Designation: E3324 – 22

Standard Test Method for Lipid Quantitation in Liposomal Formulations Using Ultra-High-Performance Liquid Chromatography (UHPLC) with Triple Quadrupole Mass Spectrometry (TQMS)[1]

This standard is issued under the fixed designation E3324; 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 the determination of lipid components in liposomal formulations, which includes sample solubilization in methanol followed by separation of the analytes using ultra-high-performance liquid chromatography (UHPLC) and detection with tandem mass-spectrometry (MS/MS). This test method adheres to multiple reaction monitoring (MRM) mass spectrometry on a triple quadrupole mass spectrometer (TQMS).

1.2 This test method is specific 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 of cholesterol, DSPE-PEG 2000, and HSPC and their ratio (DSPE-PEG 2000: HSPC: cholesterol) in liposomal formulations. Assessment of the stability of the analytes in terms of their degradation as a result of oxidation or hydrolysis is beyond the scope of this test method.

1.4 This test method includes calibration and standardization, sample preparation, UHPLC-TQMS instrumentation, potential interferences, method validation with acceptance criteria, sample analysis, and data reporting.

1.5 The detection limits for cholesterol, DSPE-PEG 2000, and HSPC using this test method are 5.3, 0.5, and 0.5 ng/g, respectively. In addition, the quantitation limits for cholesterol, DSPE-PEG 2000, and HSPC are 10.6, 0.8, and 0.5 ng/g, respectively.

1.6 This test method is intended for concentration ranges of 8-1600 ng/g for cholesterol, and of 2-400 ng/g for DSPE-PEG 2000 and HSPC.

1.7 All observed and calculated values shall conform to the guidelines for significant digits and rounding 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

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

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

[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.

2.2 *Federal Standard:*

21 CFR 211.194(a)(2) Code of Federal Regulations Title 21, Food and Drug administration, Department of Health and Human Services, Drugs: Current Good Manufacturing Practice for Finished Pharmaceuticals, Laboratory Records[3]

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 component and a common systematic error or bias component. **E177**

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

3.1.3 *analytical instrument qualification, n*—collection of documented evidence that an instrument performs suitably for its intended purpose (**1**).[4]

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

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

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

3.1.5.1 *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.

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

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

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

3.1.9 *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 *electrospray ionization, ESI, n*—sensitive ionization technique in which a solvent spray is formed by the application of a high-voltage potential held between a stainless-steel capillary containing a solution and the instrument orifice and the axial flow of a nebulizing gas (typically nitrogen).

3.1.10.1 *Discussion*—Solvent droplets from the spray evaporate in the ion source of the mass spectrometer releasing ions to the gas phase for analysis in the mass spectrometer (**2**).

3.1.11 *fragment ion, n*—product ion that results from the collision-induced dissociation of a preselected precursor ion (**3**).

3.1.12 *internal standard, ISTD, n*—chemical substance that is added in a known amount to calibration standards and samples with unknown concentrations to correct for analyte loss during sample preparation, ion suppression effects, source fouling, or matrix effects during analysis (**2**).

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 or test samples 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.

3.1.15 *ionization efficiency, n*—ratio of the number of ions generated to the number of molecules introduced into the ion source of a mass spectrometer (**3**).

3.1.16 *ion suppression, n*—matrix effect in liquid chromatography-mass spectrometry that results in a diminished analytical signal independent of the sensitivity or selectivity of the mass analyzer (**2**).

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

3.1.17.1 *Discussion*—The limit of detection is usually expressed as the concentration of the analyte in the sample (**4**). Different approaches to determine detection limits are possible that are based on the visual evaluation, signal-to-noise ratio, or standard deviation of the response and the slope. In the case of instrumental analytical procedures, a common approach is to compare the measured signals from samples having a known low concentration of analyte with that of blank samples. The minimum concentration at which the analyte can be reliably detected is established. Typically, acceptable signal-to-noise ratio is 3:1 (**4**, **5**).

3.1.18 *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**).

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

3.1.19.1 *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.

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

3.1.20.1 *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] Available from www.govinfo.gov.

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

3.1.21 *liposome, n*—synthetic vesicle composed of one or more bilayers formed by amphipathic molecules such as phospholipids that enclose a central aqueous compartment. Adapted from (**6**).

3.1.22 *lower limit of quantitation, LOQ, n*—least amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy.

3.1.22.1 *Discussion*—The LOQ is usually expressed as the concentration of analyte in the sample (**4**). This is also known as lower limit of quantitation. Different approaches to determine detection limits are possible, which are based on the visual evaluation, signal-to-noise ratio, or standard deviation of the response and the slope. In the case of instrumental analytical procedures, a common approach is to compare the measured signals from samples having known low concentrations of analyte with the signals from blank samples. Using this approach, the minimum concentration at which the analyte can be reliably quantified is established. A typically acceptable signal-to-noise ratio is 10:1 (**4, 5**).

3.1.23 *mass spectrum, n*—plot of the relative abundances of ions forming a beam or other collection as a function of their m/z values (**2**).

3.1.24 *mass transition, n*—specific pair of m/z values associated with precursor and product ions.

3.1.25 *matrix blank, n*—substance that closely matches the samples being analyzed with respect to matrix components but has none of the analyte(s) of interest.

3.1.25.1 *Discussion*—A matrix blank does not contain the analyte(s) of interest but is subjected to all sample-processing operations. A matrix blank is used to determine the selectivity of a test method and determine the absence of significant interference caused by matrix components.

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

3.1.26.1 *Discussion*—Matrix effects may be observed as enhanced or suppressed detector responses compared with those produced by simple solvent solutions of the analyte (**7, 8**).

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

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

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

3.1.29 *multiple reaction monitoring, MRM, n*—application of selected reaction monitoring to multiple product ions from one or more precursor ions (**3**).

3.1.30 *m/z values, n*—dimensionless quantity representing the ratio of the mass of an ion (m) in atomic mass units (amu) to its formal charge (z).

3.1.30.1 *Discussion*—The term "mass-to-charge ratio" has occasionally been used for *m/z* in the literature to represent the horizontal axis in a plot of a mass spectrum; however, the quantity measured by mass spectrometry is not the ion's mass divided by its electric charge (SI units kg C^{-1}).

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

3.1.32 *peak resolution (R_s), n*—measure of chromatographic separation of two components in a mixture. It is defined as the difference between the retention times of the two peaks divided by their average peak width.

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

where:

t_{R1} and t_{R2} = 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 (**2**).

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

3.1.34 *precursor ion, n*—ion that reacts to form particular product ions or undergoes specified neutral losses.

3.1.34.1 *Discussion*—The reaction can be of different types, including unimolecular dissociation, ion/molecule reaction, and change in charge state, possibly preceded by isomerization (**3**).

3.1.35 *quadrupole, n*—device used to separate ions according to their m/z using two pairs of parallel, equidistant poles (rods) biased at equal, but opposite, electric potentials.

3.1.36 *qualifier transition, n*—serves to confirm the identity of the analyte thereby enhancing the selectivity of the measurement (**2**).

3.1.37 *quality control sample, QC, n*—sample of known concentration that falls within the range of the calibration curve; it is prepared similar to calibration standards and injected at frequent intervals to check the performance of the instrument throughout the batch (see 3.2.1).

3.1.38 *quantifier transition, n*—typically the most selective transition; it is used for quantitation of the analyte (**2**).

3.1.39 *recovery, n*—extraction efficiency of an analytical process reported as a percentage of the known amount of an analyte carried through the sample extraction and processing steps of the method (**5**).

3.1.40 *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 variability conditions controlled at the same levels. **Adapted from E177**

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

3.1.42 *response factor, n*—measure of the relative mass spectral response of an analyte compared to its internal standard.

3.1.43 *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**

3.1.44 *selected reaction monitoring, SRM, n*—data acquired from one or more specific product ions corresponding to m/z selected precursor ions recorded via two or more stages of mass spectrometry (**3**).

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

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

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

3.1.46.1 *Discussion*—Typically, such components might include impurities, degradants, matrix material, and so forth (**5**).

3.1.47 *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.

3.1.48 *tandem mass spectrometry, MS/MS, n*—acquisition and study of the spectra of the product ions or precursor ions of m/z selected ions or precursor ions of a selected neutral mass loss (**3**).

3.1.48.1 *Discussion*—MS/MS can be accomplished using instruments incorporating more than one mass analyzer (tandem mass spectrometry in space) or in trap instruments (tandem mass spectrometry in time). In this test method, a TQMS has been used, which contains two quadrupole mass analyzers in a series, separated by a collision cell. Precursor ions are selected by the first quadrupole mass analyzer. The selected precursor ion(s) is then fragmented in the collision cell in the presence of inert gas, such as nitrogen or argon by a process known as collision-induced dissociation. Specific product ions are selected by the final quadrupole mass analyzer and passed to the detector. Thus, only analyte ions having a specified mass transition (precursor/product ion pair) can reach the detector, resulting in the high specificity of TQMS methods (**2**).

3.1.49 *total ion current chromatogram, TIC, n*—chromatogram created by plotting the total ion current in a series of mass spectra recorded as a function of retention time.

3.1.49.1 *Discussion*—Total ion current is the sum of all the separate ion currents carried by the ions of different m/z values contributing to a complete mass spectrum (**3**).

3.1.50 *triple quadrupole mass spectrometry, TQMS, n*—tandem mass spectrometer comprising two transmission quadrupole mass spectrometers in series with a non-selecting transmission quadrupole (or other multipole) between them to act as a collision cell (**3**).

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *batch, n*—series of uninterrupted measurements within which the trueness and precision of the measuring system are expected to be stable.

3.2.2 *test sample, n*—final form of the test unit that is used for single or multiple observations.

3.2.2.1 *Discussion*—In this test method, a sample solubilized with methanol, spiked with a known concentration of ISTD, and diluted within the bracketed range is referred to as a test sample.

3.2.3 *test unit, n*—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.

3.2.3.1 *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 *CoA, n*—certificate of analysis

3.3.2 *CRM, n*—certified reference material

3.3.3 *DMPC, n*—1,2-Dimyristoyl-sn-glycero-3-phosphocholine

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

3.3.5 *DSPE-PEG 550, n*—1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-550]

3.3.6 *ESI, n*—electrospray ionization

3.3.7 *HSPC, n*—hydrogenated (soy) L-α-phosphatidylcholine

3.3.8 *IS, n*—intermediate stock

3.3.9 *ISTD, n*—internal standard

3.3.10 *LC-MS, n*—liquid chromatography-mass spectrometry

3.3.11 *LOD, n*—limit of detection

3.3.12 *LOQ, n*—limit of quantitation

3.3.13 *MRM, n*—multiple reaction monitoring

3.3.14 *MS/MS, n*—tandem mass spectrometry

3.3.15 *m/z, n*—mass-to-charge ratio

3.3.16 *OSHA, n*—Occupational Safety and Health Administration

3.3.17 *PS, n*—primary stock standard

3.3.18 *QC, n*—quality control

3.3.19 *RCF, n*—relative centrifugal force

3.3.20 *RSD, n*—relative standard deviation

3.3.21 *SD, n*—standard deviation

3.3.22 *SDSs, n*—safety data sheets

3.3.23 *S/N, n*—signal-to-noise ratio

3.3.24 *SRM, n*—selective reaction monitoring

3.3.25 *TIC, n*—total ion chromatogram

3.3.26 *TQMS, n*—triple quadrupole mass spectrometry

3.3.27 *UHPLC, n*—ultra-high-performance liquid chromatography

3.3.28 *ULOQ, n*—upper limit of quantitation

3.3.29 *WISM, n*—working internal standard mixture

3.3.30 *WSM, n*—working standard mixture

4. Summary of Test Method

4.1 Cholesterol, DSPE-PEG 2000, and HSPC in a liposomal formulation are solubilized in methanol at 1:100 dilution by volume followed by vortex mixing. The solubilized sample is further diluted to appropriate concentrations to fit the calibration range and amended with ISTDs. The diluted sample is subjected to quantitative analysis using UHPLC-TQMS. Before sample analysis, the test method is validated for linearity, precision, accuracy, specificity, LOD, and LOQ.

4.2 Calibration curves are established for DSPE-PEG 2000 and HSPC in the range of 2–400 ng/g. Because of the low ionization efficiency, the range of the calibration curve for cholesterol (8–1600 ng/g) was expanded to be about four times greater than the calibration range for DSPE-PEG 2000 and HSPC. The analytes are separated on a bridged ethylsiloxane/silica hybrid (BEH) C18 column using UHPLC, ionized using an ESI positive source and quantified using MRM mode on a TQMS.

4.3 The target analytes and ISTDs are identified by the retention time and two MRM transitions. HSPC and DMPC use only one MRM transition because of less-sensitive or nonexistent secondary transition. The analytes are quantitated in calibration standards and sample using the primary MRM transition with ISTD calibration. The final report issued for each sample lists the absolute concentration (ng/g) of the individual lipids and their mass ratio (DSPE-PEG 2000: HSPC: cholesterol).

5. Significance and Use

5.1 Liposomes are vesicles of nanoscale dimensions, composed of lipid bilayers, which are used for various diagnostic and therapeutic applications (**9**). The growing interest in liposomal formulations in the delivery of various drugs, antisense oligonucleotides, cloned genes, or recombinant proteins by the biopharmaceutical industry, warrants QC and thorough characterization of the constituent lipids. Lipid structure, composition, and concentration are key attributes in determining the quality and efficacy of a liposomal drug product as they influence the stability of liposomes, drug loading, release kinetics, biodistribution, and pharmacokinetic properties (**9**). Cholesterol modulates the lipid membrane fluidity, elasticity, and permeability; hence, it plays a key role in controlled drug release and increased stability of the liposome (**10**).

5.2 This test method provides a rapid and reliable protocol for the determination of cholesterol, DSPE-PEG 2000, and HSPC in liposomal formulations using UHPLC-TQMS. Assessment of the stability of the analytes in terms of their degradation profiles is not included in this test method (**11**). This test method will benefit the biopharmaceutical industry in ascertaining quality assessment of liposomal formulations and monitoring batch-to-batch consistency for large-scale production, thereby facilitating safe and efficient drug development and regulatory review.

5.3 UHPLC-MS/MS measurements are analytically more sensitive and specific for lipid analysis compared to other contemporary techniques using universal detectors, such as a charged aerosol or an evaporative light-scattering detector. For liposomes, MS/MS has further advantages over ultraviolet detectors, as lipids lack chromophores for detection. In this test method, TQMS has been used as the MS/MS technique of choice because of its high selectivity, sensitivity, S/N, accuracy, and broad linear range of quantitation, thereby allowing reproducible quantitation of the analytes, especially at low concentrations.

5.4 According to the Current Good Manufacturing Practice regulations [21 CFR 211.194(a)(2)], users are required to verify the suitability of the test method under actual conditions of use. Validation should assess the suitability of the test method for the product matrix, recovery of the analytes from the product matrix, suitability of chromatographic conditions and column, appropriateness of the detector signal response, specificity, limit of detection and quantitation, accuracy, and precision. The user may need to optimize method parameters and cross validate if a different chromatography column, ionization method, or mass analyzer is used.

6. Interferences

6.1 Method interferences may be introduced by impurities present in solvents, reagents, glassware, and other apparatus used during sample preparation and instrumental analysis. These impurities may result in elevated baseline noise or interfering peaks. The presence and magnitude of such interferences are determined by routine analysis of solvent and laboratory blanks under the same conditions as the samples.

6.2 Matrix interferences may be caused by sample components and contaminants from sampling devices and storage containers. The extent of matrix interferences may vary considerably depending on the source and application of the liposomal formulations. The analysis of matrix spikes is critical for determining the impact of matrix interferences. If the user observes any matrix effects under the recommended test conditions, it may be necessary to modify the sample preparation procedure to remove the interfering compounds from the sample or to optimize the chromatographic parameters to avoid the co-elution of target analytes and interfering excipients. The Bligh-Dyer method, as described in Appendix X2, could be adopted to remove water-soluble excipients in liposomal formulations, if necessary (**12**).

6.3 This test method requires the use of high-quality consumables and LC-MS grade reagents and solvents.

6.4 It is necessary to use a high-quality nitrogen source (>95 % purity) that is free from water vapor, particles, and nonvolatile hydrocarbons, such as compressor oils. For the collision cell, a high purity (>99 %) gas source, in this case argon, is required.

6.5 To minimize baseline noise and ion-suppression effects, it is recommended to clean the autosampler injection port after

each injection for 10 s, wash the column frequently with appropriate solvents, and keep the ESI source clean. The auto-sampler injection port, column, solvent pumps, and the MS should be maintained as per the manufacturer's instructions.

6.6 It is recommended to clean solvent reservoir bottles routinely and use freshly prepared mobile phase solutions to avoid any contamination.

6.7 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. For isotope-labelled ISTDs, the use of a stable analyte with high isotopic purity is recommended. The presence of unlabeled analyte should be checked, and if detected, the potential influence should be evaluated during method verification **(13)**.

7. Apparatus

7.1 *LC/MS/MS System:*

7.1.1 *LC System*—A UHPLC system is required to analyze samples. It should include a sample injection system, a solvent pumping system capable of mixing solvents, an in-line degasser module, a sample compartment capable of maintaining the required temperature, and a temperature-controlled column compartment. Any UHPLC system that can perform at the flow rates, pressures, controlled temperatures, sample volumes, and other requirements of the test method shall be used.

7.1.2 *Analytical Column*—A reversed phase C-18 column, 13 nm pore size, 1.7 µm particle size, 2.1 mm × 50 mm (internal diameter x length) or equivalent column that can resolve all analytes of interest with $R_s \geq 1.5$ is acceptable. The retention times and order of elution may change depending on the column being used.

7.1.3 *Guard Column*—A C-18 pre-column, preferably with the same packing as the analytical column, shall be installed between the injector and the analytical column.

7.1.4 *MS/MS System*—An MS/MS system capable of MRM analysis and performing at the requirements specified in this test method shall be used (see 11.5).

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

7.3 Vortex mixer.

7.4 Mechanical pipettes and disposable pipette tips ranging from 0.002 mL to 10 mL.

7.5 Ultrasonic water bath.

7.6 Solvent reservoir bottles, 1 L.

7.7 Polypropylene tubes, 15 mL.

7.8 Amber glass vials, 10 mL and 20 mL.

7.9 Amber autosampler vials (2 mL) with polytetrafluoroethylene silicone septa-fitted caps.

8. Reagents and Materials

8.1 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.[5] 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.

8.2 Ammonium formate deliquescent crystals, LC-MS grade.

8.3 Acetonitrile, LC-MS grade.

8.4 Formic acid, LC-MS grade.

8.5 Methanol, LC-MS grade.

8.6 Water, LC-MS grade.

8.6.1 *Purity of Water*—Unless otherwise indicated, references to water shall be understood to mean reagent water conforming to Type I of Specification D1193. It shall be confirmed that this water does not contain contaminants at concentrations that would interfere with the analysis. LC-MS-grade water prefiltered with a ≤0.2 µm filter was used to develop this test method.

NOTE 1—Pre-mixed LC-MS-grade solvents with 0.1 % formic acid may be used, if commercially available. For example, "methanol with 0.1 % formic acid."

8.7 *Gases*—Ultrapure nitrogen and argon.

8.8 *Lipid Standards:*

8.8.1 Cholesterol, ≥99 % pure, powder form.

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

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

8.9 *ISTDs:*

8.9.1 Cholesterol-d7, powder form.

8.9.2 DMPC, powder form.

8.9.3 DSPE- PEG 550, powder form.

9. Hazards

9.1 Standard protective measures are required while handling liposomal formulations and organic solvents. This test method uses flammable organic solvents, such as methanol and acetonitrile. Precautions shall be taken to avoid direct contact with skin and inhalation of solvents. The user is advised to conduct a hazard assessment to determine appropriate safety protocols and personal protective equipment (PPE), and follow applicable regulations (for example, OSHA) , supplier SDSs, institutional requirements, and recommended procedures pertaining to safe handling and disposal of all chemicals used in this test method.

10. Preparation of Mobile Phases

10.1 *Mobile Phase A — 60 % Acetonitrile: 40 % Water Containing 0.1 % Formic Acid and 10 mmol/L Ammonium Formate:*

[5] *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.

10.1.1 Rinse an empty and clean solvent reservoir bottle (1 L) thoroughly with LC-MS-grade water and dry. Label the bottle to include the mobile phase composition and date of preparation.

10.1.2 To prepare 1 L of mobile phase A, weigh 0.630 ± 0.006 g of LC-MS-grade ammonium formate and transfer it to the rinsed and dried solvent reservoir bottle.

10.1.3 Measure 400 mL of LC-MS-grade water using a measuring cylinder and transfer it to the solvent reservoir bottle.

10.1.4 Measure 600 mL of LC-MS-grade acetonitrile using a measuring cylinder. Transfer to the solvent reservoir bottle and mix thoroughly.

10.1.5 Add 1 mL of LC-MS-grade formic acid to the bottle and mix thoroughly. Skip this step if using 0.1 % formic acid premixed LC-MS-grade water and acetonitrile (see Note 1).

10.1.6 Degas mobile phase A for 10 min using an ultrasonic bath at atmospheric pressure.

NOTE 2—The use of an in-line degasser module in the UHPLC stack helps to achieve a stable baseline during the analytical run. Sparging with helium can also be used as an alternative to the ultrasonic and in-line vacuum degassing combination as used in this test method.

10.2 *Mobile Phase B — Methanol Containing 0.1 % Formic Acid and 10 mmol/L Ammonium Formate:*

10.2.1 Rinse an empty and clean solvent reservoir bottle (1 L) thoroughly with LC-MS-grade methanol and dry. Label appropriately with necessary details (see 10.1.1).

10.2.2 To prepare 1 L of mobile phase B, weigh 0.630 ± 0.006 g of LC-MS-grade ammonium formate and transfer it to the rinsed and dried solvent reservoir bottle.

10.2.3 Measure 1 L of LC-MS-grade methanol in a measuring cylinder. Transfer to the solvent reservoir bottle and mix thoroughly.

10.2.4 Add 1 mL of LC-MS-grade formic acid to the bottle and mix thoroughly (see 10.1.5).

10.2.5 Degas mobile phase B for 10 min using an ultrasonic bath at atmospheric pressure.

10.3 Use an inlet-line frit (0.2 µm pore size) for each mobile phase and keep the inlet at the bottom of the reservoir to remove large particles.

10.4 Prepare fresh mobile phase solutions before UHPLC-TQMS analysis.

10.5 Prime the solvent lines and the pump system with freshly prepared Mobile Phases A and B for 2 min to eliminate air bubbles and ensure a steady flow out of the vent tube. Any glassware used to prepare or store a mobile phase shall be rinsed with respective LC-MS grade solvent before use (**14**), for example, water for mobile phase A and methanol for mobile phase B.

11. Preparation of Apparatus

11.1 *Analytical Instrument Qualification*—The UHPLC-TQMS system shall be maintained periodically following the manufacturer's recommendation to obtain reliable quantitative data. Typically, the vacuum systems, high-voltage power supplies, computers, and data collection systems are maintained in a powered-on state unless recommended otherwise by the manufacturer. The TQMS is typically maintained in standby mode.

11.2 *LC Chromatograph Operating Conditions*—The injection volume of all calibration standards and samples is 5 µL. The flow rate of the mobile phase should be set at 0.4 mL/min and the column temperature adjusted to 60.0°C. The LC gradient conditions are shown in Table 1.

11.3 *LC Sample Manager Conditions:*

11.3.1 *Needle Wash*—Prime the sample syringe and needle with LC-MS-grade acetonitrile/water/methanol, 1:1:1 (v/v/v), to remove contaminants from inside and outside the needle and the injection port. This also helps purge air from the lines.

11.3.2 *Seal Wash*—90 % water, 10 % acetonitrile; time: 2 min.

11.3.3 *Autosampler Purge*—60 % acetonitrile and 40 % water containing 0.1 % formic acid.

11.3.4 *Temperature*—Column, 60ºC; Sample compartment, 12ºC.

11.3.5 Wash and purge specifications from specific instrument manufacturers should be followed to eliminate sample carryover in the analysis of lipids.

11.4 *Column Conditioning*—Once the desired column temperature (60ºC) is achieved, equilibrate the column using a 40:60 mixture of mobile phases A:B at a flow rate of 0.4 mL/min for at least 30 min before starting the batch with the elution set to waste.

11.5 *MS Parameters:*

11.5.1 To acquire the maximum number of data points per MRM channel with optimum sensitivity, the tunable parameters shall be optimized for the instrument being used for the analysis. Each peak requires at least ten scans per peak for adequate quantitation. This test method contains three target analytes and three ISTDs for respective lipids, which are split into different MRM experiment windows to optimize the number of scans and sensitivity. MS tune parameters used in the development of this test method are provided in Table 2 for information only. These conditions shall be optimized by the user. The instrument is set in the electrospray positive source setting. Variable parameters including retention times, MRM transitions, cone voltage, and collision energy used to develop this test method are shown in Table 3. As HSPC is composed of two compounds HSPC-1 and HSPC-2 at unknown ratios in the calibration standards and samples, it was quantified by

TABLE 1 Gradient Conditions for LC

Time (min)	Flow Rate (mL/min)	Mobile Phase A (%)	Mobile Phase B (%)	
0.0	0.4	40	60	Diverted to waste
1.0	0.4	20	80	
2.0	0.4	15	85	Directed to MS
5.2	0.4	15	85	
5.3	0.4	10	90	
6.2	0.4	5	95	
6.5	0.4	0	100	
8.5	0.4	0	100	Diverted to waste
8.8	0.4	40	60	
10.0	0.4	40	60	

TABLE 2 Typical MS Tune Parameters

Electrospray Positive Mode	
Capillary voltage	2.85 kV
Cone	Variable depending on analyte (Table 3)
Source temperature	150°C
Desolvation temperature	500°C
Desolvation gas flow	1000 L/h
Cone gas flow	150 L/h
Low mass resolution 1	2.52
High mass Resolution 1	14.96
Ion energy 1	0.0
Entrance energy	30 V
Collision energy	Variable depending on analyte (Table 3)
Exit energy	30 V
Low mass resolution 2	2.86
High mass resolution 2	14.86
Ion energy 2	0.9
Multiplier	504.76
Inter-scan delay	0.02 s
Dwell time	0.007 s

adding the response of the two individual compounds using their respective MRM transitions.

11.5.2 *TQMS Stabilization*—Turn on the nitrogen and argon gas source and change the TQMS system from standby mode to operate mode. Open the optimized MS method file for this test method. The TQMS system shall be allowed time to stabilize with all predefined parameters set for this test method. The user shall verify that all the parameters are within the set range.

11.6 After stabilizing the TQMS and equilibrating the column to obtain the stable desired temperature and pressure (pressure fluctuations should be <3 bar), direct the UHPLC flow to the TQMS ESI source.

11.7 *System Suitability Check:*

11.7.1 After conditioning and stabilizing the UHPLC-TQMS system and before beginning each batch analysis, inject a QC sample three to five times through the column.

11.7.2 Process the data and make sure the analyte/ISTD peak area ratios and retention times are within the expected ranges. The system suitability acceptance criteria for the test method are set at a relative standard deviation (RSD) <6 % for analyte/ISTD peak area ratios and ±0.5 min for retention time. The S/N for the detector signal response shall at least be 10:1.

11.7.3 Once the UHPLC and MS systems pass the system suitability check, the sample batch containing the set of calibration standards and test samples shall be run on the instrument.

12. Calibration and Standardization

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

NOTE 4—All solutions in this test method are prepared gravimetrically using an analytical balance (≤0.0001 g accuracy). Hence, the user is responsible for recording weights carefully at every step of preparation of calibration standards and test samples. Although the volumetric sample preparation closely agrees with the gravimetric sample preparation, it is known that a 1–5 % error can be introduced during small volume transfers, especially for volatile or semi-volatile organic solvents which may bias the quantitation. The analytical balance provides better measurement resolution (that is, more significant figures) than mechanical pipettes and offers better accuracy (**15**).

NOTE 5—The user is required to provide a CoA or an equivalent alternative information on the source, purity, storage conditions, retest/expiration date, and lot number of the reference standard or high-purity chemical to ensure quality and stability (**13**). A full scan MS of standard solutions could be used to check for decomposition or conversion products. When using expired reference standards or a high-purity chemical, the user should provide an updated CoA or reestablish the identity, purity, and stability of the chemicals. For ISTDs, the user does not have to provide a CoA or evidence of purity if the suitability for use is demonstrated, for example, a lack of interference with an analyte. Stock and working solutions can only be prepared from reference standards that are within the stability period as documented in the CoA, either expiration date or retest date.

NOTE 6—In the working standard mixtures (WSMs) and calibration standards, concentrations of cholesterol should be four times higher than the concentrations of DSPE-PEG 2000 and HSPC, because of its low ionization efficiency with the ESI. In the working ISTD mixtures (WISMs) and calibration standards, cholesterol-d7 should also be four times higher than DSPE-PEG 550 and DMPC.

12.1 *Preparation of Calibration Standards*—To prepare the calibration curve, analyze eight calibration standards named Levels (LV) 1–8 which contain cholesterol, DSPE-PEG2000, and HSPC at nominal concentrations ranging from 8–1600 ng/g for cholesterol and 2–400 ng/g for DSPE-PEG 2000 and HSPC as shown in Table 4 (rows 1–3). ISTDs are used to account for experimental drift in the test method caused by matrix interferences that may result in ion enhancement or suppression. Three ISTDs—cholesterol-d7, DSPE-PEG 550, and DMPC—are added to the analytical calibration standards and samples at nominal concentrations of ≈ 100, 25, and 25 ng/g, respectively, as shown in Table 4 (rows 4–6). The following steps were used to produce calibration standards with the concentration levels (LV1-LV8) shown in Table 4.

12.2 *Preparation of Stock Solutions—Primary stock standard (PS) solutions* are prepared for each analyte from standard materials or purchased as certified solutions (see Note 5). These solutions are then diluted to prepare *individual intermediate stock standard (IS) solutions*. The IS solutions are used to prepare WSMs containing cholesterol, DSPE-PEG 2000, and HSPC, at three concentration levels and a WISM containing cholesterol-d7, DSPE-PEG 550, and DMPC. Aliquots of three WSMs (A, B, and C) are mixed with the WISM solution and diluted with solvent to prepare calibration standards (Table 5).

12.2.1 Powder stocks of the chemicals stored in the freezer at –20ºC or per manufacturer's recommendation should be allowed to equilibrate at ambient temperature for 15 min before weighing.

12.2.2 *PS Solutions*—Prepare 10 mL of individual PS solutions (≈1000 µg/g) of cholesterol, DSPE-PEG 2000, HSPC, cholesterol-d7, DSPE-PEG 550, and DMPC in 20 mL amber glass vials.

12.2.2.1 Rinse six empty 20 mL amber glass vials with deionized water and dry thoroughly.

12.2.2.2 Label each vial with the corresponding analyte *i* (where *i* = cholesterol, DSPE-PEG 2000, HSPC, cholesterol-d7, DSPE-PEG 550, or DMPC) and the estimated concentration of the analyte.

12.2.2.3 Weigh each empty capped vial on an analytical balance and record the mass to the nearest 0.0001 g as W_{0i}.

TABLE 3 Retention Times, MRM Ions, and Analyte-Specific MS Parameters

Analyte	Retention Time, min	Primary/ Confirmatory	MRM Transition (Parent >Product)	Cone, V	Collision, eV
DMPC	2.56	Primary	678.60 > 184.02	38	30
Cholesterol-d7	2.68	Primary	376.41 > 147.08	38	24
		Confirmatory	376.41 > 160.96	38	22
		Confirmatory	376.41 > 95.24	38	30
Cholesterol	2.70	Primary	369.55 > 161.15	38	22
		Confirmatory	369.55 > 95.10	38	26
		Confirmatory	369.55 > 147.25	38	24
DSPE-PEG 2000	3.82	Primary	607.55 > 95.16	76	30
		Confirmatory	607.55 > 109.02	76	32
		Confirmatory	726.30 > 607.55	40	10
HSPC-1	4.62	Primary	762.61 > 184.14	32	30
DSPE-PEG 550	4.66	Primary	607.45 > 94.99	70	30
		Confirmatory	607.45 > 71.11	70	32
		Confirmatory	751.06 > 607.45	40	10
HSPC-2	5.80	Primary	790.59 > 184.08	32	30

TABLE 4 Concentrations of Calibration Standards, ng/g

Analyte/ISTD	LV1	LV2	LV3	LV4	LV5	LV6	LV7	LV8
Cholesterol	8	20	40	100	200	400	800	1600
DSPE-PEG 2000	2	5	10	25	50	100	200	400
HSPC	2	5	10	25	50	100	200	400
Cholesterol-d7	100	100	100	100	100	100	100	100
DSPE-PEG 550	25	25	25	25	25	25	25	25
DMPC	25	25	25	25	25	25	25	25

TABLE 5 Preparation of Calibration Standards for Cholesterol, DSPE-PEG 2000, and HSPC from Working Standard Mixture and Working ISTD Mixture Solutions (1 mL Final Volume)

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.

Solution	LV1	LV2	LV3	LV4	LV5	LV6	LV7	LV8
Working standard mixture solution[A]	A	A	B	B	B	C	C	C
Volume of Working standard mixture solution (mL)	0.020	0.050	0.020	0.050	0.100	0.100	0.200	0.400
Volume of Working ISTD mixture solution (mL)[B]	0.050	0.050	0.050	0.050	0.050	0.050	0.050	0.050
Volume of solvent, mL[C]	0.930	0.900	0.930	0.900	0.850	0.850	0.750	0.550

[A] Working standard mixture solutions A ≈ 400,100, and 100 ng/g, B ≈ 2000, 500, and 500 ng/g, and C ≈ 4000, 1000, and 1000 ng/g of cholesterol, DSPE-PEG 2000, and HSPC, respectively. The values reported here are estimated based on rounded values; absolute concentrations for the working standard mixtures based on their exact weights are needed to calculate the volumes.
[B] Working ISTD mixture solution ≈ 1000, 500, and 500 ng/g of cholesterol-d7, DSPE-PEG 550, and DMPC, respectively.
[C] LC-MS-grade methanol is used as the solvent.

12.2.2.4 Weigh 10 ± 2 mg of cholesterol, DSPE-PEG 2000, HSPC, cholesterol-d7, DSPE-PEG 550, and DMPC individually in the respective capped vials on an analytical balance and record the masses to the nearest 0.0001 g as W_{1i}.

12.2.2.5 Add 10.0 mL of LC-MS-grade methanol using a mechanical pipette to each vial and record the mass of each capped vial (W_{2i}).

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

12.2.2.7 The final PS concentrations of the analytes (C_i) are calculated using Eq 1. The percent purity ($\%\ P$) for each analyte should be the value from the manufacturer's CoA.

$$C_i\ (\mu g/g) = \frac{\text{Mass of the analyte}}{\text{Total mass of solution}} = (\%\ P)_i \times \frac{W_{1i} - W_{0i}}{W_{2i} - W_{0i}} \times 10^6 \quad (1)$$

where:

i = cholesterol, DSPE-PEG 2000, HSPC, cholesterol-d7, DSPE-PEG 550, or DMPC.

12.2.3 *IS Solutions*—Dilute the individual PS solutions (≈1000 µg/g) with LC-MS-grade methanol to obtain 10 mL of the respective IS solutions with estimated concentrations of ≈100 µg/g.

12.2.3.1 Rinse six empty 20 mL amber glass vials with deionized water and dry thoroughly.

12.2.3.2 Label the vials with the corresponding analyte i (where i = cholesterol, DSPE-PEG 2000, HSPC, cholesterol-d7, DSPE-PEG 550, or DMPC) and the estimated concentration of the analyte.

12.2.3.3 Weigh each empty capped vial on an analytical balance and record the mass to the nearest 0.0001 g as W'_{0i}.

12.2.3.4 For each analyte, the volume of each PS solution required to prepare respective 10 mL of the respective 100 µg/g IS solution is calculated as:

$$\text{Volume of PS (mL)} = \left[\frac{100\ \mu g/g \times 10\ mL}{C_i\ (\mu g/g)}\right] \quad (2)$$

where:

i = cholesterol, DSPE-PEG 2000, HSPC, cholesterol-d7, DSPE-PEG 550, or DMPC.

12.2.3.5 Add the estimated volume of PS solution to each vial and record the masses of the capped vials (W'_{1i}).

12.2.3.6 Add the estimated volume of LC-MS-grade methanol to achieve 10 mL of each IS solution. Record the masses of the capped vials after methanol addition (W'_{2i}).

12.2.3.7 Vortex mix each vial for 1 min to obtain a homogenous solution.

12.2.3.8 The final IS solution concentrations for the analytes (C'_i) are calculated as:

$$C'_i\ (\mu g/g) = \frac{[(W'_{1i} - W'_{0i}) \times C_i]}{W'_{2i} - W'_{0i}} \quad (3)$$

where:

i = cholesterol, DSPE-PEG 2000, HSPC, cholesterol-d7, DSPE-PEG 550, or DMPC.

12.2.4 *WSM Solutions*—The WSM solutions are prepared by mixing aliquots from the IS solutions of each analyte in a 4:1:1 mass ratio (cholesterol: DSPE-PEG 2000: HSPC). Different concentration levels of WSM solutions can be prepared to cover a wide calibration range (8–1600 ng/g cholesterol and 2–400 ng/g DSPE-PEG 2000 and HSPC) depending on the expected analyte concentrations in the test samples (see Table 4). To cover the above-mentioned calibration range, three estimated levels of WSM solutions are recommended: m = A, B, and C where A is a mixture of ≈ 400, 100, and 100 ng/g; B is a mixture of 2000, 500, and 500 ng/g; and C is a mixture of ≈ 4000, 1000, and 1000 ng/g of cholesterol, DSPE-PEG 2000, and HSPC, respectively (see Table 5).

12.2.4.1 Label three 10 mL amber glass vials with the respective WSM_m, where m = A, B, or C, and their estimated concentrations.

12.2.4.2 Weigh each empty capped vial on an analytical balance and record the masses to the nearest 0.0001 g as M_0^m.

12.2.4.3 To achieve 5 mL of the desired estimated concentrations of cholesterol, DSPE-PEG 2000, and HSPC in each WSM solution, C_i^m (ng/g), the requisite volume of IS solution for each analyte is calculated as:

$$\text{Volume of } IS_i\ (mL) = \frac{C_i^m \times 5}{C'_i \times 1000} \quad (4)$$

where:

i = cholesterol, DSPE-PEG 2000, or HSPC, and
m = A, B, or C.

12.2.4.4 Add sequentially the calculated volumes of IS solutions of cholesterol, DSPE-PEG 2000, and HSPC to the vials, recording the masses of the capped vials after addition of each analyte IS solution as M_i^m where i = cholesterol, DSPE-PEG 2000, or HSPC and m = A, B, or C.

12.2.4.5 Add LC-MS-grade methanol to the vials to achieve the desired estimated concentrations of cholesterol, DSPE-PEG 2000, and HSPC in each WSM. Record the masses of the capped vials after the addition of methanol as M_1^m, where m = A, B, or C.

12.2.4.6 Vortex mix each vial for 1 min to obtain a homogenous solution.

12.2.4.7 The final concentrations of the analytes in the WSM solutions C_i^m, where i = cholesterol, DSPE-PEG 2000, and HSPC are calculated as:

$$C_i^m\ (ng/g) = \frac{[(M_i^m - M_0^m) \times C'_i]}{M_1^m - M_0^m} \quad (5)$$

where:

i = cholesterol, DSPE-PEG 2000, or HSPC, and
m = A, B, or C.

12.2.5 *WISM Solution*—Prepare a WISM solution of ≈2000, 500, 500 ng/g cholesterol-d7, DSPE-PEG 550, and DMPC, respectively, by following steps 12.2.4.1 – 12.2.4.7. The final concentrations of the ISTD in the WISM solutions $C_j^{ISTD\ mix}$, where j = cholesterol-d7, DSPE-PEG 550, and DMPC are calculated using an equation similar to Eq 5, step 12.2.4.7.

NOTE 7—The preparation of the WSM and WISM solution can be accomplished using appropriate volumes and concentrations of PS and IS solutions as per the laboratory's standard procedure. Depending on the prepared stock solution concentrations, the solubility of each analyte will have to be ensured.

12.2.6 *Calibration Curve*—An eight-point calibration curve has been used to develop this test method 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. Depending on the instrument type, the sensitivity and calibration curve responses may vary. At a minimum, a six-point calibration curve shall be obtained for all the analytes.

12.2.6.1 Use aliquots of the WSM and WISM solutions and dilute with LC-MS-grade methanol to prepare 1 mL of the eight calibration standards ranging from 8–1600 ng/mL cholesterol and 2–400 ng/g DSPE-PEG 2000 and HSPC in 2 mL amber autosampler vials as described in Table 5. In Table 4, the levels of calibration standards represent target concentrations of cholesterol, DSPE-PEG 2000, and HSPC. The target concentrations of the ISTDs, namely, cholesterol-d7, DSPE-PEG 550, and DMPC, in the calibration standards are ≈ 100, 25, and 25 ng/g, respectively. As all measurements are gravimetric, the volume presented in Table 5 are approximate and will not affect quantitation. A solvent blank and a calibration blank (solvent blank spiked with ISTD at a similar concentration as in the calibration standards) are also included in the calibration curve; however, the blanks should not be included in the determination of the regression equation of the calibration curve.

12.2.6.2 Label 2 mL amber autosampler vials with the date of preparation and the respective level number (LV1–LV8), or concentration, or both.

12.2.6.3 Weigh each empty capped vial on an analytical balance and record the mass to the nearest 0.0001 g as Z_{0i}.

12.2.6.4 Add sequentially the WISM solution, the WSM solution, and methanol to the vials according to Table 5, recording the masses using an analytical balance to the nearest 0.0001 g as Z_{1i}, Z_{2i}, and Z_{3i}, respectively, after each addition. Vortex each vial to obtain homogenous solutions.

12.2.6.5 The estimated analyte concentration (CC_i) for each calibration level is calculated as:

$$CC_i\ (\text{ng/g}) = \frac{\left[(Z_{2i} - Z_{1i}) \times C_i^m\right]}{Z_{3i} - Z_{0i}} \quad (6)$$

where:

i = cholesterol, DSPE-PEG 2000, or HSPC.

12.2.6.6 The estimated ISTD concentration (CC_j) for each calibration level is calculated as:

$$CC_j\ (\text{ng/g}) = \frac{\left[(Z_{1j} - Z_{0j}) \times C_j^{\text{ISTD mix}}\right]}{Z_{3j} - Z_{0j}} \quad (7)$$

where:

j = cholesterol-d7, DSPE-PEG 550, or DMPC.

12.2.7 Run each calibration standard using the established LC-MS method and obtain a chromatogram for each one. An ISTD calibration technique is used for monitoring the primary and confirmatory MRM transition of each analyte. Calibration software is used to conduct the quantitation of the target analyte using the primary MRM transition (Table 3). The primary/confirmatory MRM transition area ratio shall be within 35 % of the individual laboratory's accepted primary/confirmatory MRM transition area ratio. The primary MRM transition of each analyte is used for quantitation and the confirmatory MRM transition for confirmation. This gives added confirmation by isolating the parent ion, forming one or two product ions via fragmentation, and relating it to the retention time in the calibration standard. Cholesterol and DSPE-PEG 2000 have a primary MRM transition and at least one confirmatory MRM transition; however, HSPC only has a primary MRM transition (Table 3). The laboratory is responsible to generate the optimum MS conditions for the parent and confirmatory ions as well as their ion area ratio acceptance criteria, which shall meet or exceed the acceptance criteria in Section 14 in this test method.

12.2.8 The calibration software manual should be consulted to use the software correctly. The quantitation method is set as an ISTD calibration using the peak areas in ng/g units. Concentrations may be calculated using the data system software to generate linear regression calibration curves. Forcing the calibration curve through the origin (X, Y = 0) is not recommended.

12.2.9 A calibration curve is generated by plotting the ratio (analyte peak area/ISTD peak area) against the ratio (analyte concentration/ISTD concentration) using the calibration software, yielding a slope that corresponds to the response factor. The response factor (RF) is calculated as:

$$RF = \frac{\text{Peak area}_A}{\text{Concentration}_A} \times \frac{\text{Concentration}_I}{\text{Peak area}_I} \quad (8)$$

where:

A = analytes (cholesterol, DSPE-PEG 2000, or HSPC), and
I = internal standards (cholesterol-d7, DSPE-PEG 550, or DMPC).

12.2.10 Linear calibration shall be used, the point of origin is excluded, and a fit weighting of $1/x$ is used to give more emphasis to the lower concentrations. If one of the calibration standards other than the highest or lowest point on the curve causes the r^2 of the curve to be <0.98, this standard shall be run or a new calibration curve shall be generated. An initial eight-point curve over the calibration range is suggested. If the lowest or highest point (or both) is excluded to obtain a coefficient of determination >0.98, minimally a six-point curve is acceptable, but the reporting range shall be modified to reflect this change. In this event, the reporting range shall be modified to reflect this change. The retention time window of the MRM transitions shall be within 5 % of the retention time of the analyte in a midpoint calibration standard. If this is not the case, reanalyze the calibration curve to determine if there was a shift in retention time during the analysis and run the sample again. If the retention time is still incorrect in the sample, refer to the analyte as an unknown.

12.3 *Quality Control*—A mid-level calibration check standard must be analyzed at the end of each batch of 12 samples or less and shall be within ±15 % of the calculated concentration. Based on the eight-point calibration curve generated from the calibration standards in Table 4, LV5 (≈ 200, 50, and 50 ng/g cholesterol, DSPE-PEG2000, and HSPC, respectively) was used as the mid-level check. The same standard solution that was used to generate the calibration curve should be used as the calibration check. Instrument stability may be verified by comparing the midpoint of the calibration standard before and after the batch. If the mid-level calibration check does not meet the acceptance criteria, the problem must be corrected and either all samples in the batch must be re-analyzed against a new calibration curve, or the affected results must be qualified with an indication that they do not fall within the performance criteria of the test method.

12.4 *Preparation of QC, Accuracy, and Precision Samples*—QC samples for accuracy and precision studies should be prepared using a similar procedure as for the calibration standards by mixing the WSM and WISM solutions, as described in 12.2.6. Three mixtures of cholesterol, DSPE-PEG2000, and HSPC should be prepared to represent low-QC, med-QC, and high-QC samples, as follows:

Low-QC: ≈ 50, 12.5, and 12.5 ng/g cholesterol, DSPE-PEG2000, and HSPC, respectively.

Med-QC: ≈ 200, 50, and 50 ng/g cholesterol, DSPE-PEG2000, and HSPC, respectively.

High-QC: ≈ 600, 150, and 150 ng/g cholesterol, DSPE-PEG2000, and HSPC, respectively.

12.5 To assess matrix effects, spike test samples at three known analyte concentrations (same as low-QC, med-QC, and high-QC, see 12.4) with matrix blank containing components that are present in the liposomal formulation of interest (Table 4). The ratio of concentration of matrix components to concentration of analytes in the test samples should be close to that