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Ion quantification in liposomal drug products using high performance liquid chromatography

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Abstract

A simple, straightforward analytical method based on liquid chromatography has been optimized to quantify total, internal, and external ions in drug-loaded liposomal products. The quantification of ammonium and sulfate ions in Doxil is detailed; although, the methodology has been extrapolated to quantitate a variety of ions, including calcium, acetate, and others in several different liposomal formulations. Total ion concentrations were measured after disruption of the liposome via lyophilization, to liberate all components. External ion concentrations were made following membrane centrifugation, without disruption of the liposome structure, where the permeate fraction was analyzed for external ion quantities. The internal ion fraction was derived from mass balance of the total and external ion measurements. High performance liquid chromatography (HPLC), equipped with different separation columns, and coupled to a charged aerosol detector, was employed for all ion quantifications. The analytical measurements were confirmed using simple stoichiometry based on the drug crystallization of doxorubicin within the liposome interior. The method presented herein is quick, highly accurate, and has significantly improved lower limits of detection and quantification over other traditional methods. As more follow-on versions of Doxil are being developed, this facile approach to ion quantitation can be used to help establish compositional similarity to the reference listed drug.

Keywords

ion concentration; ammonium; sulfate; liposome; nanoparticle; Doxil

1. Introduction

Liposomes have been used as drug delivery vehicles for decades. Numerous types of drugs, including cytotoxic oncology drugs, anti-microbial agents, proteins, and peptides have been

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Notes

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successfully encapsulated in liposomes, in either the aqueous core or hydrophobic lipid bilayer [1, 2]. The combination of this protected aqueous core and the hydrophobic bilayer allows for high efficiency encapsulation of both water-soluble and water-insoluble drugs, affording systemic delivery of drugs that would otherwise have limited therapeutic value. Furthermore, liposome-based drug delivery has improved the biodistribution and pharmacokinetic properties of these drugs, minimizing off-target toxicities and enhancing overall therapeutic efficacy [3–6].

Drugs are traditionally loaded into liposomes one of two ways—passively loaded or actively loaded (also referred to as remote loading). Compared to traditional passive drug loading methods, which can have relatively low encapsulation efficiencies, active drug loading is simple and provides high drug encapsulation efficiencies. The strategy of active drug loading was developed based on the principle that uncharged drugs diffuse into the vesicle by crossing the lipid bilayer and become protonated. The protonated drugs are unable to redistribute through the liposome bilayer, thereby becoming entrapped inside the liposome [4, 7, 8].

Active/remote loading was first demonstrated by Deamer and coworkers to achieve the loading of amphipathic weak bases (e.g., catecholamines) by creating a pH gradient [9, 10]. It has since been widely adapted for liposomal loading of weakly basic drugs, such as doxorubicin [4, 7, 8]. The encapsulation of doxorubicin, an anthracycline drug and one of the most commonly used drugs for treatment of certain cancers, was improved upon by Barenholz et al. who developed a novel loading method using a transmembrane ammonium sulfate gradient as the driving force for remote loading of the drug into the liposome interior (Figure 1) [7]. The exterior doxorubicin exchanges with the interior ammonia and then precipitates as a sulfate salt, becoming entrapped inside the liposome. This discovery eventually led to the production of Doxil, the first liposomal drug product approved by the U.S. Food and Drug Administration (FDA) in 1995. In addition to ammonium sulfate, other salts have also since been used for active loading of weak bases in liposomes, including citrate, phosphate, and acetate sodium salts and ammonium salts, as well as a variety of metal salts (e.g., MnSO_4 , CuCl_2 , etc.) [11–13].

Techniques for characterizing the main physicochemical properties of liposomes (e.g., lamellarity, size distribution, surface charge, drug loading, lipid composition, etc.) are well established. For example, the lamellarity of liposomes can be determined via ^31P nuclear magnetic resonance (NMR) depending on the extent of phospholipid exposure to the external medium; dynamic light scattering (DLS) and microscopy techniques are frequently used to assess size distribution and morphology; and liquid/gas chromatography and field-flow fractionation coupled to various detectors are used to evaluate drug loading and composition [14–20]. The chemistry, manufacturing, and controls (CMC) section in FDA's regulatory guidance document for liposome drug products states, for the submission of an Investigational New Drug (IND) application, the characterization scope should include the drug substance, lipids, nonlipid components of the liposome, and the nonliposome inactive ingredients [21]. However, published techniques to quantify nonlipid components, especially residual ions from remote drug loading, are currently limited.

Ion concentrations within a liposomal formulation are one of the most important criteria for establishing a stable and efficient drug encapsulation process. Ammonium has been found to effectively control not only the efficiency of the drug loading process, but also the stability of the product and its drug release profile [22]. Furthermore, liposomal drugs for anti-cancer therapy can also release inorganic ion pairs into the body, potentially affecting the further metabolism of nitrogen. Ammonium plays an important role in regulating nitrogen metabolism in vivo, and excess ammonia can potentially lead to severe neurodevelopmental and neurodegenerative complications (normal ammonium levels for a healthy adult range $0.5\sim 1\times 10^{-4}$ M) [23–26]. Thus, the quantification of ammonium, sulfate, as well as other residual transmembrane gradient ions has been recognized as one of the most significant aspects of the physicochemical characterization repertoire of liposomal drug products. However, quantification of residual inorganic ions within liposomal drugs is always challenging due to the non-chromophore/fluorophore nature of the ions, limited analyte volume, and efficiency of the detachment/release of ions from the liposome structure.

Several analytical methods have been used to quantitatively determine the concentration of ammonium and sulfate ions in various sample matrices. For example, methodologies for ammonium ion quantification include traditional colorimetric (e.g., indophenol and nesslerization colorimetric methods, etc.) and titrimetric methods, ammonia-selective electrode detection, ion chromatography, Fourier transform infrared spectroscopy (FTIR), as well as several newer methods developed in recent years (e.g., laser photothermal detection) [27–33]. Most commonly, quantification of liposomal ammonium ions is achieved via conductivity measurements using an ammonia combination electrode [22]. This analytical method is highly pH dependent ($\text{NH}_4^+ \rightleftharpoons \text{NH}_3 + \text{H}^+$); thus, calibration curves are required under specific pH values, and require close pH monitoring. Furthermore, higher sample volumes (e.g., 5 mL) are often required for this method.

Sulfate ions, generally, can be quantified via ion chromatography, inductively coupled plasma mass spectrometry atomic emission spectroscopy (ICP-AES), fluorescence, Raman and surface enhanced Raman spectroscopy (SERS), and turbidimetric methods [34–36]. Capillary zone electrophoresis and ion chromatography are the two most commonly used techniques for liposomal sulfate ion quantification. Capillary zone electrophoresis uses a fused silica capillary for sample separation, and a background electrolyte with an added chromophore for indirect UV detection [37]. This technique requires at least 1 mL sample and requires a multi-step sample preparation process, including an extraction to disrupt the liposome for the quantification of total sulfate. In addition, due to the poor UV response of ions, a chromophoric additive is required for measurement. The ion chromatography method uses a lysing agent (Triton-X) for disruption of the liposome for total sulfate analysis [38]. Incomplete disruption of the liposome, however, will lead to poor recovery of the total ions in the system.

Overall, these methods are time-consuming and require larger analyte volumes or complicated sample pre-treatments before analysis, which could lead to lower recovery efficiencies and poor accuracy. Furthermore, the inherently low concentration of external ions in the liposomal system require analytical methods with low limits of detection and quantification (LOD and LOQ). Herein, we describe a high-performance liquid

chromatography (HPLC)-based analytical method using charged aerosol detection (CAD) for the quantification of both total and external ion concentrations of ammonium and sulfate, using the commercial Doxil formulation as a representative test material. For quantification of total ion concentrations, the procedure uses a simple lyophilization-reconstitution process; whereas, for quantification of external ion concentrations, a facile centrifugal filtration is used. This HPLC-based method uses low sample volumes ($\sim 100 \mu\text{L}$), has a simple preparation process, and results in high ion recovery efficiencies with low detection/quantification limits.

2. Materials and Methods

2.1 Total and External Doxorubicin Concentration

Doxil (PEGylated liposomal doxorubicin; Janssen Product, NDC 59676-960-01) was purchased from the NIH pharmacy. The total doxorubicin concentration was quantified using an Agilent HPLC (Infinity 1260, Agilent, CA) equipped with a degasser (Agilent G4225A), capillary pump (Agilent G1312B), well-plate auto sampler (Agilent G1329B), C18 reversed-phase column (Zorbax 300SB-C18, $4.6 \times 150 \text{ mm}$, particle size $5 \mu\text{m}$), UV detector (Agilent G1315C), fluorescence detector (Agilent G1321B) and charged aerosol detector (CAD, Corona Veo RS, Thermo Scientific, MA). Doxil, $10 \mu\text{L}$, was lyophilized overnight at -81°C under vacuum (0.003 mbar) using a Freeze Dryer (Labconco, FreeZone, MO). The lyophilized powder was reconstituted with 1 mL 25% (v/v) acetonitrile (ACN, 99.8% for HPLC, ACROS Organics) to achieve complete disruption of the liposome structure and release of doxorubicin. The mobile phase used a two-solvent system. Solvent A was comprised of water (HPLC gradient grade, ACROS Organics) with 0.1% (v/v) trifluoroacetic acid (TFA, ReagentPlus, 99%, Sigma Aldrich). Solvent B was comprised of ACN with 0.1% (v/v) TFA. The gradient elution profile was 25% B for 3 min , ramp to 100% B in 10 min , hold at 100% B for 3 min , ramp down to 25% B in 5 min , and hold at 25% B for 5 min . The flow rate was 1 mL/min . The retention time of doxorubicin was identified as approximately 6.2 min , monitored by UV detection at 234 nm . Doxorubicin standards were prepared by dissolving doxorubicin hydrochloride (DXR-HCl, HPLC grade, Fluka, Lot 087K1263) in 25% ACN and diluting accordingly to provide standards over the range of 5 to $60 \mu\text{g/mL}$ ($8.7 \times 10^{-6} \text{ M}$ to $1.0 \times 10^{-4} \text{ M}$).

The external doxorubicin concentration was quantified using a fluorescence spectrometer (Fluoromax-4, Horiba Jobin Yvon; $\lambda_{\text{excitation}} = 482 \text{ nm}$ and $\lambda_{\text{emission}} = 593 \text{ nm}$). Doxil, $25 \mu\text{L}$, was diluted 10-fold in 0.025 M NaCl , then centrifuged to dryness using a Microcon DNA Fast Flow centrifugal device (100 kDa MWCO , MRCFOR100, Millipore Sigma) at $14,000 \text{ rpm}$ (17968 g-force), 24°C using a Microfuge 20R centrifuge with a FA361.5 rotor (Beckman Coulter). The filtrate was analyzed directly by fluorescence spectrometry to determine the external concentration of doxorubicin. Calibration standards were prepared by dissolving DXR-HCl in 0.025 M NaCl from 0.5 to $5 \mu\text{g/mL}$ ($8.7 \times 10^{-7} \text{ M}$ to $8.7 \times 10^{-6} \text{ M}$). The standards were also run through the centrifugal filtration devices to dryness using similar conditions.

The internal doxorubicin fraction was calculated by subtraction of the external drug concentration from the total drug concentration. While this approach does not provide a true

“internal concentration” within the interior of the liposome, this is a standard approach to deriving the quantity of an analyte entrapped within the liposomal compartment [7, 37]. Furthermore, describing the internal content as a concentration of the total sample volume allows for a stoichiometric validation of internal vs. external components. This approach was also used to calculate the internal ammonium and sulfate content as well.

2.2 Total and External Ammonium Concentration

The total ammonium concentration in Doxil was quantified by HPLC using charged aerosol detection (CAD, Corona Veo RS, Thermo Scientific, MA). The HPLC was equipped with a SIELC Primesep 100 column (4.6×150 nm, 5 µm particle size, SIELC Technologies, IL) which has embedded ion-pairing groups to separate ammonium through an ion exchange process. Doxil, 10 µL, was lyophilized overnight at –81°C under vacuum (0.003 mbar). The lyophilized sample was reconstituted with 100 µL 25% ACN with 0.1 % (v/v) TFA. The mobile phase used a two-solvent system. Solvent A was comprised of water with 0.2% (v/v) TFA. Solvent B was comprised of ACN with 0.2% (v/v) TFA. The isocratic elution profile was 70% B for 30 min, with a flow rate of 0.5 mL/min. Ammonium standard solutions were made by dissolving ammonium sulfate ((NH₄)₂SO₄, molecular biology, 99.0%, Sigma Aldrich) in water to 0.1 M and diluting to concentrations ranging from 2.5×10^{–4} M to 2.0×10^{–3} M with 25% ACN containing 0.1% (v/v) TFA, 0.001 M L-Histidine (ReagentPlus, 99%, Sigma Aldrich) and 1% (w/v) sucrose (BioXtra, 99.5%, Sigma Aldrich). This buffer system was used to ensure consistency of the sample conditions between the standards and the Doxil test sample. Ammonium ions could form adducts in the presence of sucrose and/or histidine, which could affect the accuracy of ammonium quantification [39–41].

The external ammonium concentration was quantified following centrifugal filtration. Doxil, 100 µL was centrifuged to dryness using a Microcon DNA Fast Flow centrifugal device (100 kDa MWCO) at 14,000 rpm, 24°C. The filtrate was diluted 10-fold with water containing 0.1% (v/v) TFA and analyzed by HPLC-CAD using identical conditions as specified for total ammonium ion concentration. Ammonium standards were prepared at concentrations ranging 1.0×10^{–3} M to 1.4×10^{–2} M by dissolving ammonium sulfate in the native buffer for Doxil (10% sucrose with 0.01 M histidine). The standards were then run through the centrifugal filtration devices (centrifuged to dryness using similar conditions), and were then diluted 10-fold with 0.1% TFA to yield final concentrations ranging from 1.0×10^{–4} M to 1.4×10^{–3} M.

2.3 Total and External Sulfate Concentration

The total sulfate concentration in Doxil was also quantified by HPLC with CAD. The HPLC was equipped with an Agilent acclaim trinity P2 column (Agilent, 3.0×100 nm, 3 µm particle size), a strong anion exchange column with weak cation exchange capacities. Doxil, 10 µL, was lyophilized overnight at –81°C under vacuum (0.003 mbar). The lyophilized sample was reconstituted with 100 µL 25% ACN with 0.6% (v/v) formic acid. The mobile phase used a two-solvent system. Solvent A was comprised of water. Solvent B was comprised of 0.1 M ammonium formate (HPLC grade, 99.0%, Fluka, Honeywell Research Chemicals) with 0.3% (v/v) formic acid (FA, LC-MS Grade, Pierce, ThermoFisher

Scientific), with a measured pH of 3.65 ± 0.05 (ORION 3 STAR, ThermoFisher Scientific). The gradient elution profile was 10% B for 2 min, ramp to 100% B in 10 min, hold at 100% B for 5 min, ramp down to 10% B in 5 min, and hold at 10% B for 3 min. The flow rate was 0.6 mL/min. Sulfate standard solutions were made by dissolving ammonium sulfate ((NH₄)₂SO₄, molecular biology, 99.0%, Sigma Aldrich) in 25% ACN with 0.6% (v/v) formic acid to cover the range of 1×10^{-4} to 8×10^{-4} M. Recognizing there could be adduct interference derived from sucrose in the native buffer of Doxil, sulfate standards were also analyzed in the presence of 10% native buffer (1% (w/v) sucrose with 0.001 M histidine); however, no interference with sulfate quantification was observed.

The external sulfate concentration was quantified following centrifugal filtration. Doxil was diluted 3-fold in native buffer (10% sucrose with 0.01 M histidine), and 100 μ L was centrifuged to dryness using a Microcon DNA Fast Flow centrifugal device (100 kDa MWCO) at 14,000 rpm, 24°C. The filtrate was analyzed by HPLC-CAD using identical conditions as specified for total sulfate ion concentration. Sulfate standards were prepared at concentrations ranging 1.0×10^{-4} M to 5.0×10^{-4} M by dissolving ammonium sulfate in the native buffer of Doxil (10% sucrose and 0.01 M histidine). The standards were then run through the centrifugal filtration devices to dryness using similar conditions.

3. Discussion

3.1 Doxorubicin Quantification

The total and external doxorubicin (DXR) concentrations in Doxil were measured, and the internal DXR content calculated, such that the ammonium and sulfate ion concentrations could be stoichiometrically validated (Figure 1). Total DXR concentration was measured following lyophilization of the sample. Lyophilization of liposomes with subsequent solubilization in a dilute organic solvent, such as 25% ACN, enables complete disruption of the liposome bilayer structure. When reconstituted in dilute organic solvents (e.g., ACN), rather than pure water, the liposome bilayer is disrupted, affording a complete release of DXR and other internal components into the reconstitution solution [42].

External DXR concentration was measured following centrifugal filtration. An appropriate membrane was selected such that non-liposome encapsulated DXR and other small molecular components could pass through. The larger liposome molecules, containing the internally encapsulated DXR and associated ion salts, could not permeate the membrane and were retained on the filter. To afford complete recovery of the external components, the centrifugal filtration devices were spun to dryness. As a result, recovery of the retentate fraction for measurement of the internal DXR was not possible. Rather, the internalized fraction was deduced by simple mass balance, i.e. subtraction of the measured external DXR concentration from the measured total DXR concentration.

Following the appropriate sample preparations, DXR was quantified by HPLC using UV-Vis detection at 234 nm (total) or fluorescence detection at 482 nm excitation and 593 nm emission (external). The analyte eluted at approximately 6.2 min. The measured total and external DXR concentrations, as well as calculated internal DXR fraction, are summarized in Table 1. The total DXR in the sample was 1.99 ± 0.08 mg/mL (n=5), or $3.7 \pm 0.1 \times 10^{-3}$

M. The labeled drug loading of Doxil is 2 mg/mL as specified in the product's prescribing information [43], indicating 99% extraction efficiency from this lyophilization-reconstitution protocol. The external DXR in the sample was 0.05 ± 0.005 mg/mL ($n=3$), or $1.0 \pm 0.1 \times 10^{-4}$ M. Less than 3% of the total DXR in the formulation was free and unencapsulated, in agreement with the <10% free drug specified in the product informational insert.

3.2 Ammonium Ion Quantification

The total, external, and internal ammonium sulfate ($(\text{NH}_4)_2\text{SO}_4$) concentrations were determined similarly, except that charged aerosol detection was necessary to visualize/detect the ion peaks. Importantly, for quantification of the ammonium ions it was necessary to prepare the ammonium sulfate standards in 25% ACN with 0.1% (v/v) TFA, 1% (w/v) sucrose, and 0.001 M histidine, such that the standards were evaluated in the same buffer composition as the Doxil test sample (native buffer contains 10% (w/v) sucrose and 0.01 M histidine) [43]. As depicted in Figure 2, ammonium sulfate standards prepared solely in 25% ACN with 0.1% (v/v) TFA had a lower CAD response than standards prepared with the sucrose and histidine additives. In general, the response was about 20% lower in the absence of the native Doxil buffer. Ammonium sucrose adducts or complexes of different molecular weights, sizes, and/or surface areas could form, thus amplifying the CAD response over free ammonium [39–41]. It has also been reported that basic analytes (such as ammonia) can affect the CAD signal when ion-paired with acidic species, wherein adducts with increased molecular weights produce greater CAD responses.

The HPLC chromatogram for ammonium concentration determination is shown in Figure 3. The ammonium ion peak (peak 3) eluted at approximately 6.0 min. Other peaks identified in the chromatogram were sucrose (peak 1) at 3.5 min, doxorubicin (peak 4) at 7.8 min, and histidine (peak 6) at 15.5 min. Peaks 2 (5.5 min) and 5 (11.5 min) were identified as background signals resulting from the presence of histidine and sucrose, and likely derived from adducts of histidine with sucrose (control analysis of histidine and sucrose in the absence of ammonium sulfate showed similar peaks, data not shown). The linear range for ammonium concentration measurements (in 25% ACN with 1% sucrose and 0.001 M histidine) was from 1.0×10^{-4} M to 1.0×10^{-2} M with an R^2 value 0.95. The limit of detection (LOD), calculated as three times the noise level, was 5.0×10^{-5} M ammonium. The lower limit of quantification (LLOQ) for ammonium, identified as having a signal to noise ratio higher than 10, was found to be as low as 1.0×10^{-4} M ($S/N \sim 15$). The total concentration of ammonium in Doxil was $8.9 \pm 0.9 \times 10^{-3}$ M ($n=5$). According to the product specification sheet, each mL of Doxil contains approximately 0.6 mg ammonium sulfate ($[\text{NH}_4^+] = 9.1 \times 10^{-3}$ M) [43], indicating >95% recovery efficiency was achieved with the method described herein. The external concentration of ammonium, measured from the permeate fraction following centrifugal filtration, was $3.5 \pm 0.2 \times 10^{-3}$ M ($n=3$), or roughly 40% of the total ammonium concentration. The internal, or entrapped ammonium fraction was calculated to be $5.4 \pm 1.1 \times 10^{-3}$ M of the total ammonium concentration (Table 1).

As depicted in Figure 1, the remote loading process of Doxil results in precipitation of doxorubicin ammonium sulfate ($(\text{DXR-NH}_3)_2\text{SO}_4$) inside the liposome at a 1:1 molar ratio

of DXR to NH_3 . The internal DXR to NH_4^+ ratio calculated herein was 1:1.5, in close agreement with the theoretical prediction. DXR is the limiting reagent in this equilibrium; therefore, it is expected that NH_4^+ be in excess over DXR, and that the ratio will show greater than one equivalent of ammonium.

3.3 Sulfate Ion Quantification

The HPLC chromatogram for sulfate ion quantification is shown in Figure 4. The sulfate ion peak eluted at 14.2 min. Sucrose (peak 1, 1.0 min) and histidine (peak 4, 10.6 min), as well as sodium (peak 2, 6.0 min) and chloride (peak 3, 7.3 min) ions were also observed under this elution profile. Notably, the sulfate CAD response was not sensitive to the presence of sucrose and histidine, as was observed for ammonium. Standards were prepared both with and without the additives, and the resulting calibration curves were nearly superimposable (data not shown). For sulfate ion concentration measurements of Doxil, the lyophilized samples were reconstituted in 25% ACN with 0.6% (v/v) formic acid. A higher concentration of acid (0.6% FA, pH ~2.0) was required to achieve an equivalent pH as the reconstitution solutions for ammonium and doxorubicin measurements (0.1% TFA, pH ~1.8).

The total measured sulfate concentration in Doxil was $4.2 \pm 0.2 \times 10^{-3}$ M (n=5, Table 1). Theoretically, total sulfate in the system should be 1:2 as compared to ammonium. Indeed, the measured ratio was 1: 2.1 sulfate to ammonium. Furthermore, the total mass of ammonium sulfate in the sample was calculated to be 0.56 ± 0.04 mg/mL, which is approximately 93% of the total ammonium sulfate mass specified in the product documents, 0.6 mg/mL [43], indicating high recovery efficiency for both ammonium and sulfate ions.

The measured external sulfate concentration was $0.8 \pm 0.1 \times 10^{-3}$ M (n=3), or about 20% of the total sulfate detected. A lower fraction of total sulfate was found external to the liposome as was observed for ammonium (40% of the total ammonium). Due to the higher transmembrane diffusion coefficient of ammonia as a non-electrolyte ($1.3 \times 10^{-1} \text{ cm} \cdot \text{s}^{-1}$) compared to cations/anions (e.g., sulfate, protons; 10^{-3} to $10^{-8} \text{ cm} \cdot \text{s}^{-1}$) [44, 45], ammonia can diffuse back out of the liposome bilayer dependent upon the transmembrane pH gradient (demonstrated in Figure 1). Sulfate, however, becomes entrapped in the interior and cannot efficiently re-cross the lipid bilayer.

The calculated internal sulfate fraction was $3.4 \pm 0.2 \times 10^{-3}$ M of the total sulfate concentration. The theoretical stoichiometry expected for $(\text{DXR-NH}_3)_2\text{SO}_4$ is 2:1 DXR to sulfate. The measured molar ratio for DXR to sulfate was nearly 1:1. Although, sulfate concentrations are expected to be in excess, as DXR is the limiting reagent in the equilibrium process, and sulfate cannot readily escape the lipid bilayer once entrapped. The LOD of sulfate by this method was 2.0×10^{-6} M, the LLOQ was 5×10^{-6} M (S/N ~10), and the linear range (in 25% ACN with 0.6% formic acid) was from 5.0×10^{-6} M to 1.0×10^{-2} mM with an R^2 value 0.99. The LLOQ of sulfate in this method was found to be an order of magnitude lower than the ion chromatography method developed previously ($\sim 3.0 \times 10^{-5}$ M) [38].

4. Conclusions

Liposomes are one of the most widely used nanoscale drug delivery platforms and are used for delivery of a variety of therapeutic agents. Among the physicochemical characterization requirements for liposomes, techniques for ion composition analysis have been hindered by less than ideal sample preparation requirements, analyte volumes, LOD and LLOQ. The HPLC-based analytical methodology described herein requires extremely low analyte volumes ($< 100 \mu\text{L}$), has a facile 2-step sample preparation (lyophilization and reconstitution), entails relatively short HPLC runs for each sample analyses (ca. 20 mins for each run), and has favorably low LOD and LLOQ values. Furthermore, this approach can be applied to quantitate the ion fractions both inside and outside of the liposome structure with excellent reproducibility and high accuracy. The theoretical molar ratio of DXR:ammonium:sulfate in the internal DXR crystal precipitate is 2:2:1. This measured ratio was 2:3.1:1.9 using the analytical methodology described herein. Ammonium and sulfate are expected to be in excess as compared to DXR; thus, the measurements concur with the expected results. Additionally, all measurements were in very good agreement with published values, further demonstrating the accuracy of the method. This technique is also easily amenable to quantification of a variety of cations and anions (e.g., calcium, acetate, etc.) used for remote loading of liposomes. Furthermore, the full elution chromatograms (Figure 3 and 4) indicate that the method (including sample preparation and HPLC elution profile) developed here could be applied to quantify other components in Doxil (e.g., sucrose, histidine, sodium and chloride ions), further improving the characterization methodologies for the “nonliposome inactive ingredients”, a requirement in FDA’s guidance for industry of liposome drug products [21].

The ion quantification method developed here supports the FDA regulatory guidance for doxorubicin hydrochloride liposomal injection products [46]. To date, two follow-on versions of Doxil have been approved by the FDA (Sun Pharma Global FZE and Dr. Reddy’s Laboratories Ltd.), one is undergoing regulatory review through the Abbreviated New Drug Application (ANDA) pathway (Actavis, Inc.) [18], more are in clinical evaluation (e.g., NCT01715168, www.clinicaltrials.gov), and still others are continuing preclinical development. These follow-on products are required to use an ammonium sulfate remote-loading process, and to quantitate the total, internal, and external ion concentrations as part of the product composition comparison. As more and more follow-on versions of Doxil are being developed, this method will prove instrumental in speeding the clinical translation of these therapies.

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Highlights:

- Ions in liposomal products were quantitated using HPLC and charged aerosol detection
- Total, internal, and external concentrations were quantified with simple workups
- High recovery efficiency, low analyte volumes, and better LLOQ than other techniques
- Ideal for comparison of upcoming Doxil generics, supporting FDA requirements

Figure 1. Doxorubicin remote loading with ammonium sulfate.
Figure adapted from Yingchoncharoen et al.[47]

Figure 2. Ammonium ion standard curve.

Comparison of the ammonium ion calibration curve (2.5×10^{-4} M – 2×10^{-3} M) in the presence (●) or absence (△) of 1% (w/v) sucrose and 0.001 M histidine. The CAD response was about 20% greater in the presence of sucrose and histidine, components of the buffer for Doxil.

Figure 3. HPLC chromatogram for ammonium ion detection in Doxil.

The lyophilized and reconstituted Doxil sample for total ammonium ion quantification is shown. The ammonium ion peak (peak 3) eluted at 6.0 min. Several other peaks were also detected, namely sucrose (peak 1) at 3.5 min, doxorubicin (peak 4) at 7.8 min, and histidine (peak 6) at 15.5 min. The peaks at 5.5 and 11.5 min (peaks 2 and 5, respectively) were background signals, likely a result of histidine and sucrose adducts in the buffer. (Inset: Full HPLC chromatogram for ammonium ion detection in Doxil. For reference, the largest peak is sucrose.).

Figure 4. HPLC chromatogram for sulfate ion detection in Doxil.

The lyophilized and reconstituted Doxil sample for total sulfate ion quantification is shown. The sulfate ion peak (peak 5) eluted at 14.2 min. Several other peaks were also detected, namely sucrose (peak 1) at 1.0 min, sodium (peak 2) at 6.0 min, chloride (peak 3) at 7.3 min, and histidine (peak 4) at 10.6 min (Inset: Full HPLC chromatogram for sulfate ion detection in Doxil. For reference, the largest peak is sucrose.)

Table 1.

Drug and ion (ammonium and sulfate) quantification of Doxil.

	Measured Total Concentration ($\times 10^{-3}$ M)	Measured External Concentration ($\times 10^{-3}$ M)	Calculated Internalized Content ^a ($\times 10^{-3}$ M)
DXR	3.7 $\pm$ 0.1	0.1 $\pm$ 0.01	3.6 $\pm$ 0.1
NH ₄ ⁺	8.9 $\pm$ 0.9	3.5 $\pm$ 0.2	5.4 $\pm$ 1.1
SO ₄ ²⁻	4.2 $\pm$ 0.2	0.8 $\pm$ 0.1	3.4 $\pm$ 0.2

^a Internalized content was calculated by subtraction of the measured external concentration from the measured total concentration, and is represented as a concentration based on the total sample volume.