.

11.6.5 Label each vial with the corresponding calibration level (1 to 8).

11.6.6 Add appropriate volumes of the three individual stock solutions (cholesterol, DSPE-PEG 2000, and HSPC) and methanol as given in Table 2 for the calibration level. Place a cap on each vial.

11.6.7 Record the mass of each capped vial after the addition of each analyte solution.

11.6.8 Add the appropriate volume of methanol to each vial as given in Table 2 for the calibration level. Place a cap on each vial.

11.6.9 Record the mass of each capped vial after the addition of methanol.

11.6.10 The final concentration of an individual analytes j (where j = cholesterol, DSPE-PEG 2000, or HSPC) in a calibration standard mixture can be calculated as:

$$C_j^{cal}(\mu g/g) = [(\text{mass of analyte added, } \mu g)/(\text{total mass of analyte+methanol, g})] \quad (6)$$

Where the mass of analyte j added (µg) = C_j × (mass of the individual stock solution added, g).

11.6.11 Homogenize the mixture by vortex mixing the solution. Store these calibration standard mixtures at 0 °C to 4 °C (for one day) or –20 °C for longer storage. Stock solutions are stable up to one month at –20 °C.

12. Preparation of Samples for QC, Method Specificity, Recovery Experiment, and Method Precision

12.1 QC samples are to be prepared by mixing cholesterol, DSPE-PEG 2000, and HSPC from the 500 µg/g stock solutions. A mixture of 25 µg/g (cholesterol), 75 µg/g (DSPE-PEG 2000), and 75 µg/g (HSPC) in methanol will serve as QC samples.

12.2 To prepare ≈ 2.0 g of QC sample, aliquots of 100 µL, 300 µL, and 300 µL of cholesterol, DSPE-PEG 2000, and HSPC, respectively, from individual stock solutions (concentration ≈500 µg/g) is transferred to a 10 mL glass amber vial. To this vial, add ≈1.60 mL of LC/MS-grade methanol and mix thoroughly using vortex mixer. Masses in each step shall be recorded. The component concentrations in the QC sample are determined based on the masses of each of three analytes and the mass of methanol solvent that have been added.

12.3 To assess the specificity: (*1*) prepare a mixture of cholesterol, DSPE-PEG 2000, and HSPC having each analyte concentration of 50 µg/g in methanol as described in Table 2; (*2*) prepare a matrix blank which is a methanol solution containing possible matrices that are expected to be present in the test sample (that is, liposomal formulation); (*3*) prepare a solvent blank containing only methanol; and (*4*) prepare three independent solutions of cholesterol (50 µg/g), DSPE-PEG (50 µg/g), and HSPC (50 µg/g) in methanol as described in Table 1.

12.4 For the accuracy study, prepare three replicates in methanol of a set of samples with the following analyte concentrations: (*1*) (low) mixture of cholesterol, DSPE-PEG 2000, and HSPC with each analyte concentration of 25 µg/g; (*2*) (medium) mixture of cholesterol, DSPE-PEG 2000, and HSPC with each analyte concentration of 50 µg/g; and (*3*) (high) mixture of cholesterol, DSPE-PEG 2000, and HSPC with each analyte concentration of 100 µg/g (see Table 2 for preparation parameters). The exact analyte concentrations for each set are calculated from the measured masses of the analyte(s) present in a measured amount of solution (analyte + methanol).

12.5 For repeatability (that is, method precision), the same set of solutions that have been prepared in 12.4 can be used.

12.6 To assess matrix effects, prepare three samples in methanol that contain all of the expected matrix components at expected concentration levels as in the test sample. The analyte concentrations chosen for this study were (low, medium, and high): (*1*) cholesterol (25 µg/g), DSPE-PEG 2000 (25 µg/g), and HSPC (25 µg/g); (*2*) cholesterol (50 µg/g), DSPE-PEG 2000 (50 µg/g), and HSPC (50 µg/g); and (*3*) cholesterol (100 µg/g), DSPE-PEG 2000 (100 µg/g), and HSPC (100 µg/g).

12.7 Store the solutions at –20 °C.

12.8 Prepare a solvent blank for a control experiment.

13. Sample Preparation

13.1 Two different sample preparation procedures have been tested during the development and verification of this test

method: methanol solubilization (single phase) and Bligh-Dyer extraction (two phase) (**21**). Methanol solubilization involves one-step dilution of the liposomal lipids in methanol, whereas the Bligh-Dyer method involves multiple steps to extract the analytes (lipids) into an organic solvent layer via two-phase extraction and reconstitution of the analytes in methanol, as described in Appendix X3. Unlike methanol solubilization in which all excipients are dissolved in the solvent, the Bligh-Dyer extraction method minimizes undesirable water-soluble excipients including salts, sugars, and hydrophilic drug components present in liposomal formulations. However, the Bligh-Dyer method is a cumbersome and multistep procedure with potential for sample loss or contamination. A variation of <3 % was noted in the quantitation of lipids between the two methods during the method development, however, that may vary depending on variables introduced during the extraction procedure. Thus, in this test method, methanol solubilization is recommended because of the ease of the sample preparation procedure and no apparent interferences affecting the quantitation of lipids. The user may adopt the Bligh-Dyer method only in the case in which baseline noise or a matrix effect is noted (see Appendix X3).

13.2 *Methanol Solubilization Protocol for Preparation of a Test Sample:*

13.2.1 To obtain an aliquot for the preparation of a test sample, the original container of the liposomal formulation with a valid expiration date (as indicated by the manufacturer) shall be used. Sampling shall be performed in a biological safety cabinet with necessary precautions.

13.2.2 Swirl the closed container of the liposomal formulation gently.

13.2.3 Fill a 1 mL syringe outfitted with a needle and dispense carefully into a clean 2 mL amber glass vial. Maintain a positive pressure in the syringe before inserting the needle into the original container of the liposomal formulation through the rubber seal to avoid introducing air into the sample.

13.2.4 Label an empty and clean 15 mL polypropylene tube and record the mass of the capped polypropylene tube (w_1) to the nearest 0.0001 g.

13.2.5 Aliquot 100 µL of the liposomal formulation from the test unit into the weighed polypropylene tubes. Record the mass after sample addition (w_2) to the nearest 0.0001 g.

13.2.6 Add 10.00 mL of methanol (LC-MS grade) to achieve a 1:100 (*v/v*) sample dilution. Record the mass after methanol addition (w_3) to the nearest 0.0001 g.

NOTE 7—The solubility range of an unknown formulation (test unit) in methanol shall be determined before analysis to ensure accuracy of the results.

13.2.7 Vortex mix the solution for 15 min with continuous shaking at room temperature.

13.2.8 Use this test sample for characterization and data analysis. The final concentration of the sample (in µg/g) after methanol dilution can be calculated using the weights recorded during the sample preparation:

$$\text{Sample Concentration} = \frac{w_2 - w_1}{w_3 - w_1} \times 10^{-6} \quad (7)$$

Where w_1, w_2, and w_3 indicates mass (in gram) of the empty tube, mass of the tube + test sample, and mass of tube + test sample + methanol, respectively.

13.2.9 Repeat 13.2.4 – 13.2.8 to obtain three independent methanol solubilized samples and label as 1, 2, and 3.

13.2.10 Samples may need to be diluted with LC/MS-grade methanol appropriately to ensure that the response of the detector is within the calibration range for all analytes. The dilution factor can be calculated as:

$$\text{Dilution factor} = \frac{\text{Original sample concentration}}{\text{Concentration of diluted sample for analysis}} \quad (8)$$

13.2.11 The diluted sample (that is, the test sample) is transferred to a 2.00 mL LC vial for analysis.

13.2.12 Test samples should be stored at 0 °C to 4 °C for short-term storage (≈one day) or at –20 °C for longer-term storage (≤seven days).

14. Preparation of Apparatus

14.1 *Analytical Instrument Qualification*—Instrument performance is critical to reliable quantitative data. Instrument performance should be qualified following the manufacturer's guidelines to ensure each component of the instrument functions as expected.

14.2 *Column Conditioning:*

14.2.1 The HPLC-ELSD system, including the nitrogen flow, should be turned on at least for 45 min in advance of sample analysis. After turning on the instrument, make sure the solvent elution through the HPLC column is set to waste.

14.2.2 After powering the instrument, purge the mobile phase to waste for 15 min (column is bypassed).

14.2.3 After purging the system with the desired solvent, the column should be equilibrated with 40 % mobile Phase B at a flow rate of 0.60 mL/min for at least 15 min.

14.3 *Detector Stabilization*—During the column conditioning, the ELSD detector will be stabilized with all necessary parameters set on the instrument. Make sure the nebulizing gas, temperature and pressure are in the set range.

14.4 *System Suitability Check:*

14.4.1 After the column is equilibrated to obtain a constant column pressure, temperature, and a stable detector baseline, a standard of cholesterol and lipid mixture of known concentration (for example, a QC sample) should be injected at least three times to condition the column.

14.4.2 Process the data from the QC sample and make sure the retention time remains unaltered and the peak area for analytes in the chromatogram satisfy the quality criteria (that is, variation of area <5 %).

14.4.3 Once the LC and ELSD detector pass the system suitability check and a stable baseline is achieved, samples may be analyzed.

15. Procedure

15.1 *HPLC Method Parameters:*

15.1.1 Use a pre-mixed acetonitrile/water (90/10 *v/v*) + 5 mmol/L ammonium acetate (mobile Phase A) and methanol + 5 mmol/L ammonium acetate (mobile Phase B) as solvents for HPLC.

15.1.2 The following parameters shall be used for the separation and quantification of cholesterol and lipids in liposomal formulation: HPLC with the flow rate of 0.60 mL/min and an injection volume of 15 µL at a column temperature of 35 °C. Total runtime of separation was 19 min as shown in Table 3.

15.1.3 The elution gradient is summarized in Table 3.

NOTE 8—UHPLC can also be used for separating the analytes with an appropriate choice of column. However, method transfer from HPLC to UHPLC can introduce a difference in peak retention time and, in some cases, peak order as a result of lower system volume and reduced band spreading. Therefore, optimization of the gradient elution conditions may be required for appropriate peak resolution. Also, if UHPLC is used, the ELSD parameters should be optimized, and the method should be re-validated.

15.1.4 The solvent blanks (methanol solvent only) should be run between two different samples or one in every six consecutive injections or both to avoid any carryover effect.

15.2 *ELSD Method Parameters*—Maintain the ELSD evaporation temperature at 50 °C, nebulization temperature at 50 °C, nitrogen gas flow rate at 1.70 SLM, data rate at 80 Hz, and smoothing at 50 (5.0 s).

NOTE 9—In principle, the temperature of the nebulizer and evaporation tube and nitrogen flow rate can influence the detector response. In this test method, we have observed that the ELSD responses for cholesterol and HSPC are reduced significantly with increases in temperature of the evaporation tube from 60 °C to 80 °C. However, no significant effect has been observed as the nebulization temperature varies in the range of 40 °C to 55 °C. ELSD response varies inversely with the flow rate of nitrogen, and thus, gas flow rate should be constant throughout the test method. The nitrogen flow rate can be set within the range of 1.50 SLM to 1.70 SLM without losing peak resolution, LOD, or ULOQ. Gain is adjusted automatically.

15.3 A needle wash (one time) with methanol shall be programmed for each injection to avoid contamination.

15.4 The above conditions including the mobile phase composition, nebulization temperature, evaporation temperature, and nitrogen flow rate have been optimized to obtain the best separation of analytes through HPLC and the response of ELSD detector.

15.5 *Method Validation Parameters:*

15.5.1 *Specificity*—To demonstrate the ability of the test method to measure an analyte of interest in the presence of other components that are expected to be present in the test sample matrix, a representative chromatogram shall be generated from a mixture of cholesterol, DSPE-PEG 2000, and HSPC, (12.3), with identified peaks. Compare this chromatogram to the chromatograms obtained from independent analyte solutions, a solvent blank, and a matrix blank (possible sample matrix other than cholesterol, DSPE-PEG 2000, and HSPC and the concentrations are subjected to sample preparation as described in 13.2) to ensure peak integrity of all three target analytes, including peak shape and elution profile. Here, methanol has been used as the solvent blank to assess any peak interference from potential impurities in the solvent.

TABLE 3 Elution Gradient with Mobile Phase A, Acetonitrile/Water (90/10 *v/v*) with 5 mmol/L Ammonium Acetate and Mobile Phase B, Methanol with 5 mmol/L Ammonium Acetate

Time (min)	Mobile phase, A(%)	Mobile phase, B(%)	Flow rate (mL/min)
0	60	40	0.6
5	60	40	0.6
7	10	90	0.6
17	10	90	0.6
18	60	40	0.6
19	60	40	0.6

15.5.2 *Linearity*—Use calibration standards of mixture of analytes ranging from 5 µg/g to 300 µg/g for linearity measurements (perform triple injections for each calibration level). Determine the peak areas for cholesterol, DSPE-PEG 2000, and HSPC by integration using the chromatography software. ELSD responses with analyte concentration are nonlinear, and the response is often modelled empirically with a power law function of the form:

$$A = \alpha(M_{inj})^{\beta} \tag{9}$$

where:

A = response (peak area),
M_{inj} = mass of analyte injected, and
α and β = constants indicating sensitivity and curve shape, respectively.

15.5.2.1 To obtain a linear fit, logarithmic transformation of the peak area (the detector's response) and the mass of the injected analyte over a suitable concentration range can be performed for providing a linear regression model. Because the mass of the analyte is proportional to the concentration of analyte injected, it is convenient to replace mass (M_{inj}) by concentration (C) in Eq 9. Individual calibration curves for each lipid component are constructed from the plot of log (peak area, A) versus log (concentration, C). The logarithmic regression model for a bracketed range of analyte concentrations is applied and fitted to the equations for cholesterol, DSPE-PEG 2000, and HSPC as:

$$\log(\text{peak area}) = \beta \times \log(\text{conc.}) + \log(\alpha) \tag{10}$$

where:

β = slope, and
$\log(\alpha)$ = intercept.

15.5.3 The coefficient of determination (also called as regression coefficient) (r^2), slope, intercept, and equation of the calibration standard curves were determined. The acceptance criteria for the r^2 value should be greater than or equal to 0.995.

NOTE 10—*(1)* The HSPC component shows two peaks in the chromatogram corresponding to two fatty acid distributions, that is, 16:0 (HSPC 1) and 18:0 (HSPC 2). Hence, for the HSPC calibration curve, the peak areas from HSPC 1 and HSPC 2 are added before the logarithmic transformation. *(2)* The use of bracketed calibrants over a concentration range that encloses the concentration of the unknown sample provides more accurate results.

15.5.4 *Precision: Repeatability*—Nine independent replicates of lipid mixtures should be tested with the set parameters of HPLC (15.1) and ELSD (15.2). One set of three replicates is prepared at a concentration close to the target levels of the analytes, and the other two mixtures (low and high) are prepared at a different concentration within the range encompassed by the calibration standard curve. Using the mean peak areas (n = 3), SD, RSD, and % RSD should be computed.

15.5.5 *Intermediate Precision*—For an intermediate precision study, a QC sample should be analyzed by a single operator for five consecutive days with the same instrument. Each analyte concentration should be determined as detailed in Section 16. Calculate mean (*M*), SD, and % RSD as in Eq 11-13 in compliance with Practice E177 and Terminology E456 and report the computed data.

$$M = \frac{\sum_{i=1}^{N} X_i}{N} \tag{11}$$

$$SD = \sqrt{\frac{\sum_{i=1}^{N} (X_i - M)^2}{N - 1}} \tag{12}$$

$$\%RSD = 100 \times \frac{SD}{M} \tag{13}$$

where:

N = number of measurements and
X_i = value of measurements indexed by i.

15.5.6 *Accuracy*—For an accuracy study, nine samples (three concentration levels and three replicates of each concentration level, see 12.4) containing mixture of three analytes were used. The concentration of each analyte in the injected sample using this method should be calculated as described in Section 16. Analyze the data and determine each analyte concentration (mean recovery) with triplicate injection, SD, RSD, and percent recovery for all three sets of samples. Recovery % for low, medium, and high concentration samples are calculated using:

$$\%\text{recovery} = \frac{\text{Mean recovery}}{\text{Injected conc.}} \times 100 \tag{14}$$

15.5.7 *Limit of Detection*—Run six replicates for the lowest analyte concentration that the detector can reliably differentiate from the baseline. A mixture of calibration standards should be used. Signal-to-noise ratio, S/N ≥ 3, is generally considered acceptable for the estimation of LOD.

15.5.8 *Limit of Quantitation*—Similarly, analyze six replicates of analyte mixtures for cholesterol, DSPE-PEG 2000, and HSPC to determine the LOQ. It is expressed as the lowest analyte concentration that can be reliably quantified. The typical signal-to-noise ratio is S/N ≥ 10.

15.5.9 *Matrix Effect*—To evaluate the matrix effect, nine sample mixtures at each concentration level of 25 µg/g (low), 50 µg/g (medium), and 100 µg/g (high) were prepared by spiking stock solutions in methanol, see 12.6. Methanol should be used for dilution to reach the target concentration level, while the amount of matrix blank should be kept the same as the equivalent concentration that is present in the test samples. Determine each analyte concentration (mean recovery) with triplicate injection and percent recovery for all three sets of samples as described in 15.5.6.

15.5.10 *Quality Criteria*—Analyte peaks in the chromatogram should be well resolved. For linearity, coefficient of determination (r^2) values should be greater than 0.995. RSD values for repeatability and intermediate precision studies should be less than 5 %. For accuracy studies, data should pass acceptance criteria of mean recovery of ±20 % for cholesterol, DSPE-PEG 2000, and HSPC (**3**).

15.6 *Sample Analysis:*

15.6.1 After preparation of the sample in methanol as described in 13.2, the test sample should be run, and the data should be analyzed. Peak areas were determined for cholesterol, DSPE-PEG 2000, and HSPC by manual (when needed) or automatic integration using HPLC software. Concentrations were calculated from respective calibration curves (see 16.1).

15.6.2 To determine the absolute concentration in the test sample, multiply the concentration by the dilution factor. The sum of the absolute concentration values represents the total concentration of the three components in the liposomal formulation.

15.6.3 The report should include method validation parameters and sample analysis.

16. Calculation and Interpretation of Results

16.1 Peak areas were determined for cholesterol, DSPE-PEG 2000, and HSPC by automatic integration in the software package provided with the instrument. Manual integration was performed when deemed necessary by the user, but consistency should be maintained. Each chromatogram shall be inspected to ensure that the baseline is correctly drawn. The analyte concentration, *C*, can be determined using respective calibration curves as:

$$\text{Analyte concentration}\left(\frac{\mu g}{g}\right) = 10^{\left(\frac{\log(\text{peak area}) - \log(\alpha)}{\text{slope}}\right)} \tag{15}$$

16.2 The intercept and slope were obtained from the calibration curve, and the peak area is the integrated peak area of the unknown analyte present in the test sample.

16.3 Three independent test samples prepared by following the steps described in 13.2 were analyzed according to the test method. The concentration of each analyte was calculated from the average values of those three samples (n = 3).

16.4 To determine the absolute lipid concentration (mg/g) in the test sample, multiply the concentration by the dilution factor. The sum of the absolute concentrations of cholesterol, DSPE-PEG 2000, and HSPC gives the total lipid content in the liposomal formulation.

17. Report

17.1 For each test sample, report individual and total concentrations of each analytes, cholesterol, DSPE-PEG 2000, and HSPC in mg/g up to one decimal place and report the component ratio (DSPE-PEG 2000: HSPC: cholesterol) for each test sample. The report format should include method validation parameters. Refer to the example Sample Report in Appendix X2.

NOTE 11—If the user needs the sample concentration to be reported in mg/mL, a standard protocol for density measurement should be followed to measure the density of the test sample and used for conversion. The density used in the calculation needs to be documented (**22**).

18. Precision and Bias

18.1 *Precision*—The repeatability standard deviation from a single operator has been determined to be <5 %.