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Designation: E3297 − 21

Standard Test Method for Lipid Quantitation in Liposomal Formulations Using High Performance Liquid Chromatography (HPLC) with a Charged Aerosol Detector (CAD)[1]

This standard is issued under the fixed designation E3297; 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 is for the separation of lipids in liposomal formulations through high performance liquid chromatography (HPLC) and their quantitation using a mass-flow sensitive charged aerosol detector (CAD).

1.2 This test method is specifically for liposomal formulations containing cholesterol, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG 2000) and hydrogenated soy L-α-phosphatidylcholine (HSPC).

1.3 This test method is applicable to report the absolute concentrations and ratio of cholesterol, DSPE-PEG 2000, and HSPC in liposomal formulations. Assessment of the stability of the analytes in terms of their degradation profiles as a result of oxidation or hydrolysis is beyond the scope of this test method.

1.4 This test method includes calibration standards preparation, sample preparation, method validation, and sample analysis. This method also contains specifications for instrumentation and the chromatography experimental procedure.

1.5 The detection limit and quantitation limit for the analytes in this test method is in the range of 0.1–2.0 µg/g and 1.0–5.0 µg/g respectively. The analytical measurement range for cholesterol, DSPE-PEG 2000, and HSPC is 5–300 µg/g.

1.6 All observed and calculated values shall conform to the guidelines for significant digits and rounding as established in Practice D6026.

1.7 *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.

1.8 *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.*

1.9 *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

3. Terminology

3.1 *Definitions:*

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

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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.

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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. It 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 a component 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 this test method, the charged aerosol detector (CAD) response expressed as current (pA) is plotted against elution time (min). For analysis, the characteristic detector response of the eluting analyte is typically evaluated from the peak area recorded in the chromatogram. This peak area (*A*) can be expressed mathematically as the integral of detector response for analyte over an elution time interval from t_1 to t_2:

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

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:

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

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

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

3.1.13 *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**

3.1.14 *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**).

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

3.1.15.1 *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.

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

3.1.16.1 *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.

3.1.17 *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**).

3.1.18 *matrix blank, n*—substance that closely matches the samples being analyzed with regard to matrix components, but has none of the analytes of interest.

3.1.18.1 *Discussion*—Ideally, the matrix blank does not contain the analyte(s) of interest but is subjected to all sample-processing operations including all reagents used to analyze the test samples. The matrix blank is used to determine the absence of significant interference as a result of the matrix, reagents, and equipment used in the analysis.

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

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

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

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

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

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

3.1.22 *3.1.22 peak resolution (R_s), 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:

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.

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

3.1.24 *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.

3.1.24.1 *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 predictor variable is CAD signal (pA) and the response variable is mass concentration. **E3080**

3.1.25 *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.

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

3.1.27 *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.

3.1.28 *solvent blank, n*—solution containing all reagents used in sample dissolution in the same quantities used for preparation of blank and sample solutions. **D7439**

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

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

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

3.1.30 *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 test determination.

3.2.1.1 *Discussion*—In this test method, a test unit solubilized with methanol and diluted to within the bracketed range is defined as test sample.

3.2.2 *test unit, n*—portion of a material that is sufficient 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 a test unit.

3.3 *Acronyms:*

3.3.1 *CAD*—Charged aerosol detector

3.3.2 *CRM*—Certified reference material

3.3.3 *DSPE-PEG*—1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]

3.3.4 *HPLC*—High performance liquid chromatography

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

3.3.6 *ID*—Inside diameter

3.3.7 *LC-MS*—Liquid chromatography-mass spectrometry

3.3.8 *LOD*—Limit of detection

3.3.9 *LOQ*—Limit of quantitation

3.3.10 *QA*—Quality assurance

3.3.11 *QC*—Quality control

3.3.12 *RCF*—Relative centrifugal force

3.3.13 *RSD*—Relative standard deviation

3.3.14 *SD*—Standard deviation

3.3.15 *µg/g*—Parts per million

3.3.16 *UHPLC*—Ultra high performance liquid chromatography

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4. Summary of Test Method

4.1 Cholesterol, DSPE-PEG 2000, and HSPC in the liposomal formulation are solubilized in methanol at 1:100 dilution by volume followed by vortex mixing. This solubilized sample is further subjected to quantitative analysis using reversed-phase HPLC with CAD. Before sample analysis, the test method is validated for linearity, precision, accuracy, specificity, LOD, and LOQ.

4.2 Calibration curves of concentrations ranging from 5–300 µg/g are established for cholesterol, DSPE-PEG 2000, and HSPC. For analysis, as CAD response is nonlinear in the measured range, the linear regression model is applicable only after logarithmic transformation. Hence, log (peak area) versus log (concentration) is plotted for each analyte to obtain the corresponding calibration curve. Linear regression analysis gives the slope and intercepts necessary to quantify cholesterol, DSPE-PEG 2000, and HSPC in the test samples. Lipid component ratio (DSPE-PEG 2000: HSPC:cholesterol) shall be reported along with total lipid content in milligrams/grams.

5. Significance and Use

5.1 The growing interest in liposomal formulations in the pharmaceutical industry requires QC and thorough characterization and quantification of lipids that form liposomes (**6**). Lipid composition has proven to be a critical attribute of the liposomal formulation; it directly influences the stability of the formulation, drug loading, performance, size, and surface characteristics of the liposome. Cholesterol plays a key role in controlled drug release by adding stability to the liposome (**7**). Significant variation in the lipid composition and ratio of the components will influence the safety, biodistribution, drug efficacy, and drug release kinetics of the liposomal formulation (**8-11**).

5.2 This test method is a fast and reliable procedure for the quantification of cholesterol, DSPE-PEG 2000, and HSPC in liposomal formulations using HPLC-CAD.

5.3 This test method can be used for QC and QA and to ascertain variations in component profiling of liposomal formulations.

6. Interferences

6.1 Interferences caused by solvent impurities could lead to high baseline noise; hence, in this test method, LC-MS grade solvents are recommended.

6.2 Use a high-quality nitrogen source (≥95 % purity) that is free from water vapor, particles, and nonvolatile hydrocarbons such as compressor oils. The use of gases that allow either combustion of solvents or oxidation of analytes should be avoided.

6.3 Keep the auto sampler injection port and column clean as per the manufacturer's recommendations to avoid carryover or ghost peaks. Contamination of glass containers or vials used for this test method should be avoided.

6.4 If the user observes any matrix effects under the recommended test conditions, optimization of the sample preparation or modification in the chromatographic parameters (for example, solvent gradient or eluent flow rate) may be required. The Bligh-Dyer method described in Appendix X3 could potentially be adopted to remove water soluble excipients in test liposomal formulations if deemed necessary.

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

6.6 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) be acquired for calibration standards. If reference materials are not available, high-quality crystalline or lyophilized chemicals of known purity can be used.

7. Apparatus

7.1 HPLC with in-line degasser module.

7.2 CAD.

7.3 Reversed-phase HPLC BEH (Bridged Ethylene Hybrid) column-C18, 13 nm pore size, 3.5 µm particle size, 3 mm × 150 mm (ID × length) or equivalent column that can resolve all analytes of interest with $R_s \geq 1.5$.

7.4 Analytical balance that can accurately weigh with readability up to 0.0001 g.

7.5 Vortex mixer.

7.6 Mechanical pipettes with disposable tips covering from 0.002 to 10 mL.

7.7 Solvent reservoir bottles, 1 L.

7.8 Volumetric flask, 10 mL and 1 L.

7.9 Measuring cylinder, 0.5 and 1 L.

7.10 Polypropylene tubes, 15 mL.

7.11 Glass amber vials, 5, 10, and 20 mL.

7.12 Autosampler amber vials, 2 mL.

7.13 Ultrasonic bath.

7.14 0.2 µm bottle top vacuum filter.

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 reagent is of sufficiently high purity to permit its use without lessening the accuracy of the determination.

8.1.1 Acetonitrile, LC-MS grade.

8.1.2 Methanol, LC-MS grade.

8.1.3 Ammonium acetate deliquescent crystals, LC-MS grade.

8.1.4 *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 (>18 MΩ cm) high-purity (Type 1) water.

8.2 *Materials:*

8.2.1 Cholesterol, >99 % purity, powder form.

8.2.2 HSPC, >99 % purity, consisting of C16:0 (HSPC 1) and C18:0 (HSPC 2) fatty acids, powder form.

8.2.3 DSPE-PEG 2000, >99 % purity, powder form.

9. Hazards

9.1 Proper protective measures are required while handling liposomal formulations. This test method uses methanol and acetonitrile, which are flammable. All solvents should be handled in a chemical fume hood to avoid inhalation. Organic

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[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.

solvent waste should be disposed of according to local regulations. The CAD outlet should be connected to an exhaust as per the manufacturer's recommendation to avoid aerosol exposure.

10. Mobile Phase Preparation

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

10.1.1 Deionized (>18 MΩ cm) water must be filtered through a 0.2 µm bottle top vacuum filter. 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 reservoir bottle (1 L) with deionized water thoroughly and dry.

10.1.3 Weigh 3.85 ± 0.02 g of LC-MS-grade ammonium acetate deliquescent crystals and transfer to an empty volumetric flask (1 L).

10.1.4 Add ≈800 mL of 0.2-µm filtered deionized water and dissolve the salt thoroughly. Then, fill the volumetric flask up to the 1L graduation mark with deionized water to make a homogenous 50 mmol/L solution of ammonium acetate.

10.1.5 To prepare 1 L of mobile phase A, mix 100 mL of 50 mmol/L ammonium acetate and 900 mL of LC-MS-grade acetonitrile in the reservoir bottle (1 L) using a measuring cylinder.

10.1.6 Degas mobile phase A for 10 min using an ultrasonic bath at atmospheric pressure. Use of an in-line degasser module further helps to achieve a stable baseline during an analytical run.

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

10.2.1 To prepare 1 L of mobile phase B, weigh 385 ± 10 mg of LC-MS-grade ammonium acetate deliquescent crystals and transfer it to an empty volumetric flask (1 L).

10.2.2 Add ≈500 mL LC-MS-grade methanol to a volumetric flask and dissolve the salt thoroughly. Then, fill the flask up to the graduated mark with LC-MS-grade methanol.

10.2.3 Transfer all contents to a clean and dry solvent reservoir bottle.

10.2.4 Degas mobile phase B for 10 min using an ultrasonic bath at atmospheric pressure. Use of an in-line degasser module further helps to achieve a stable baseline during an analytical run.

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

11. Preparation of Calibration Standards

NOTE 2—*Gravimetric Measurements*—All working standards in this test method are prepared gravimetrically using an analytical balance (0.0001 g accuracy). 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 (**12**).

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—Chemicals stored in the freezer at –20ºC or as per manufacturer's recommendation should be allowed to equilibrate at ambient temperature before weighing.

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

11.2 *Preparation of Primary Stock Solutions:*

11.2.1 Rinse three empty 20 mL glass amber vials with deionized water and dry thoroughly. Cap the vials.

11.2.2 Label each vial with the corresponding component (that is, analyte) j, where j = cholesterol, DSPE-PEG 2000, or HSPC.

11.2.3 Weigh each capped vial on an analytical balance and record the mass to ± 2 mg as W_{0j}.

11.2.4 Individually weigh 10 ± 2 mg of each component (cholesterol, DSPE-PEG 2000, and HSPC) on an analytical balance. Record the masses as W_{1j}. Transfer the components to the appropriately labeled vials.

11.2.5 Rinse an empty volumetric flask with methanol and dry thoroughly.

11.2.6 Measure 10 mL of LC-MS-grade methanol in the clean volumetric flask. Add to the vial from 11.2.4labeled cholesterol. Cap the vial.

11.2.7 Repeat 11.2.5 and 11.2.6 for DSPE-PEG 2000 and HSPC.

11.2.8 Weigh each capped vial on an analytical balance and record the mass to ± 2 mg as W_{2j}.

11.2.9 Dissolve the solids in each vial thoroughly by vortex mixing for 1 min followed by bath sonication for 5 min.

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

11.2.11 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:

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

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

11.3 *Preparation of Intermediate Stock Solutions:*

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

11.3.2 Label each vial with the corresponding component (analyte), that is, j = cholesterol, DSPE-PEG 2000, or HSPC.

11.3.3 Rinse three empty volumetric flasks with deionized water and dry thoroughly.

11.3.4 Follow 11.2.5 – 11.2.11 (with lesser amount of W_{1j} to achieve a target concentration C_j = 500 µg/g).

11.3.5 Store the vials of the intermediate stock (I-stock) solutions at –20°C until needed. Intermediate stock solutions are stable up to four months under this condition.

11.4 *Calibration Curve:*

11.4.1 The calibration curve shall have six levels with analyte concentrations ranging from 5 (Level 1) to 300 (Level 6) µg/g (specifically 5, 25, 50, 100, 150, and 300 µg/g).

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11.4.2 Each calibration standard (level) is obtained by mixing either the stock solutions or the I-stock solutions of the three analytes in a 1:1:1 mass ratio with methanol in a 20 mL vial. Table 1 shows the estimated volumes of each analyte stock solution or I-stock solution and methanol for the various calibration levels. Use a gravimetric method similar to that in 11.2 and 11.3 to obtain the desired analyte concentrations.

11.4.3 *Preparation of Calibration Standards of Analyte Mixtures:*

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

11.4.3.2 Rinse one empty volumetric flask with deionized water and dry thoroughly.

11.4.3.3 Start with calibration Level 1 (target concentration = 5 µg/g). Label the 10 mL vial as Level 1.

11.4.3.4 Add appropriate volumes of each of the three stock solutions (cholesterol, DSPE-PEG 2000, and HSPC) and methanol according to Table 1.

11.4.3.5 Record the mass using an analytical balance in each step; mass of the vial with cap (A_0, g), mass of the vial + each stock solution of analyte j added (= A_{1j}, g), and mass of the vial + stock solutions + methanol (= A_2, g).

11.4.3.6 The final concentration of the calibration standard can be calculated as in Eq 5. The concentrations of the both primary and intermediate stock solutions in µg/g calculated in 11.2 and 11.3 are used here:

$$(C_j)_{cal}(\mu g/g) = C_j \times (\text{mass of the individual stock solution of analyte } j \text{ added}) / (A_2 - A_0) \quad (5)$$

Where mass of individual stock solution of analyte j added (µg) can be calculated using the masses recorded in the step 11.4.3.5.

11.4.4 Homogenize the mixture by vortexing.

11.4.5 Repeat 11.4.3.1 – 11.4.3.6 for the remaining five calibration levels.

11.4.6 Store the calibration solutions at 0–4°C (for one day) or –20°C (for longer storage). Calibration solutions are stable up to one month at –20°C.

NOTE 5—Since CAD sensitivity is comparable for all analytes, namely, cholesterol, DSPE-PEG 2000, and HSPC, calibration standards are prepared in 1:1:1 ratio. For example, a 100 µg/g calibration standard contains 100 µg/g each of cholesterol, DSPE-PEG 2000, and HSPC.

NOTE 6—The concentrations provided in Table 1 are examples of target concentrations for various calibration levels. The appropriate formula shall be used as described earlier to obtain mass-based concentrations (µg/g) for any calibration level, which are then used to plot a calibration curve. The bracketed concentration range should pass the quality criterion ($r^2 \geq 0.995$). The user is not limited to example target concentrations recommended in this test method and any six concentrations (evenly spaced) can be used to establish a linear bracketed concentration range that satisfies the above quality criterion.

12. Preparation of QC, Accuracy, and Precision Samples

12.1 A QC sample should be prepared similarly to calibration standards (11.4.3) by mixing cholesterol, DSPE-PEG 2000, and HSPC stock solutions with a final concentration of 75 µg/g of each analyte. Mixtures of 75 µg/g will serve as the QC sample.

12.2 Mixtures of 25 µg/g (low), 75 µg/g (medium), and 150 µg/g (high) should be prepared for precision and accuracy studies. The final concentration calculation shall use gravimetric measurements as described in 11.2.

12.3 To assess the matrix effect, test samples shall contain all possible matrix components from the test unit. The ratio of concentration of matrix components to analytes in the test sample should be close to that of the test unit. The three test samples should contain all three analytes, each with a concentration of 25 µg/g (low), 75 µg/g (medium), and 150 µg/g (high).

12.4 LC-MS-grade methanol will serve as a solvent blank for this test method.

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13. Test Sample Preparation

NOTE 7—Two sample preparation procedures were considered for this test method, namely, methanol (single-phase) and Bligh-Dyer (two-phase) extraction. Methanol solubilization as described in this section involves a one-step dilution of a liposomal sample in methanol, whereas the Bligh-Dyer method is a multistep procedure to extract lipids into an organic phase as described in Appendix X3. In comparison with the Bligh-Dyer extraction method, methanol solubilization is recommended in this test method because it reduces the complexity of the experimental procedure (**13**).

13.1 The Bligh-Dyer method minimizes undesirable water-soluble excipients present in a liposomal formulation, such as salts and sugars. However, it is a cumbersome, multistep procedure with potential for sample contamination. In the best-case scenario, the variation in quantitation of lipids was found to be <3 % between the two sample preparation methods for liposomal formulations. The Bligh-Dyer method should be adopted only when elimination of water-soluble excipients is necessary and such excipients are found to affect the quantitation of lipids. In the case when the Bligh-Dyer extraction is adopted, the matrix blanks should be subject to the same extraction procedure.

TABLE 1 Six Calibration Standards for Cholesterol, DSPE-PEG 2000, and HSPC Prepared from Individual Stock Solutions

NOTE 1—For the estimation of volumes of analyte stock solutions, it is assumed that the density of each stock solution is equal to the density of methanol.

Calibration Level	Target Concentration, µg/g	Approximate Volume of Each Analyte Added, µL		Approximate Volume of Methanol added, µL	Total Volume, mL
		Stock	I-Stock		
1	5	...	40	3880	4
2	25	...	200	3400	4
3	50	...	400	2800	4
4	100	400	...	2800	4
5	150	600	...	2200	4
6	300	1200	...	400	4

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

13.2.1 Swirl the closed container of liposomal formulation gently.

13.2.2 Use a 0.5 mL syringe to aliquot approximately 100 µL. Maintain a positive pressure in the syringe before inserting the needle into the closed container of the liposomal formulation through the rubber seal to avoid introducing air into the sample. Sampling may be performed in a biological safety cabinet with necessary precautions.

13.2.3 Add 100 µL of the liposomal formulation to a clean 15 mL polypropylene tube. Record the mass of the tube covered with a lid before (WE) and after the addition of the sample (W_S).

13.2.4 Add 10 mL of LC-MS-grade methanol to achieve 1:100 (v/v) sample dilution. Record the mass of the tube covered with a lid after methanol addition (W_F).

13.2.5 Vortex the sample at room temperature for 15 min.

13.2.6 Use this sample for further HPLC-CAD analysis. The mass concentration shall be calculated and expressed in µg/g using mass recorded as:

$$\text{Mass concentration } (\mu g/g) = (W_S - W_E)/(W_F - W_E) \quad (6)$$

13.2.7 Samples shall be diluted further by the user with LC-MS-grade methanol appropriately to ensure that detector response falls close to the middle of the calibration range (5–300 µg/g) for all analytes. Diluted mass concentration in µg/g should be calculated using gravimetric measurements as described in Section 11. Calculate the dilution factor as:

$$\text{Dilution factor} = \frac{\text{Mass concentration}}{\text{Diluted mass concentration for analysis}} \quad (7)$$

13.2.8 Repeat 13.2.1 – 13.2.7 to obtain three independent test samples for analysis.

13.2.9 The test samples should be analyzed within 24 h of methanol solubilization for optimum results.

14. Preparation of Apparatus

14.1 *Analytical Instrument Qualification*—The HPLC system and CAD performance are critical to obtain reliable quantitative data. Hence, the user shall perform analytical instrument qualification according to the manufacturer's recommendation.

14.2 *Column Conditioning:*

14.2.1 The HPLC-CAD system including the nitrogen flow should be powered up for at least 60 min in advance of sample analysis. After powering up the instrument, ensure that the solvent elution through the HPLC column is set to waste.

14.2.2 Run the mobile phase mixture (60:40, A:B) through the column at 0.7 mL/min for at least 30 min to condition the column and then direct the flow to the CAD.

14.3 *Detector Stabilization*—After conditioning the column for 30 min, run the same mobile phase mixture through the CAD to stabilize the detector baseline.

14.4 *System Suitability Check:*

14.4.1 After the column is equilibrated to obtain constant column pressure, temperature, and a stable detector baseline, inject the QC sample three times through the column.

14.4.2 Peak resolution for the analytes shall satisfy the quality criteria outlined in this test method (15.3.7).

14.4.3 Once the HPLC-CAD system passes the system suitability check, the system is ready to run test samples.

15. Procedure

15.1 *HPLC Method Parameters:*

15.1.1 The following parameters shall be used for the separation and quantification of the liposomal lipids. HPLC with a flow rate of 0.7 mL/min, injection volume of 5 µL, and temperature of 35°C gives ideal separation of cholesterol, DSPE-PEG 2000, and HSPC on this column. The auto sampler temperature should be set at 10°C. The total runtime of separation is 19 min followed by equilibration of the column under the elution gradient starting conditions (60:40 mobile phases A:B at a flow rate of 0.7 mL/min as shown in Table 2) for 2 min.

15.1.2 The elution gradients shown in Table 2 should be used for lipid separation, including equilibration of the column under the elution gradient starting conditions for 2 min.

15.1.3 Maintain the CAD evaporation temperature at 35°C, the CAD power function value at 1.00, the data collection rate at 10 Hz, the full-scale range at 100 pA, and the digital filter setting at 5 s.

15.1.4 Solvent blank (methanol only) should be run during HPLC-CAD analysis between two different samples, or one in every six injections, or both, to avoid any carryover effect.

15.1.5 LC/MS grade methanol should be used for HPLC needle washes between each injection to avoid contamination.

15.1.6 Ultra high performance liquid chromatography (UHPLC) can also be used for separation purposes. However, method transfer from HPLC to UHPLC can introduce a difference in peak retention time and, in some cases, peak order because of lower system volume and reduced band spreading. Also, if UHPLC is used, the CAD parameters should be optimized and the method should be re-validated.

15.2 *CAD Response:*

15.2.1 CAD is ideal for lipid analysis as it enables the analyte detection of non-chromophoric compounds. CAD and other aerosol detectors involve similar steps of primary spray droplet formation from the HPLC eluent stream and evaporation of volatile solvents from the droplets to form residue particles containing the analyte of interest. The size and number of residue particles formed increases proportionally with the amount of analyte present. These residue particles are

TABLE 2 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	% A	% B	Flow Rate, mL/min
0.0	60	40	0.7
6.0	60	40	0.7
6.1	10	90	0.7
16.0	10	90	0.7
16.1	60	40	0.7
19.0	60	40	0.7
21.0	60	40	0.7

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