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Ion chromatography for the determination of sulfate in STEALTH® liposomes

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Abstract

An ion chromatography (IC) method has been validated for the assay of sulfate ion content in the STEALTH® liposome drug-delivery system (ALZA, Mountain View, CA, USA), which contains ammonium sulfate as an excipient. This method contains two assays. One assay determines the total sulfate ion content; the other determines the external sulfate ions. The total sulfate ion analysis measures the sulfate ion content of the formulation, inside and outside of the liposome. The analysis includes the disruption of the liposome bilayer with Triton-X, followed by dilution with 10% sucrose, and analysis using IC. The external sulfate analysis measures sulfate ions outside the liposome without disrupting the liposome structure. A neat sample of STEALTH® liposome drug-delivery system is filtered through a 0.02 μm filter, and the filtrate is analyzed by IC. Sulfate ion is resolved on an anion exchange column and detected by a conductivity detector. Quantitation is performed by linear regression analysis of peak areas from a standard curve of sulfate ion containing at least five standard points. The method was validated for specificity, linearity, accuracy/recovery, precision, and stability of the standard and the sample. The validated method has been applied to the quantification of sulfate ion in STEALTH® liposomes for product release and stability testing.

Keywords: STEALTH® drug-delivery system; Pharmaceutical analysis; Liposomes; Sulfate

1. Introduction

The STEALTH® liposome drug-delivery system (ALZA, Mountain View, CA, USA), which delivers cytotoxic compounds to tumor cells, contains ammonium sulfate as an excipient (Fig. 1). Because sulfate ion is one of the critical elements for drug encapsulation and encapsulation stabilization, it is essential to quantify and monitor the internal sulfate content during product lot release and stability testing. While traditional ion conductivity probes can allow direct measurement of sulfate ions in solutions [1–4], difficulties were encountered when applying this approach to quantify internal sulfate ion, within a liposome, without disrupting the liposome bilayer. Therefore, a new method was developed that utilizes ion chromatography (IC) with suppressed conductivity detection to indirectly measure the internal sulfate ion content of STEALTH® liposomes. In the first analysis, the total sulfate ion content of the liposome formulation was measured, i.e., sulfate ions inside and outside of the liposome. The second asssay, external sulfate analysis, measured only sulfate ions outside of the liposome, without disrupting the liposome structure. Internal sulfate ion content could then be calculated as the difference of the two measurements.

2. Experimental

2.1. Chemicals and reagents

All solutions were prepared using deionized water (>18 MΩ) purified by a Milli-Q Gradient system (Millipore, Billerica, MA USA). AS14 concentrate (350 mM sodium carbonate/100 mM sodium hydrogen carbonate) purchased from Dionex (Sunnyvale, CA, USA). Ammonium sulfate (ACS grade) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Sucrose (GR grade) was purchased from EM Science (Gibbstown, NJ, USA). Triton-X (electrophoresis grade) was purchased from Fischer Chemicals (Springfield, NJ, USA). Doxil® (STEALTH® liposomal doxorubicin·HCl; ALZA, Mountain View, CA, USA) was used in method development and validation as an example of a STEALTH® liposome drug-delivery system.

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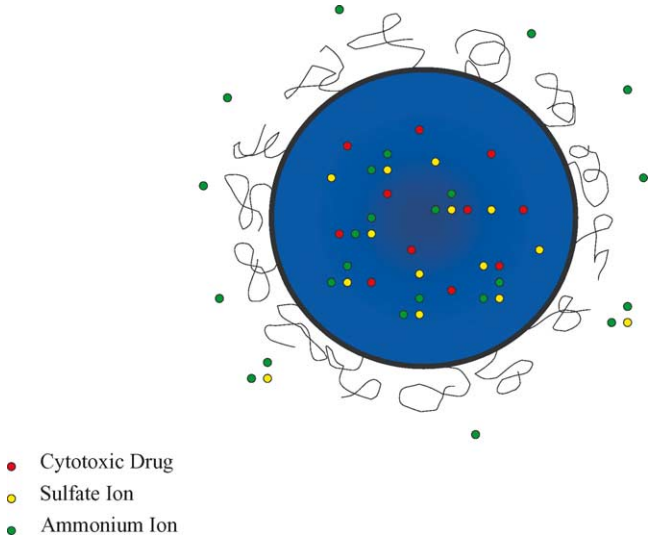

Fig. 1. STEALTH® liposome drug-delivery system.

2.2. Materials and equipments

Syringes (1 ml) were purchased from BD (Franklin Lakes, NJ, USA). Anotop Plus syringe filters (0.02 μm) were purchased from Whatman (Clifton, NJ, USA). The syringe pump used in the sample preparation was purchased from Harvard Apparatus (Holliston, MA, USA).

2.3. IC instrumentation and chromatographic conditions

The IC system utilized in the method development and validation was a Dionex DX500 IC system (Sunnyvale, CA USA) equipped with both conductivity and UV detectors, in-line degasser, and an AS3500 autosampler. Separation and resolution of ions was carried out on a Dionex IonPac AS14 separation column (4 mm × 250 mm) attached with a corresponding AG14 guard column. Conductivity was suppressed with Dionex ASRS II suppressor. The suppressor voltage, flow rate, and run time were set at 100 mA, 1.2 ml/min, and 8 min, respectively. Injection volumes on the AS3500 were set to 10 μl for total sulfate analysis and 5 μl for external sulfate analysis. Instrument control and data reduction were carried out with Dionex Peaknet 5.1 software. Data collection rate and cell temperature were set at 5 Hz/s and 35 °C, respectively.

2.4. Eluent, diluent, lysing agent, and standard solutions

Eluent was prepared from the AS14 concentrate by dilution with Milli-Q water to achieve a final concentration of 7.0 mM sodium carbonate/2.0 mM sodium hydrogen carbonate. A solution of 10% (w/w) sucrose in water was prepared and used as the diluent. The lysing agent was an aqueous solution of 25% (w/w) Triton-X. Sulfate standard solutions were prepared by weighing an appropriate amount of ammonium sulfate into volumetric flasks and diluting it to achieve the desired concentration.

2.5. Sample preparation

Total sulfate analysis: equivalent volume of STEALTH® liposome formulation and the lysing agent were transferred to a volumetric flask utilizing the appropriate pipettor and diluted by 25-fold with sample diluent. Samples were mixed well after dilution.

External sulfate analysis: an appropriate volume of STEALTH® liposome formulation was filtered through a 0.02 μm syringe filter using a syringe pump at a flow rate of 0.54 ml/min for a 5 ml syringe. Sufficient volume (minimally 100 μl) of filtrate was collected for IC analysis.

3. Results and discussion

3.1. Method development

3.1.1. Detergent selection

The selection of a suitable lysing agent for the disruption of the liposomes for total sulfate analysis was a challenge. Lysing agent is necessary to break or disrupt the liposome bilayers, thus releasing the contents into the dilution media. Incomplete lysing results in a low recovery of sulfate ion from the liposomes. Typically, either detergent or organic solvent can act as a lysing agent. In this method, detergent was selected because the life of the conductivity suppressor decreases with prolonged interactions with organic solvents in auto-suppression recycle mode. Detergent also aids lipid dissolution by forming micellar structures with the lipids in aqueous solution, and thus prevents the lipids from aggregation and precipitation in the dilution solvent. Triton-X was selected for these purposes. Liposomes are completely lysed in an aqueous solution of 1% Triton-X. Due to the 25-fold dilution in the preparation of total sulfate sample, a stock solution of 25% Triton-X was prepared.

3.1.2. Chromatographic optimization

Methods for quantitation of sulfate ions in aqueous media utilizing IC have been reported in the literature [5–7]. Application of one of these methods for our needs has shown promising potential. Because there was no chromatographic interference from the chloride ion and the Triton-X detergent peak, we were able to increase the concentration of the eluents from 3.5 mM sodium carbonate/1.0 mM sodium hydrogen carbonate to 7.0 mM sodium carbonate/2.0 mM sodium hydrogen carbonate. With this modification, the run time was shortened to 8 min with full resolution of all observed peaks (Fig. 2).

3.1.3. Filter selection

The most challenging aspect of this method development was to analyze external sulfate ion content without disrupting the liposome bilayer. Several approaches were tried, ranging from size-exclusion chromatography (SEC) to dialysis. The most successful approach was to

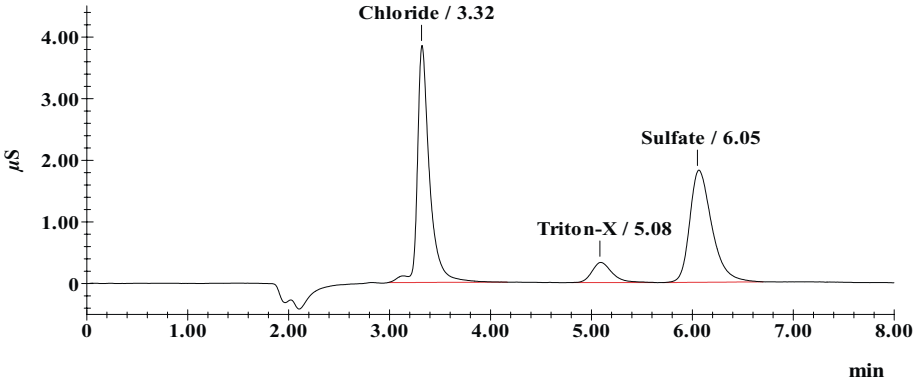

Fig. 2. Elution profile of a lysed STEALTH® liposome product.

filter the sample using syringe filters. This approach is the least time consuming way to separate liposomes from the dilution buffer. Due to the intrinsic size of the liposomes (∼100 nm), a syringe filter with the pore size of 0.02 μm was selected. Using a syringe pump, the liposomes were separated from the dilution buffer without any detectable liposome disruptions (unpublished internal study results).

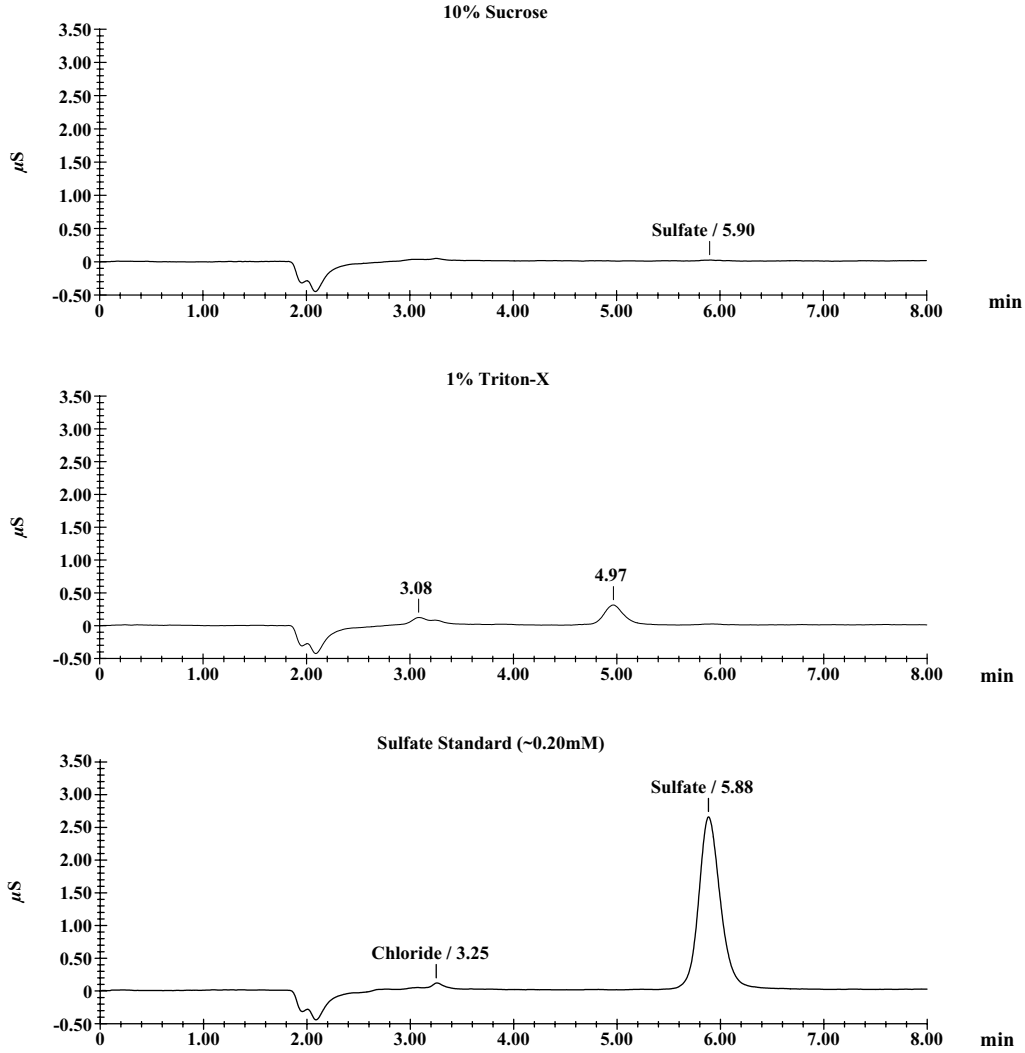

Fig. 3. Elution profiles of 10% sucrose, 1% Triton-X, and sulfate standard (∼0.20 mM), respectively.

3.2. Method validation

The optimized method was validated for specificity, linearity, accuracy/recovery, and precision following ICH [8] guidelines and system suitability criteria as recommended in USP XXIII [9].

3.2.1. Specificity

Specificity is demonstrated by analyzing blanks, excipients, and standards to show the absence of peaks, or no more than 1% detectable, within 5% of the retention time for the analyte of interest, sulfate ion. Fig. 3 shows chromatograms of 10% sucrose (diluent), 1% Triton-X, and a 0.20 mM sulfate standard. Minor detection of sulfate ion in the 10% sucrose injection is due to sulfate impurity found in sucrose crystals. However, it is only less than 1% of peaks detectable, as compared to the 20 mM sulfate standard. Therefore, the method is satisfactory in resolving sulfate ion from other excipient or reagent-related peaks.

3.2.2. Linearity

The linearity of the standard sulfate calibration curve was established using seven concentration levels, ranging from 0.075 to 0.35 mM sulfate ion. Three injections were performed at each concentration level. A regression line was obtained by plotting peak area (μS) of the sulfate ion versus the standard concentration (mM sulfate) using the least square method. The relationship between peak response and concentration was found to be linear between the ranges of 0.075–0.35 mM sulfate ion, with a coefficient of determination (r^2) of 0.999 (Table 1). The slope and intercept of the regression line were 2,011,758 ± 14,824 and −16,309 ± 3388, respectively. Standard error ($S_{y/x}$) was 6559. The relative standard deviation (R.S.D.) of the response factor was 2.61%. Back-calculated concentrations for each calibration standard were within 10% of prepared concentrations. The origin was not included in the 95% confidence interval (CI). However, this was not considered a problem because approximately 0.20 mM sulfate ion concentrations were expected to be in the samples, well above the lowest point on the calibration curve.

3.2.3. Accuracy

In this validation, placebo samples were not available, therefore standard additions of ammonium sulfate to STEALTH® liposome products were used. The accuracy of

Table 1
Linearity of sulfate ion in 10% sucrose

Sample name	Prepared concentration (mM)	Injections	Peak area (μS)	Calculated concentration (mM)	Response factor	Bias (%)
Standard 1	0.076	1	141133	0.078	1849463	−2.56
		2	144289	0.080	1890824	−4.61
		3	141151	0.078	1849705	−2.57
Standard 2	0.102	1	186304	0.101	1831160	1.01
		2	187043	0.101	1838420	0.65
		3	192841	0.104	1895411	−2.18
Standard 3	0.153	1	286568	0.151	1877762	1.35
		2	283314	0.149	1856434	2.41
		3	286348	0.150	1876315	1.42
Standard 4	0.203	1	394093	0.204	1936744	−0.26
		2	389987	0.202	1916566	0.75
		3	396855	0.205	1950319	−0.93
Standard 5	0.254	1	486989	0.250	1914622	1.64
		2	493353	0.253	1939639	0.40
		3	498546	0.256	1960057	−0.62
Standard 6	0.305	1	588449	0.301	1927929	1.51
		2	598525	0.306	1960942	−0.13
		3	591342	0.302	1937408	1.04
Standard 7	0.356	1	694572	0.353	1950530	0.77
		2	716215	0.364	2011309	−2.25
		3	709621	0.361	1992793	−1.33
Average					1912588	
R.S.D. (%)					2.61%	
Regression						
Y-Intercept	−16309 ± 3388					
Slope	2011758 ± 14824					
R^2	0.999					
Standard error ($S_{y/x}$)	6559					

Table 2
Accuracy/recovery and precision of sulfate from STEALTH® liposome product

Level	Prepared concentration (mM)	Calculated concentration (Mm)	Individual recovery (%)	Mean recovery ($n = 3$) (%)	R.S.D. ($n = 3$) (%)
Total sulfate					
1	4.136	4.077	98.57	98.46	0.46
		4.066	98.32		
		4.073	98.49		
2	4.248	4.236	99.73	99.20	1.01
		4.184	98.51		
		4.220	99.35		
3	4.359	4.353	99.86	98.95	1.48
		4.315	99.00		
		4.270	97.97		
Mean recovery (%) ($n = 9$)			98.87		
R.S.D. (%) ($n = 9$)			0.67		
External sulfate					
1	0.503	0.575	114.28	107.73	5.67
		0.505	100.33		
		0.546	108.58		
2	0.633	0.643	101.51	99.95	6.93
		0.578	91.30		
		0.678	107.04		
3	0.762	0.740	97.10	97.92	1.42
		0.744	97.65		
		0.755	99.01		
Mean recovery (%) ($n = 9$)			101.87		
R.S.D. (%) ($n = 9$)			6.84		

the method was defined as the percentage recovery between the observed sulfate concentration and the prepared sulfate ion concentration in a spike recovery study. Two concentrations, 4 and 0.6% sulfate ions, were used to determine total and external sulfate recovery, respectively. This is because external sulfate concentration was assumed to be no more than 15% of total sulfate concentration in the product. Therefore, it was necessary to determine recovery of sulfate ion at a lower concentration (0.6%) from the liposome. The results of the study are shown in Table 2. For total sulfate ion analysis, the individual percentage recovery ranged from 98.32 to 99.86% and the mean percentage recovery was 98.46% for level 1, 99.20% for level 2, and 98.95% for level 3. For external sulfate ion analysis, the individual percentage recovery ranged from 91.30 to 114.28% for all three levels, and the mean percentage recovery was 107.73% for level 1, 99.95% for level 2, and 97.92% for level 3. Mean % recoveries within 10% are satisfactory.

3.2.4. Method precision: repeatability

The precision of the method was assessed by comparing the variation between similarly prepared samples in a spike recovery experiment. Data generated from both the total and external sulfate ion analysis (all levels) in the accuracy study were used to calculate the R.S.D. Data are shown in Table 2. For the total sulfate analysis, the R.S.D.s are 0.46, 1.01, and 1.48% for levels 1 (4.1 mM sulfate), 2 (4.2 mM sulfate), and 3 (4.4 mM sulfate), respectively. Based on the percentage of recovery of all nine spiked samples, the overall precision of the total sulfate assay is 0.67%. For the external sulfate analysis, the R.S.D.s are 5.67, 6.93, and 1.42% for levels 1 (0.5 mM sulfate), 2 (0.6 mM sulfate), and 3 (0.8 mM sulfate), respectively. The overall precision of the external sulfate assay is 6.84%. Repeatability for the total sulfate assay with less than 1% is generally acceptable for an IC method. Because of the intrinsic variation observed with the use of a membrane filter, repeatability for external sulfate assay less than or equal to 10% is satisfactory.

3.2.5. Intermediate precision

Intermediate precision evaluates the variations within the laboratory: different days and different analysts.

3.2.5.1. Intermediate precision: day-to-day. The day-to-day precision of the method was evaluated by analyzing six preparations (triplicate injections) of one representative lot of STEALTH® liposomes on three different days for total and external sulfate ions. Precision was evaluated by the R.S.D. of each individual day and the R.S.D. of day-to-day variations. In the total sulfate ion analysis, R.S.D.s of each individual day ranged from 0.99 to 1.49% (Table 3). Day-to-day precision for total sulfate was 2.06%. Because of the intrinsic method variation for external sulfate ion analysis, R.S.D.s ranged from 6.15 to 7.65% for individual days. Day-to-day precision for external sulfate ion analysis was 7.44%. Day-to-day precision for total and

Table 3
Inter-day precision

Preparation	Injection	Total sulfate			External sulfate		
		Day 1, sulfate ion concentration (mM)	Day 2, sulfate ion concentration (mM)	Day 3, sulfate ion concentration (mM)	Day 1, sulfate ion concentration (mM)	Day 2, sulfate ion concentration (mM)	Day 3, sulfate ion concentration (mM)
1	1	4.028	3.919	3.892	0.395	0.370	0.434
	2	4.045	3.865	3.922	0.388	0.374	0.443
	3	4.048	3.892	4.060	0.384	0.371	0.444
2	1	4.056	4.000	3.973	0.461	0.395	0.380
	2	4.007	3.903	3.946	0.458	0.408	0.375
	3	4.040	3.934	3.925	0.461	0.403	0.379
3	1	4.017	4.001	3.860	0.410	0.444	0.439
	2	3.928	3.941	3.960	0.415	0.438	0.446
	3	4.042	3.948	3.894	0.415	0.438	0.457
4	1	4.094	3.940	3.972	0.476	0.367	0.403
	2	4.058	3.887	3.952	0.485	0.361	0.406
	3	4.150	3.928	3.897	0.475	0.368	0.412
5	1	4.123	3.893	3.935	0.409	0.427	0.408
	2	4.092	3.870	3.960	0.401	0.427	0.414
	3	4.152	3.894	3.837	0.403	0.424	0.427
6	1	4.138	3.903	3.938	0.446	0.405	0.395
	2	3.975	3.908	3.903	0.449	0.416	0.393
	3	4.101	3.888	3.966	0.447	0.426	0.397
Mean ($n = 18$)		4.061	3.917	3.933	0.432	0.403	0.414
R.S.D. (%) ($n = 18$)		1.49	0.99	1.27	7.65	7.05	6.15
Mean sulfate concentration ($n = 54$)				3.970			0.416
R.S.D. (%) ($n = 54$)				2.06			7.44

Table 4
Analyst-to-analyst precision

Preparation	Injection	Total sulfate		External sulfate	
		Analyst 1, sulfate ion concentration (mM)	Analyst 2, sulfate ion concentration (mM)	Analyst 1, sulfate ion concentration (mM)	Analyst 2, sulfate ion concentration (mM)
1	1	3.892	3.882	0.434	0.341
	2	3.922	3.871	0.443	0.332
	3	4.060	3.895	0.444	0.343
2	1	3.973	3.985	0.380	0.358
	2	3.946	3.891	0.375	0.357
	3	3.925	3.972	0.379	0.354
3	1	3.860	3.927	0.439	0.418
	2	3.960	3.890	0.446	0.413
	3	3.894	3.875	0.457	0.407
4	1	3.972	3.949	0.403	0.370
	2	3.952	3.901	0.406	0.375
	3	3.897	3.900	0.412	0.371
5	1	3.935	3.869	0.408	0.340
	2	3.960	3.939	0.414	0.351
	3	3.837	3.920	0.427	0.350
6	1	3.938	3.873	0.395	0.326
	2	3.903	3.846	0.393	0.333
	3	3.966	3.865	0.397	0.331
Mean ($n = 18$)		3.933	3.903	0.414	0.360
R.S.D. (%) ($n = 18$)		1.27	0.99	6.15	7.88
Mean sulfate concentration ($n = 36$)			3.918		0.387
R.S.D. (%) ($n = 36$)			1.19		9.90

external sulfate ion analyses of less than or equal to 5 and 10%, respectively, are satisfactory.

3.2.5.2. Intermediate precision: analyst-to-analyst. Analyst-to-analyst precision was evaluated by analyzing six preparations (triplicate injections) of STEALTH® liposomes on two different days by two different analysts. The R.S.D.s of the results obtained from the two analysts and the R.S.D. of the results obtained from the two analysts combined (analyst-to-analyst) were evaluated for both the total and the external sulfate ion. The R.S.D.s generated from analyst 1 and analyst 2 for total sulfate ion analysis were 1.27 and 0.99%, respectively (Table 4). Analyst-to-analyst variation was 1.19%. For the external sulfate ion analysis, the R.S.D.s from analyst 1 and analyst 2 were 6.15 and 7.88%, respectively. Analyst-to-analyst variation was 9.90%. Same criteria were used in the evaluation of day from the analyst-to-analyst study as in the day-to-day study. The results for this study are satisfactory.

3.2.6. Limit of detection

Limit of detection (LOD) is determined as the signal that is three times the noise level (S/N = 3). The noise level is calculated as the average of three measurements of noise at three different regions on the chromatogram from the sample injection, and is in the unit of 10^2 ps (picosecond). LOD is determined as 0.0006 mM sulfate ion, with signal-to-noise ratio of 3.2.

3.2.7. Limit of quantitation

Limit of quantitation (LOQ) was established by analyzing sulfate ion spiked samples. The individual percent recovery ranged from 105.09 to 107.08%, and the mean percent recovery was 106.02%. The variability (R.S.D.) of the six injections was 1.06%. Therefore, LOQ was determined as 0.0263 mM sulfate.

3.2.8. Stability of standard stock solutions

Evaluation of sulfate stock stability was carried out. Freshly prepared solutions at a concentration of 0.198 mM sulfate ion from the same stock solution were analyzed at different time points. The percentage ratios of sulfate ion concentration at the individual time points to that at initial (t_0) were within 2% variation during a 3-day evaluation.

3.2.9. Stability of sample solutions at 2–8 °C

To determine the stability of the prepared samples at 2–8 °C, six solutions (three for total sulfate samples and three for external sulfate samples) were prepared and stored at 2–8 °C, and analyzed at different time points. The percentage ratio of sulfate ion concentration at later time points to that at t_0 ranged from 96.75 to 97.28% for external sulfate

Table 5
Recovery of sulfate ion filtered through Anotop syringe filters

Level	Prepared concentration (mM)	Calculated concentration (mM)[a]	Mean concentration (mM)[b]	R.S.D. (%)[b]	Individual recovery (%)	Mean recovery (%)
1	0.201	0.201	0.201	0.99	99.61	99.84
	0.201	0.201			100.08	
2	0.403	0.401	0.399	0.65	99.66	99.20
	0.403	0.398			98.74	
3	0.604	0.593	0.590	0.70	98.20	97.69
	0.604	0.587			97.18	

[a] Results are average of triplicate injections.
[b] Results are average and relative standard deviation of six injections.

and 99.36 to 99.55% for total sulfate over a 3-day period. The results indicated that the samples for external sulfate ion analysis were not stable at 2–8 °C, while the samples for total sulfate ion assay could be stored at 2–8 °C for up to 3 days.

3.2.10. Autosampler stability of sample solutions

Due to the instability of the external sulfate sample at 2–8 °C, it was necessary to determine the stability of the samples under autosampler condition at room temperature and under light exposure. Replicate preparations of samples were placed in the autosampler and analyzed at different time points over 24 h. The percentage ratio of sulfate ion at later time points to that at t_0 ranged from 98.61 to 100.27% over 24 h for total sulfate samples. However, the ratio dropped below 98% after 3 h. These data indicated that samples for total sulfate ion analysis are stable for 24 h at room temperature, while the samples for external sulfate ion analysis are stable for only 3 h. Therefore, samples for external sulfate ion analysis require the IC analysis immediately following preparations.

3.2.11. Effect of filtration on sulfate recovery

To evaluate the percentage recovery of sulfate ion from the filtration, duplicate samples of sulfate ion in 10% sucrose at three different concentrations were prepared. The samples were then filtered and analyzed by IC according to the sample preparation for external sulfate ion assay in the method. The results showed that the individual percentage recovery of sulfate ion ranged from 97.18 to 100.08% (Table 5). The mean percentage recovery for all three levels ranged from 97.69 to 99.84%. No significant variation in the recovery of sulfate ion from the filtration was observed. Percentage of recovery within the 3% range is satisfactory.

3.2.12. System precision

System precision is expressed in replicate injections of a single standard solution. The R.S.D. is calculated for both peak area response and retention time. The R.S.D. of peak areas and retention time from six replicate injections of a single sulfate ion standard (~0.10 mM of sulfate ion) are 1.67 and 0.13%, respectively. Tailing factor (T), theoretical plates (N Tangential), and capacity factor (k') were also evaluated. The theoretical plate ranged from 3404 to 3926, and the capacity factor ranged from 2.02 to 2.03. In general, R.S.D. for peak areas observed to be less than 2% is satisfactory for a chromatographic assay per USP XXIII.

4. Conclusion

An IC method has been developed for the quantitation of sulfate ion content in the STEALTH® liposome drug-delivery system. The method provides a route to indirectly determine the internal sulfate ion content within a liposome without the disruption of the liposome bilayer as seen with other conventional methods. The method has been validated based on ICH guideline and is suitable for the analysis of STEALTH® liposome products in the range of 0.075–0.35 mM sulfate ion. Satisfactory recoveries were observed for both total and external sulfate analyses.

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